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Original Article

FoxD2-AS1 is a prognostic factor in glioma and promotes temozolomide resistance in a O^6-methylguanine-DNA methyltransferase-dependent manner

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ABSTRACT Glioma is the most common brain tumor with a dismal prognosis. While temozolomide (TMZ) based chemotherapy significantly improves survival in glioma patients, resistance against this compound commonly leads to glioma treatment failure. Overexpression of long-noncoding RNA (LncRNA) *FoxD2* adjacent opposite strand RNA 1 (*FoxD2-AS1*) was identified to promote glioma development, but the role in TMZ resistance remains unclear. In this paper, we found that *FoxD2-AS1* was overexpressed in recurrent glioma, high *FoxD2-AS1* expression was significantly correlated with poor patient outcome. Methylation of O^6-methylguanine-DNA methyltransferase (MGMT) is significantly less frequent in high *FoxD2-AS1* expression patients. Knockdown of *FoxD2-AS1* decreased the proliferation, metastatic ability of glioma cells and promote the sensitivity to TMZ in glioma cells. Furthermore, knockdown of *FoxD2-AS1* induced hypermethylation of the promoter region of MGMT. Our data suggested that *FoxD2-AS1* is a clinical relevance LncRNA and mediates TMZ resistance by regulating the methylation status of the MGMT promoter region.

INTRODUCTION

Glioma is the most common form of brain malignancy that produces severe damage to the brain leading to a very poor survival prognosis [1,2]. Currently, temozolomide (TMZ)-based chemotherapy significantly improves prognosis, and is recommended as a standard care for glioma patients [3]. TMZ is an alkylating agent which cause lethal DNA damage (O^6-methylation-mediated DNA base mismatching) and subsequent induce cell death and apoptosis [4]. However, a majority of patients with glioma gradually develop resistance to TMZ during treatment. The most popular mechanism of TMZ resistance is the expression of O^6-methylguanine-DNA methyltransferase (MGMT) which removes the cytotoxic O^6-methylguanine-DNA adducts [5,6]. The expression of MGMT is regulated by MGMT promoter methylation. Normally, high promoter methylation status which means low MGMT activity predicts good response to TMZ chemotherapy and results in a longer survival period in glioma patients [7,8]. This observation led to the hypothesis that MGMT inhibition may be a plausible strategy for sensitizing TMZ therapy.

In recent years, emerging evidence has regarded the functional role of dysregulated long-noncoding RNAs (LncRNAs) in the cancer formation and progression, as well as the resistance to chemotherapy [9,10]. The LncRNA *AC003092.1* promotes TMZ chemosensitivity through *miR-195/TFPI-2* signaling pathway in glioblastoma [11]. LncRNA *XIST* can amplify the chemoresistance of glioma cell lines to TMZ through directly targeting *miR-29c via SP1* and MGMT [12]. Additionally, LncRNA *H9* and *RP11-838N2.4* have been reported to enhance cytotoxic effects of TMZ in glioma cell lines [13,14]. Thus, genomic characterization

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of LncRNA alterations may provide potential therapeutic strategy for TMZ resistant in glioma.

LncRNA *FoxD2* adjacent opposite strand RNA 1 (*FoxD2-AS1*) is a newly identified LncRNA which locate on chromosome 1p33 with a transcript 2527 nucleotides in length. *FoxD2-AS1* was identified to be overexpressed in esophageal squamous cell carcinoma, nasopharyngeal carcinoma, bladder cancer, colorectal cancer and glioma [15-19]. *FoxD2-AS1* can regulate cancer progression and recurrence, but the functional role of *FoxD2-AS1* in chemoresistance remains unclear. In the present study, we analyzed the expression of *FoxD2-AS1* in glioma tissues obtained from glioma gene expression datasets, and found that *FoxD2-AS1* was a clinically relevant LncRNA, as high expression was associated with poor patient outcome. Moreover, we demonstrated that methylation of MGMT is significantly less frequent in high *FoxD2-AS1* expression patients and downregulation of *FoxD2-AS1* decreased TMZ resistance in glioma cells through regulating the methylation status of the MGMT promoter region. Our findings revealed that the dysregulation of *FoxD2-AS1* is a potential component of glioma pathogenesis and TMZ resistance, which might become a new therapeutic target for patients with glioma.

