FOXP1 has a low expression in human gliomas and its overexpression inhibits proliferation, invasion and migration of human glioma U251 cells

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Abstract. The present study aimed to examine the clinical characteristics of forkhead box protein P1 (FoxP1) in gliomas and its role in the proliferation, invasiveness, migration and apoptosis of the human glioma U251 cell line. The expression levels of FOXP1 were first studied in operation resection specimens of glioma and normal peripheral brain tissues. The enhanced green fluorescent protein (EGFP) expression vector of FOXP1 was prepared and transfected into U251 cells. MTT, cell invasion, transwell and scratch assays were utilized to investigate the cell growth activity and the rate of apoptosis of the cells was tested by flow cytometry. Western blot analysis and quantitative polymerase chain reaction assays were employed to measure the transfection efficacy. The results revealed that FOXP1 was highly expressed in glioma, as compared with low levels detected in normal brain tissues. Following transfection with pEGFP-N1-FOXP1, the proliferation, invasiveness and migration capabilities of cells significantly decreased, whilst the rate of apoptosis was markedly enhanced ($P<0.01$). Furthermore, the expression of FOXP1 in U251 cells was enhanced ($P<0.01$). In conclusion, the present study indicated that FOXP1 is closely associated with tumorigenesis and development of glioma, as demonstrated by a reduction in the proliferation, migration and invasion of glioma cells upon FOCP1 overexpression.

Introduction

Malignant gliomas, the most common primary brain malignant tumor, are aggressive, highly invasive and neurologically destructive types of cancer. Glioblastoma multiforme (GBM), the most aggressive manifestation of all gliomas, typically affects adults between 45-60 years of age. Despite therapeutic advances in surgical techniques, radiotherapy and chemotherapy, the prognosis of patients with GBM remains discouraging (1-4), largely due to recurrences as a result of tumor growth into adjacent brain regions.

Establishing the molecular basis of tumorigenesis and the progression of malignant gliomas is crucial to improve current therapies and develop novel treatment strategies. It has been suggested that gene expression profiles from glioma specimens may predict patient outcome more accurately than pathological criteria (5,6). One potential candidate gene for therapeutic targeting in glioma is the gene encoding forkhead box protein P1 (FOXP1), a transcription factor that is widely expressed and important in the development of various human tissues (7-11). Of note, FOXP1 has been suggested to be both a tumor suppressor candidate and potential oncogene, due to its differential expression levels in distinctive types of tumors. Several authors have suggested that FOXP1 is an oncogene with high expression levels and the protein overexpression is often identified in many types of B-cell lymphomas associated with a poor outcome (12-15). By contrast, loss of FoxP1 has been observed in endometrial (16), prostate (17,18) and renal cell carcinoma (19), and the loss of FOXP1 in breast cancer has been associated with lower survival rates (17,20).

To the best of our knowledge, associations between FoxP1 expression and the clinical features of gliomas, to determine its clinicopathological significance, have not been investigated. Therefore, the present study investigated the role of FOXP1 in patients with gliomas and whether it may be a potential target for novel therapeutic strategies. FOXP1 gene and protein expression in glioma samples were investigated and compared with those in tissue adjacent to the tumors. Then, an exogenous expression vector was transfected into the glioma cell line U251, in an attempt to elucidate whether its overexpression alters the biological characteristics of glioma cells.

Materials and methods

Patients. The patient population consisted of 25 adults (16 male, 9 female; mean age at sampling, 54.3 years). Written informed consent was obtained from all patients, and the study was approved by and performed according to the guidelines of the Institutional Review Board of the General Hospital of Tianjin Medical University (Tianjin, China; approval no. 85-188). GBM was verified in the histological specimens between

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July 2008 and July 2012 by a neuropathologist according to the World Health Organization criteria. All 25 cases were classified as grade 4, with 18 cases of GBM and 7 cases of glioblastoma with oligodendroglioma.

Region-specific specimen collection. Deep-seated tumors were removed using an intraoperative navigation system (Brainlab, Feldkirchen, Germany) that minimized invasiveness and maximized patient safety and accurate tumor resection. Brain tissue samples were collected from the resection zone, categorized as peripheral normal brain, tumor marginal tissue or tumor core, and stored in liquid nitrogen as described previously (21).

Quantitative polymerase chain reaction (qPCR). Fresh-frozen tumor and peritumoral tissue samples were collected from 25 patients with glioma at our hospital. Total RNA from a subset of these fresh frozen tissue samples was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Total RNA extraction, quality control and 1-step qPCR were performed as previously described (22,23). FOXP1-specific oligonucleotide primers: forward, 5'-TGCAGAGCAGCCACGCCTAC-3' and reverse, 5'-CCGTTCAGCTCTTCCCGTATT-3', were designed to give a 148-base pair (bp) PCR product. The levels of GAPDH mRNA were used to standardize the mRNA data, using GAPDH primers obtained from Invitrogen Life Technologies, and the melting curves were analyzed. Amplification conditions consisted of 30 min at 42˚C for reverse transcription and 2 min at 94˚C for Taq activation, followed by 35 cycles at 94˚C for 20 sec, 58˚C for 20 sec and elongation at 72˚C for 30 sec.

Expression of FOXP1 protein in tissue samples. Western blot analysis was used to determine protein levels in the cancerous tissues. Briefly, frozen tissues were homogenated with lysis buffer, centrifuged at 4˚C for 30 min (9,000 x g). The supernatant was collected and the bicinchoninic acid assay was used to determine protein concentration. A 10% polyacrylamide gel was prepared to load protein samples and 5% non-fat dry milk was added to block the non-specific antigen. The primary antibodies (Rabbit anti-human ATP binding cassette E1 (ABCE1) polyclonal antibody; Abcam, Cambridge, MA, USA) and the secondary antibody HRP-conjugated goat anti-rabbit IgG (H&L) antibody (Cell Signaling Technology, Beverly, MA, USA) were applied.

Cell lines and culture conditions. The human glioma cell line (U251) was purchased from American Type Culture Collection (ATCC; Mannassas, VA, USA). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 10 U/l penicillin G and 100 mg/l streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

PCR Amplification and cloning of the FOXP1 cDNA. A cDNA encoding full-length FOXP1 was amplified by PCR in a 50 μl reaction containing 1 μl human fetal brain cDNA library (1:10 dilution; Clontech Laboratories, Mountain View, CA, USA), oligonucleotide primers (forward, 5'-CTCGGATTCATGATG CAGGAATCTGCGACAGAGAC AATAAGC-3' and reverse, 5'-CTCGAATTCTCATTCCAGATCTTCAGATAAAGG CTCTTCTTC-3') at 0.5 μmol/l, deoxynucleotide triphosphates at 200 μmol/l, 1X reaction buffer and 2.5 units Pfu Turbo polymerase (Stratagene Inc., La Jolla, CA, USA). The thermal cycler was programmed as follows: 95˚C for 1 min, followed by 26 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 3 min, followed by a final 10 min extension at 72˚C. The PCR-generated full-length FOXP1 was digested with BamHI and EcoRI, ligated together and cloned to create the eukaryotic expression construct p-enhanced green fluorescence protein (EGFP)-N1-FOXP1. Plasmid DNA was prepared and the construct insert was fully sequenced.

Lipofectamine 2000 was used for transfection of $2x10^5$ cells/well (six-well plate) and 5 μg of recombinant plasmids. Empty vector was used as a control. An inverted fluorescence microscope (Axiovert 200; Carl Zeiss, Göttingen, Germany) was used for observation and obtaining images.

MTT assay. A total of $1x10^4$ U251 cells were cultured in 96-well tissue-culture plates overnight and then transfected with plasmids as described above. Following 1-5 days, glioma cells were incubated for 3 h in 100 μl MTT (0.1 mg/ml; Sigma, St. Louis, MO, USA). Cells were resuspended in 200 μl isopropanol (to dissolve the formazan) and the optical density (OD) of the solution was determined using a spectrometer (Fluostar Optima; BMG Labtech, Ortenberg, Germany) at an incident wavelength of 570 nm. Cell viabilities in experimental wells were expressed as a percentage of the viability in the control well.

Invasion assay. Invasion assay was performed using a Boyden chamber system (Neuro Probe) with a fibronectin-precoated (0.5 mg/ml) polycarbonate membrane (8 μm pore size). The lighter side of the polycarbonate membrane was precoated with 250 μg/ml Matrigel® (BD Biosciences, Franklin Lakes, NJ, USA). The bottom chambers were filled with 30 μl RPMI-1640 medium containing 2% bovine serum albumin (BSA), while the top chambers were filled with 50 μl RPMI-1640 serum-free medium containing 0.2% BSA. Cells ($5x10^4$/well) were added to the top chamber and incubated for 15 h in an incubator at 37°C with 5% CO_2. Three independent experiments were performed in triplicate. The cells were fixed in methanol and stained with haematoxylin. The top surface of the membrane was gently scrubbed with a cotton bud, the cells that had migrated to the lower side of the membrane were counted under the microscope (Carl Zeiss) and the numbers of migrated cells were calculated as the mean ± standard deviation.