METHODS

Cell culture

The human glioma cell lines U251 and A172 were obtained from Cell Resource Center of Shanghai and cultured in Dulbecco's Modified Eagle Medium (DMSO; Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FBS; Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Life Technologies, Grand Island, NY, USA) at 37°C in 5% CO_2.

Bioinformatics analysis

Glioma gene expression arrays with survival data were obtained from the National Cancer Institute Repository for Molecular Brain Neoplasia Data (NCI REMBRANDT), the Cancer Genome Atlas (TCGA), and gene expression omnibus datasets. TCGA data were extracted directly from the web site (https://portal.gdc.cancer.gov/). For assessing the overall survival (OS) of glioma patients included in REMBRANDT, we used project Betastasis (http://www.betastasis.com). For assessing the OS of glioma patients included in GSE16011, we used R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). OS was defined as the period from the date of the pathological diagnosis to death.

Cell transfection

Small interfering RNA (siRNA) targeting *FoxD2-AS1* and negative control siRNA were synthesized by Biomics Biotechnologies Co., Ltd. (Nantong, China). Sense si*FoxD2-AS1*: 5′-GCGAAGAGUACGUUGCUAUTT-3′, antisense si*FoxD2-AS1*: 5′-AUAGCAACGUACUCUCGCTT-3′; Sense siNC: 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense siNC: 5′-ACGUGACACGUUCGGAGAATT-3′. Cells were grown on six-well plates to confluency and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, each well was supplemented with 5 μl Lipofectamine 2000 and 100 pmol siRNA. About 48 h later, cells were acquired for the following experiments.

Western blot analysis

si*FoxD2-AS1* or siNC transfected cells were treated with TMZ for 48 h, and then were washed once with phosphate buffer saline (PBS) and dissolved in Protein Extraction Reagent (Boster Bioengineering, Wuhan, China) containing 1 mM phenylmethanesulfonyl fluoride (PMSF; Roche Molecular Biochemicals, Indianapolis, IN, USA). Protein samples were separated by 10%–12% SDS-PAGE, transferred onto the surface of polyvinylidene fluoride membrane and immunoblotted with the indicated primary antibodies. Using the enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ, USA), visualization of the protein bands was conducted in the Omega Lum G system (Aplegen, Pleasanton, CA, USA). The primary antibodiy to β-actin was purchased from Hua Bio (Hangzhou HuaAn Biotechnology Co., Ltd., Hangzhou, China), and the antibodys to Caspase-3, Bax, MGMT were purchased from (Proteintech Group, Chicago, IL, USA).

Isolation of DNA and methylation-specific PCR

The DNA was extracted using Takara MiniBEST Universal Genomic DNA Kit (Takara, Shiga, Japan) and then 1 μg of extracted DNA underwent bisulfite modification using a DNA Methylation Kit (CoWin Biosciences, Beijing, China) according to the manufacturer's instructions. The MS-PCR was performed under the following conditions: 95°C for 10 min; 35 cycles of 95°C for 30 sec, the annealing temperature 60°C for 30 sec, and 72°C for 30 sec; and a final extension of 10 min at 72°C. Primers for the methylated MGMT were 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ (forward) and 5′-GCACTCTTCCGAAAACGAAACG-3′ (reverse); for the un-methylated MGMT were 5′-TTTGTGTTTTGATGTTTGTAGGTTTTT GT-3′ (forward) and 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ (reverse). The PCR product was directly loaded onto 2.5% agarose gels, stained with ethidium bromide, and visualized with the aid of ultraviolet light. The density of each band was quantified using imaging analysis and the relative band density values were calculated as the ratio of methylated MGMT to that of methylated plus un-methylated MGMT.

MTT assay

Cell viability was evaluated by using MTT assay. Glioma cells were seeded into 96-well plates at the concentration of 2×10^3 cells/well. Cells were treated with different concentrations of TMZ (MedChem Express, Monmouth Junction, NJ, USA) for 48 h. Then, 10 μl MTT (5 mg/ml) was added to each well and incubated in the dark at 37°C for another 4 h. Absorbance was determined at a wavelength of 570 nm using a SpectraMax M3 microplatereader (Molecular Devices, Sunnyvale, CA, USA).

Colony formation assay

Cells were seeded ($3–5 \times 10^5$ cells/well) and then transfected with si*FoxD2-AS1* or siNC alone. After 24 h post-transfection, cells were treated with TMZ for another 24 h, washed, serially diluted, plated in triplicate into 6 well plates, allowed 6–8 days of cell growth for colony formation, stained with 5% crystal violet. Finally, colonies were photographed, and the number of colonies was counted manually.

Migration and invasion assays

In the migration assay, cells were suspended in DMEM and the density was adjusted to approximately 1×10^5 cells/ml. Cell suspension was then seed to the upper chamber (Corning, Corning, NY, USA) of the inserts (0.2 ml/well), and DMEM containing 20% FBS was added to the lower chamber. After culturing for 36 h, the cells migrated to the bottom of the membrane were fixed with 4% paraformaldehyde and stained with Giemsa violet. The invasion assay was performed in the same way as the migration assay except that the membrane was coated with matrigel (BD Biosciences, Bedford, MA, USA).

Cell apoptosis analysis

An apoptosis analysis kit (NanJing KeyGen Biotech Co., Ltd., Nanjing, China) was utilized to detect the cell apoptotic rates. Briefly, the Annexin V and PI were firstly added into the binding buffer, and the treated cells were resuspended in the binding buffer. After incubating in the dark for 15 min, the cells were washed and analyzed using a Guava easyCyte 5 Flow Cytometer (EMD Millipore, Bellerica, MA, USA).

Statistical analysis

All statistical data were analyzed using the SPSS 21.0 software (IBM Co., Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). p-values < 0.05 were considered statistically significant. Data are expressed as mean ± standard deviation. Statistical significance was determined using Student's t-test or one-way analysis of variance. The Kaplan–Meier method with the log-rank test was applied for OS.