Adhesion assay. Matched quantities of pEGFP-N1-FOXP1- and control empty vector-transfected U251 cells ($3x10^4$) were plated onto a Matrigel-precoated (50 μg/ml) 96-well plate in triplicate. The cells were washed at 30, 60 and 120 min to remove non-adherent cells. Following washing, the adhered cells were quantified using an MTT assay. The relative OD was determined at 570 nm using Wellscan MK3 ELISA (Ani Labsystems Ltd. Helsinki, Finland) and a 450 nm reference filter. The OD values reflected the proportion of cells in the Matrigel-coated 96-well plate.

Wound healing assay. The wound healing assay was conducted as previously described (24). The distance of wound closure

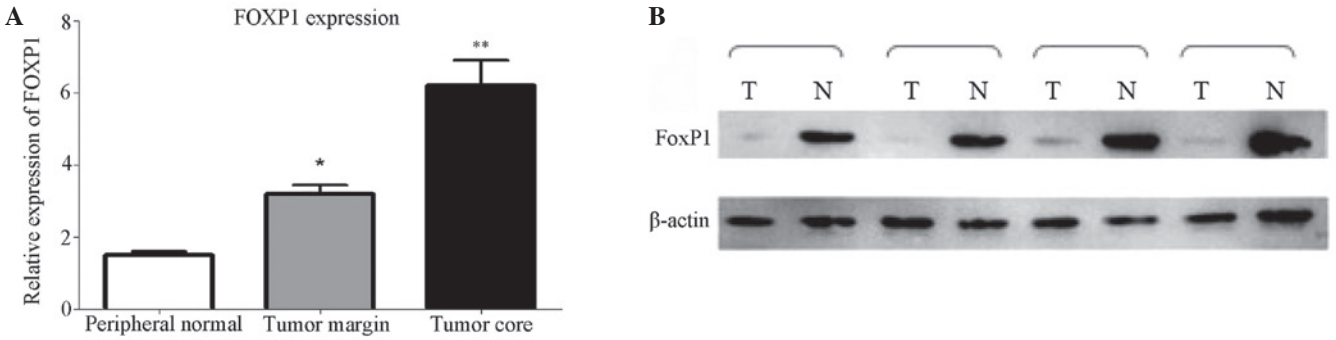

Figure 1. FOXP1 expression in surgically defined regions. A) quantitative polymerase chain reaction analysis of FOXP1 expression in samples from different regions of gliomas; B) expression of FOXP1 detected by western blot analysis. *P<0.05 compared with normal tissues. **P<0.01 compared with normal tissues; N, normal peripheral tissues; T, tumor tissues; FOXP1, forkhead box protein P1.

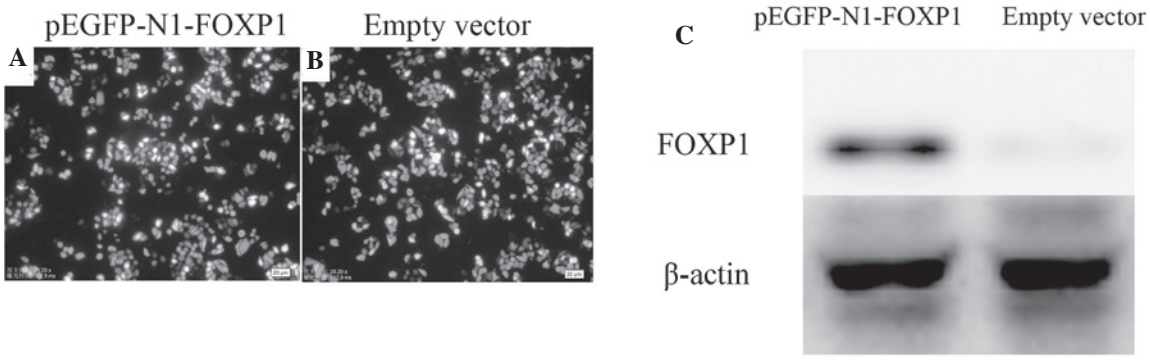

Figure 2. Expression of GFP in U251 cells 48 h following transfection. (A) pEGFP-N1-FOXP1 group (magnification, x100); (B) empty vector group (magnification, x100); (C) western blot analysis of FOXP1 protein levels in the two groups. GFP, green fluorescent protein; FOXP1, forkhead box protein P1.