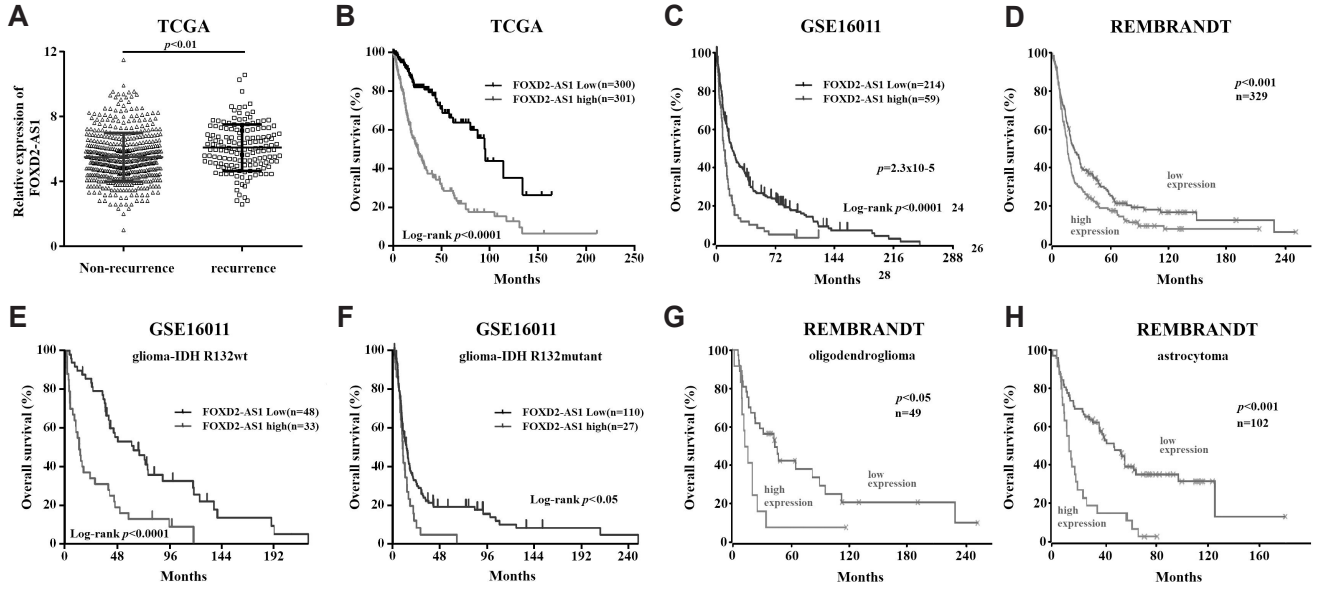

Fig. 1. *FoxD2-AS1* is associated with clinical outcome of patients with glioma. (A) *FoxD2-AS1* expression in recurrent glioma tissues and non-recurrent tissues obtained from the Cancer Genome Atlas (TCGA). (B–D) Kaplan–Meier plots of patient outcome based on *FoxD2-AS1* expression. (E–H) Kaplan–Meier plots of patient outcome based on *FoxD2-AS1* expression in glioma, stratified according to the annotated IDH mutation status and glioma subtype. Data, statistical evaluation, and visualization were obtained using the R2 website "R2: Genomics Analysis and Visualization Platform" (http://r2.amc.nl) and http://www.betastasis.com/. REMBRANDT, Repository for Molecular Brain Neoplasia Data.

RESULTS

FoxD2-AS1 is associated with clinical outcome of patients with glioma

Three independent glioma gene expression datasets (TCGA, GSE16011, and REMBRANDT) were employed to examine the association between *FoxD2-AS1* expression levels and clinical outcome of patients with glioma. First, we compared the *FoxD2-AS1* expression in recurrent glioma tissues with non-recurrent tissues obtained from TCGA and found that *FoxD2-AS1* expression is significantly higher in recurrent than in non-recurrent tissues ($p < 0.01$, Fig. 1A). Further Kaplan–Meier and log-rank test analysis showed that *FoxD2-AS1* was associated with OS of glioma patients included in TCGA, GSE16011, and REMBRANDT, patients with *FoxD2-AS1* higher expression had a significant shorter overall survival time than those with *FoxD2-AS1* lower expression (Fig. 1B–D). Moreover, high expression of *FoxD2-AS1* is significantly correlated with poor patient outcome in all tested cases, namely, IDH wildtype and mutant tumors, astrocytoma, and oligodendroglioma (Fig. 1E–H). Collectively, these data suggest that high *FoxD2-AS1* expression is associated with poor outcome in patients with glioma and potentially contributing to the aggressive tumor biology of glioma.

FoxD2-AS1 is contributed to TMZ response of patients with glioma

Evidence of a correlation between *FoxD2-AS1* expression and the clinicopathological status of patients with glioma was searched in TCGA. The results of a chi-square test indicated that methylation of MGMT is significantly less frequent in high *FoxD2-AS1* expression patients. As shown in Table 1, methylation of MGMT was found in 81.6% (266/326) of patients with low *FoxD2-AS1* expression, but in 66.7% (205/307) of patients with high *FoxD2-AS1* expression ($p < 0.001$). Significant correlations were also detected between *FoxD2-AS1* expression and certain clinicopathological features, including IDH mutation status and neoplasm histologic grade (Table 1).

Table 1. Correlation between *FoxD2-AS1* expression and glioma clinic-pathological features in TCGA

Characteristics	Total	*FoxD2-AS1* expression		χ^2	p-value
		Low	High		
Sex	607			0.078	0.780
Male	355	172	183		
Female	252	125	127		
Age (y)	607			0.641	0.423
≤60	473	250	223		
>60	134	47	87		
KPS	364			3.935	0.047*
≥70	317	157	160		
<70	47	16	31		
IDH mutation status	656			0.759	< 0.001***
IDH-mutated	422	265	157		
IDH-wildtype	234	65	169		
ATRX mutation status	652			8.829	0.003**
ATRX-mutated	193	115	78		
ATRX-wildtype	459	215	244		
BRAF V600E mutation status	652			3.089	0.079
BRAF V600E-mutated	3	0	3		
BRAF V600E-wildtype	649	330	319		
MGMT promoter status	633			18.235	< 0.001***
Methylated	471	266	205		
Unmethylated	162	60	102		
Neoplasm histologic grade	607			84.365	< 0.001***
G2	214	152	62		
G3	237	109	128		
G4	156	36	120		
Tumor recurrence	665			15.529	< 0.001***
Yes	153	55	98		
No	512	277	235		

Values are presented as number. TCGA, the Cancer Genome Atlas; KPS, Karnofsky performance score; MGMT, O^6-methylguanine-DNA methyltransferase. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

FoxD2-AS1 is contributed to TMZ resistance of glioma cells

To asked whether *FoxD2-AS1* could modulate the sensitivity of glioma cells to TMZ, the siNC or si*FoxD2-AS1* transduced A172 and U251 cells were treated with different doses of TMZ, respectively. The results of the MTT assay shown that down-regulation of *FoxD2-AS1* reduced the viability of glioma cells after treatment with TMZ (Fig. 2A). Moreover, we found that the IC_{50} values for TMZ in si*FoxD2-AS1* transduced glioma cells were much lower than that in their control cells (56.66 μg/ml in A172 cells, 52.27 μg/ml in A172/siFox cells, 49.15 μg/ml in U251 cells, and 45.31 μg/ml in U251/siFox cells). Next, the colony formation assay, flow cytometry, and western blot analysis were performed to further determine the effect of *FoxD2-AS1* on TMZ resistance of glioma cells. As shown in Fig. 2B, the colony formation for si*FoxD2-AS1* transduced cells was lower than that of the siNC group by treatment with 20 and 40 μg/ml TMZ. The results of flow cytometry analysis revealed that *FoxD2-AS1* knockdown significantly increased glioma cell apoptosis caused by TMZ treatment (Fig. 2C). Furthermore, Western blot analysis showed that TMZ-induced cellular apoptosis was greatly enhanced by *FoxD2-AS1* knockdown compared to NC (Fig. 2D). Above all, it was implied that down-regulation of *FoxD2-AS1* increased the sensitivity of glioma cells to TMZ.

FoxD2-AS1 is contributed to migration and invasion ability of glioma cells

Previous studies reported that the arisen of chemoresistance may associated with the high metastatic ability of cancer cells [20], so we used transwell system to investigate the role of *FoxD2-AS1* on the migration and invasion ability of glioma cells. As shown in Fig. 3, the cell counts of U251 and A172 were significantly de-