(compared with the control at 0 h) was measured at three independent wound sites per group. The relative cell motility was calculated as the wound width at t = 0 h minus the wound width at t = 24 h, as indicated. Values from at least three independent experiments were pooled and expressed as the mean ± standard deviation.

Statistical analysis. All experiments were repeated at least three times. Student's t-test was used to evaluate the differences between experimental and control groups. The data were analyzed by one-way analysis of variance with SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). The frequencies of FOXP1 expression among cancer samples were analyzed by the χ^2 test with modification by the Fisher's exact test to account for frequency values <5. All of the P-values reported are two-sided. P<0.05 was considered to indicate a statistically significant difference between values.

Results

Expression of FoxP1 in human glioma tissues. In 25 sets of tissue samples collected from different brain regions, expression of the FOXP1 gene was significantly downregulated in tumor core samples (P<0.05) compared with peripheral normal brain tissue, as determined by qPCR, although expression decreased progressively in samples obtained from more distal areas (Fig. 1A). Immunoblotting confirmed the reduction in FOXP1 protein expression in malignant gliomas was consistent with the reduced transcript levels (Fig. 1B).

Transfection efficiency. As demonstrated in Fig. 2A and B, under fluorescence microscopy observation, the transfection efficiency of the two groups (pEGFP-N1-FOXP1 and empty vector) was satisfactory, with all exceeding 80%.

Western blot analysis demonstrated that transfected pEGFP-N1-FOXP1 significantly increased exogenous expression of FOXP1 in U251 cells as compared with that in the empty vector control (Fig. 2C).

FOXP1 inhibited proliferation and increased apoptosis in U251 cells. The MTT assay revealed that the proliferation of cells in the empty vector group increased almost 4-fold from 24 to 96 h following transfection, while the proliferation in the pEGFP-N1-FOXP1 group was relatively slow, which increased only 1-fold in 72 h (P<0.01; Fig. 3A). Flow cytometric analysis demonstrated that the rate of apoptosis increased significantly (P<0.01) in pEGFP-N1-FOXP1-transfected cells as compared with that of the empty vector control (Fig. 3B and C).

Effect of exogenous expression of FOXP1 on the invasive, motive and adhesive abilities and proliferation of U251 cells. To study the role of FOXP1 on cell invasion, motility and

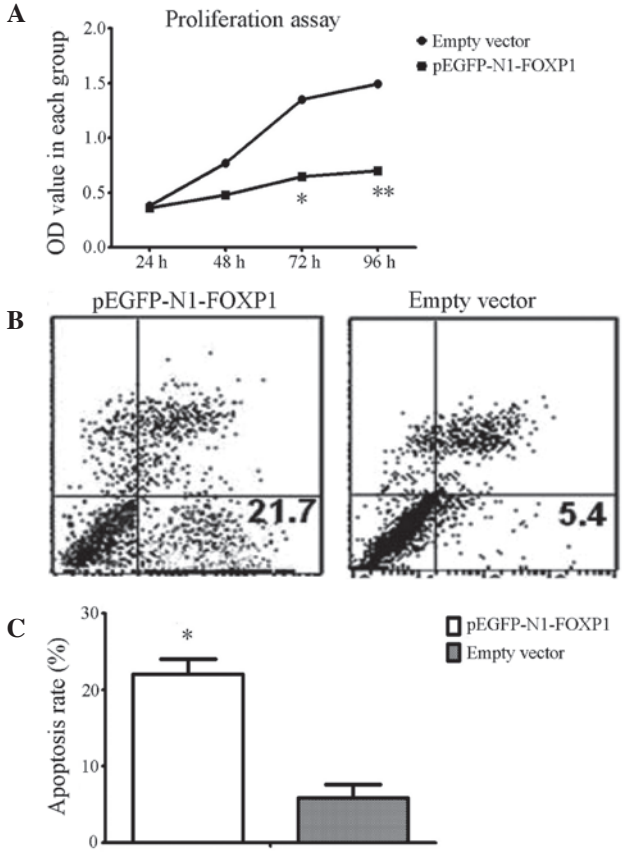

Figure 3. Proliferation assay and analysis of apoptosis. A) Growth curve as determined by an MTT assay. B) FOXP1 increased the rate of apoptosis of U251 cells. C) Quantification of the rate of apoptosis of each group of cells. *P<0.05, **P<0.01, compared with the empty vector. OD, optical density; GFP, green fluorescent protein; FOXP1, forkhead box protein P1.