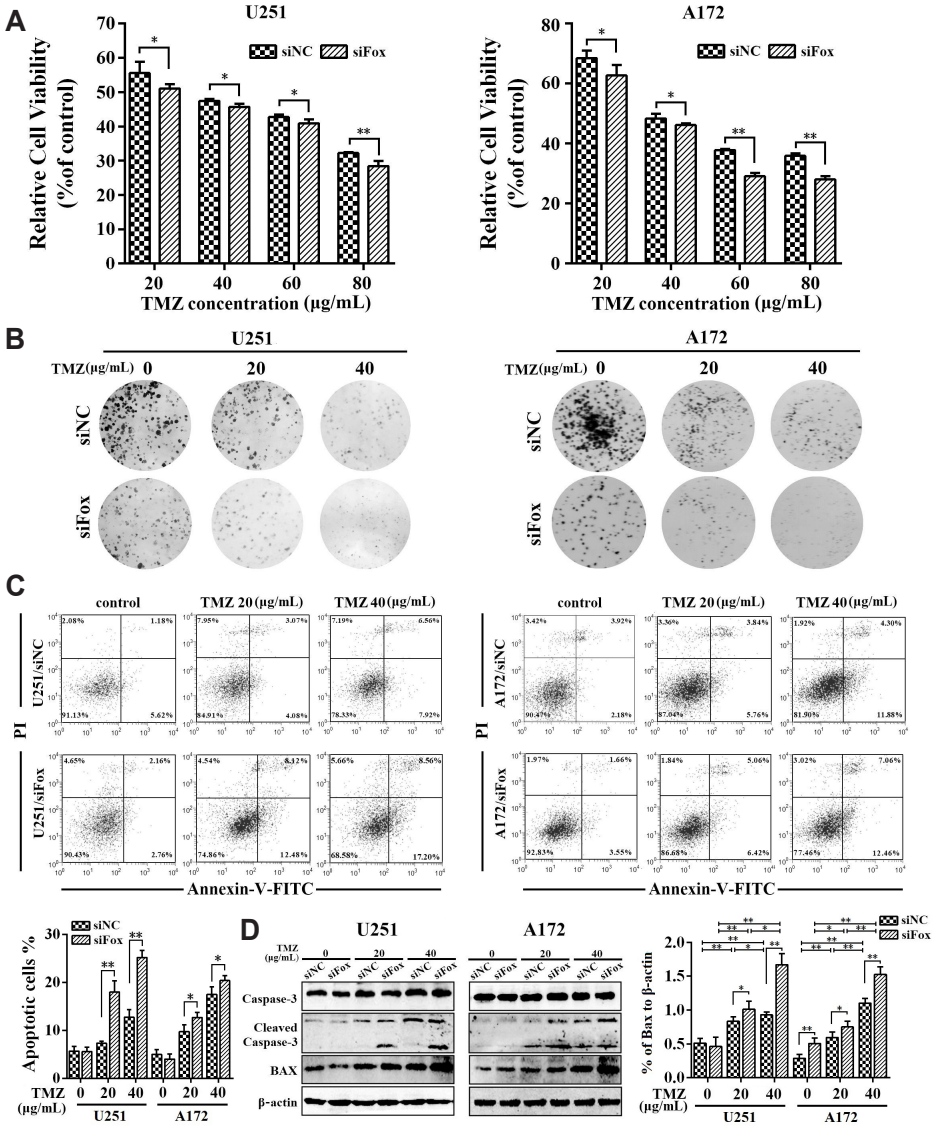

Fig. 2. *FoxD2-AS1* knockdown sensitizes glioma cells to temozolomide (TMZ) treatment. (A) Cell viabilities of si*FoxD2-as1* or siNC transducted glioma cells under treatment of indicated doses of TMZ. (B) Colony formation of si*FoxD2-AS1* or siNC transducted glioma cells after TMZ treatment. (C) Flow cytometry analysis shown the cell apoptosis of si*FoxD2-AS1* or siNC transducted glioma cells after TMZ treatment. (D) Western blot analysis of cleaved caspase-3 and Bax in glioma cells transfected with si*FoxD2-AS1* or siNC after TMZ treatment. siFox, si*FoxD2-AS1*. *p < 0.05, **p < 0.01.

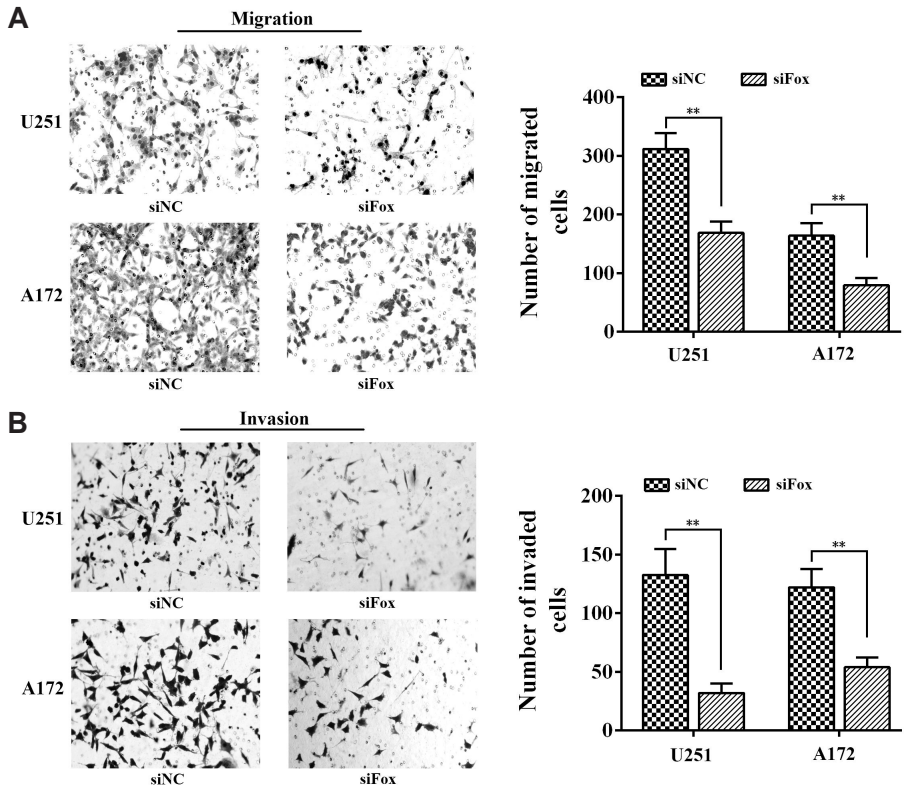

Fig. 3. *FoxD2-AS1* knockdown inhibits the metastatic ability of glioma cells. (A) Effect of *FoxD2-AS1* knockdown on the migration of glioma cells. (B) Effect of *FoxD2-AS1* knockdown on the invasion of glioma cells. Cells were counted after staining with crystal violet (magnification, ×200). Data represent means ± standard deviation. of at least three independent experiments. siFox, si*FoxD2-AS1*. **p < 0.01.

creased in both migration and invasion systems after transfection with si*FoxD2-AS1*, which suggested that *FoxD2-AS1* knockdown could inhibit the metastatic ability of glioma cells.

FoxD2-AS1 mediated TMZ resistance is associated with methylation status of the MGMT promoter region in glioma cells

MGMT promoter methylation is the key mechanism of MGMT gene silencing and predicts a favorable TMZ chemotherapy outcome in glioblastoma (GBM) patients. To further clarify the relationship between *FoxD2-AS1* expression and methylation status of the MGMT promoter in glioma cells, DNA was isolated from siNC or si*FoxD2-AS1* transduced A172 and U251 cells and MGMT methylation was determined by MS-PCR. As shown in Fig. 4A, the ratio of methylated promoter DNA was much higher in si*FoxD2-AS1* transduced than siNC transduced glioma cells. We further detected the MGMT protein expression in siNC or si*FoxD2-AS1* transduced glioma cells by western blot, the results shown that the MGMT protein level was much lower in si*FoxD2-AS1* transduced cells than that in siNC group (Fig. 4B). These results demonstrated that *FoxD2-AS1* mediated TMZ resistance in glioma cells by regulating the methylation status of the MGMT promoter region.

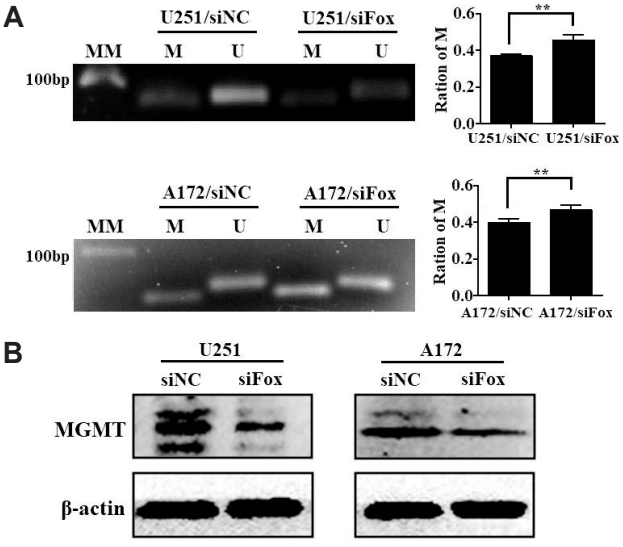

Fig. 4. *FoxD2-AS1* is associated with the methylation status of the O^6-methylguanine-DNA methyltransferase (MGMT) promoter in glioma cells. (A) MS-PCR analysis depicts the methylation status of the MGMT promoter in si*FoxD2-AS1* or siNC transducted glioma cells. The density of each band was quantified using imaging analysis and the relative band density values were calculated as the ratio of methylated MGMT to that of methylated plus un-methylated MGMT. MGMT unmethylated: 92 bp; MGMT methylated: 8l bp. (B) Western blot analysis of MGMT expression in si*FoxD2-AS1* or siNC transducted glioma cells. U, unmethylated; M, methylated; MM, molecular marker; siFox, si*FoxD2-AS1*. **p < 0.01.

DISCUSSION

Although previous studies have demonstrated the overexpression of *FoxD2-AS1* in glioma tissues and cells, the biological role and the clinical relevance of *FoxD2-AS1* in glioma are only emerging. Therefore, we interrogated three glioma gene expression datasets to clarify the clinical relevance of *FoxD2-AS1* in patients with glioma and found that high *FoxD2-AS1* expression was significantly correlated with poor patient outcome. Furthermore, we pointed out the higher expression of *FoxD2-AS1* in recurrent glioma compared with primary glioma. Both Shen and Ni groups [19,21] reported that the expression of *FoxD2-AS1* was upregulated as the grade of glioma increased. Taken together, these results suggest that *FoxD2-AS1* are overexpressed in glioma with more aggressive phenotypes and could function as a prognostic marker for glioma patients.