adhesion, the major characteristics of metastasis, all the assays were performed 48 h following transfection. In an invasion assay, the number of cells that migrated to the bottom side of the membrane on a chamber, where the cells were seeded, was calculated (Fig. 4A). The data demonstrated that the number of migrated cells in the transfected FOXP1 group decreased dramatically compared with that in the empty vector control group (Fig. 4B). These results suggested that the overexpression of FOXP1 significantly suppressed the migration ability of U251 cells.

To examine whether the depletion of FOXP1 had any effect on the motive ability of the cells, a wound-healing experiment was performed using U251 cells transfected with FOXP1 or the empty vector. The data revealed that there was a significant difference in the relative wound closure at 15 and 24 h for the two groups (P<0.01, Fig 4C and D).

The elevated FOXP1 levels that correlated with glioma metastasis in the tumor patients prompted us to examine whether FOXP1 had an effect on cell adhesion to the extracellular matrix (ECM). For this purpose, a cell adhesion assay was performed. The results demonstrated that the cells, when transfected with FOXP1, had a characteristically low adhesion ability as compared with the empty vector control (Fig. 4E). The results were consistent at different time-points, including at 30, 60 and 120 min, respectively. These results indicated that FOXP1 may inhibit tumor cell adhesion to the ECM. In

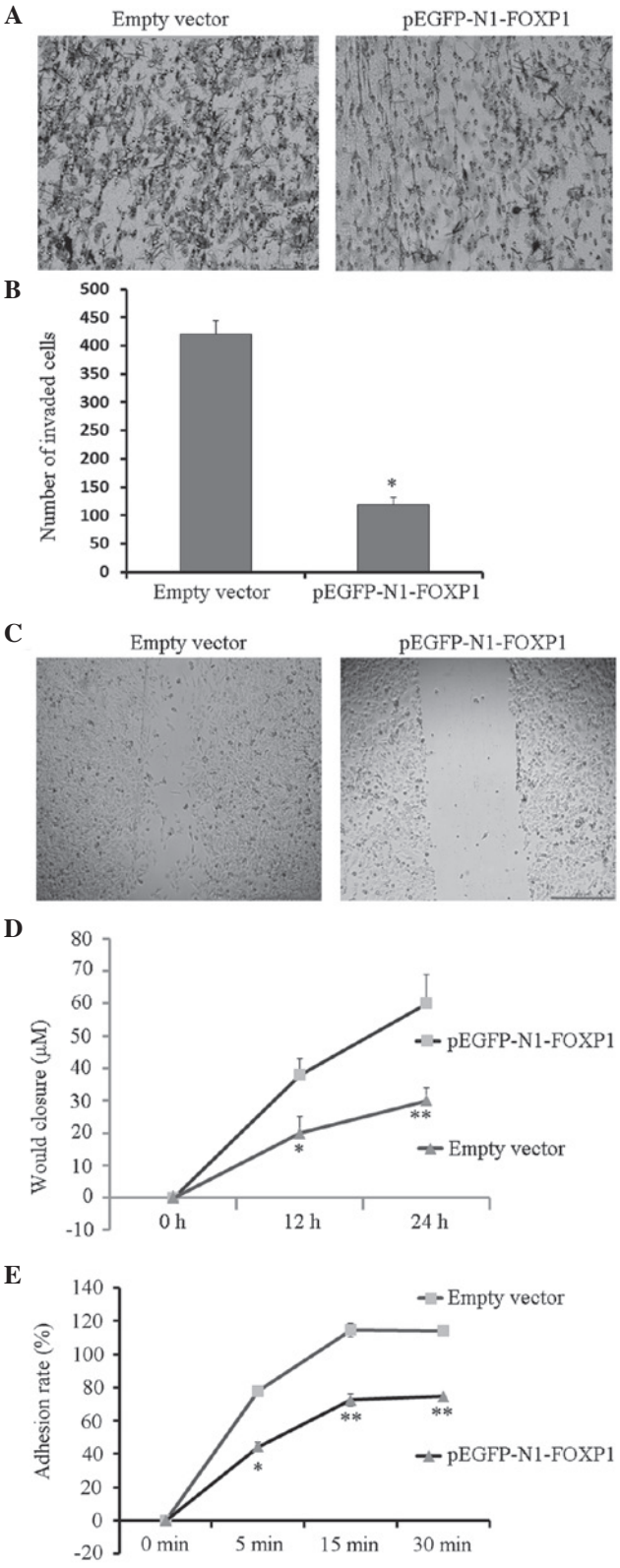

Figure 4. Exogenous expression of FOXP1 suppresses invasion, motility and adhesion of U251 cells. A) Invasion of empty vector cells and FOXP1-transfected U251 cells through reconstituted basement membrane. B) The number of invaded cells is expressed as the mean ± SD from three independent experiments. C) Cell motive capability was determined by a wound healing assay in empty vector cells and FOXP1-transfected U251 cells. D) The distance of wound closure was measured at three independent wound sites/group. E) Cell motility results were quantitated, with the graph presenting the mean ± SD of values from a representative experiment performed in triplicate. *P<0.05 compared with the control group. **P<0.01 compared with the control group. SD, standard deviation; GFP, green fluorescent protein; FOXP1, forkhead box protein P1.

conclusion, the data suggested that FOXP1 exerts its effects on metastasis by inhibiting invasion, migration and adhesion in U251 cells.

Discussion

FOXP1 was first identified in a study screening for glutamine-rich transcription factors in B cells (25). The full length of the human FOXP1 gene was initially cloned with a monoclonal antibody (JC12) that recognized a differentially expressed protein in malignant and normal B cells (26). As a member of the FOXP subset of 'forkhead' (Fox) transcription factors, FOXP1 has multiple functions, including the regulation of B-cell development (10), lung and esophagus development (11), monocyte and macrophage differentiation (27) and cardiac development (28). Of note, separate studies investigating different tumor types have suggested that FOXP1 may act as either a tumor suppressor or an oncogene (25). Therefore, the present study aimed to verify the association between FOXP1 expression and the clinical parameters of malignant gliomas.