De novo and acquired resistance to TMZ in glioma cells have emerged as a challenging problem in clinical practice [22]. To date, the bulk of evidence suggests that epigenetic silencing of the MGMT gene through hypermethylation of the cytidine phosphate guanosinedinucleotides in the promoter region is associated with greater response to the TMZ treatment of glioma patients. So we detected the relationship of *FoxD2-AS1* expression and glioma patient's MGMT promoter status. Our results shown that methylation of MGMT is significantly less frequent in high *FoxD2-AS1* expression patients, suggesting the promoting function of *FoxD2-AS1* overexpression on TMZ resistance.

To further determine whether *FoxD2-AS1* could modulate the sensitivity of glioma cells to TMZ, we knocked down the *FoxD2-AS1* expression level in two glioma cell lines using siRNA. Our results shown that downregulation of *FoxD2-AS1* could decrease the cell proliferation and promote the sensitivity to TMZ in a dose dependent manner both in A172 and U251 cells. Further western blot and flow cytometric analysis found that downregulation of *FoxD2-AS1* could promote TMZ induced activation of apoptosis. The metastasis and chemoresistance have been reported to be closely related, the arisen of chemoresistance could promote metastasis of cancer cells and the metastasis contributed to chemoresistanc [23-25]. After transfection of si*FoxD2-AS1*, we found that the migration and invasion ability were significantly decreased in both glioma cell lines. Finally we evaluated whether *FoxD2-AS1* mediated TMZ resistance is associated with methylation status of the MGMT promoter region in glioma cells, and we found that downregulation of *FoxD2-AS1* could induce hypermethylation of the promoter region of MGMT.

We also investigated the methylation status of MGMT promoter and the expression of *FoxD2-AS1* in glioma cells after treated with TMZ. The results shown that neither the methylation status of MGMT promoter nor the level of *FoxD2-AS1* in U251 and A172 cells changed significantly after treatment with 20 and 40 μg/ml TMZ for 48 h. The methylation status of MGMT promoter and the expression of *FoxD2-AS1* were associated with TMZ resistance in glioma cells. A short time (48 h) treatment with TMZ can't induce the chemoresistance of glioma cells; therefore, it is reasonable that 48 h TMZ treatment can't control MGMT methylation or *FoxD2-AS1* expression. In the future, we will develop TMZ-resistant glioma cell lines to further explore the relationship between the expression of *FoxD2-AS1* and the chemoresistance of glioma cell to TMZ.

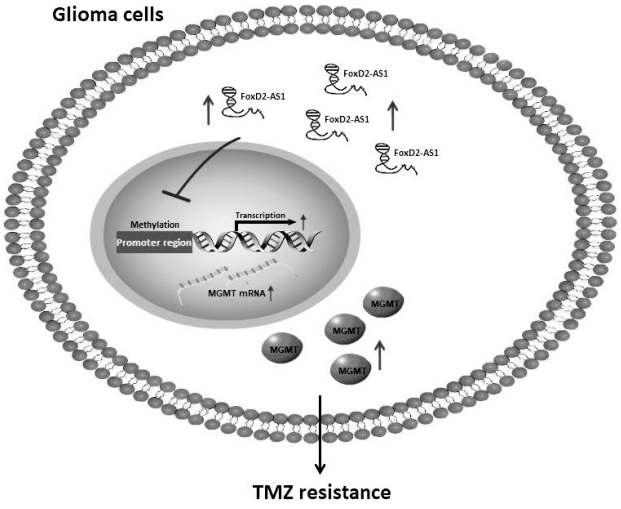

Fig. 5. A diagram summarizing the main findings of this study. *FoxD2-AS1* contributes to temozolomide (TMZ) resistance of glioma cells by regulating the methylation status of the O^6-methylguanine-DNA methyltransferase (MGMT) promoter region.

In summary, our results demonstrated that *FoxD2-AS1* was a clinically relevant LncRNA conferring TMZ resistance in glioma. Downregulation of *FoxD2-AS1* reduced the TMZ resistance of A172 and U251 cells by regulating the methylation status of the MGMT promoter region (Fig. 5). *FoxD2-AS1* is a potential target for developing new therapeutic strategies against drug resistance in glioma.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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