In the present study, it was demonstrated for the first time, to the best of our knowledge, that FOXP1 mRNA and protein expression in glioma tumors is significantly elevated compared with matched peripheral normal tissue. The immunostaining results are consistent with the qPCR results, which suggested that the expression of FOXP1 may be important in the tumorigenesis and progression of gliomas. Previous studies demonstrated that the positive expression of FOXP1 in malignant endometrium was linked with deep myometrial invasion and poor differentiation (16). Furthermore, FOXP1 expression was associated with the postoperative Gleason score of prostate cancer (29) and with tumor grading of renal cancer (19), whereas no significant correlations were identified between FOXP1 expression and other clinical characteristics in these patients. Of note, the loss of FOXP1 protein expression in breast carcinoma is closely associated with poor patient outcome (17). This accumulative evidence suggests that FOXP1 may act as a tumor suppressor gene in human cancer. The results of the present study support these findings, demonstrating that low expression levels of FOXP1 protein are more common in glioma tissues than in normal tissues.

Establishing the factors responsible for the development, proliferation, metastasis and recurrence of brain tumors may facilitate the identification of potential diagnostic markers or targets for new therapeutic regimens. In the present study, the aim was to to investigate the potential role of FOXP1 and its possible regulatory mechanism in glioblastoma. Therefore, an exogenous expression vector of FOXP1 was established and transfected it into the glioma cell line U251 in order to identify its role in the proliferation, migration and invasion of U251 cells. The results further confirm that FOXP1 acts as a tumor suppressor gene in glioma and is crucial in controlling glioma cell invasion, migration and motility. These results are consistent with evidence from earlier studies, which suggested that targeting FOXP1 may be a strategy for the development of novel glioma therapies.

To the best of our knowledge, the present study was the first report of the differential expression of FOXP1 in gliomas, indicating that FOXP1 may be used as a novel therapeutic target for glioma treatment. The results demonstrated elevated expression levels of FOXP1 in malignant glioma tissue, and that this exogenous high expression significantly reduced the proliferation, migration and invasion activity of glioma cells. Further studies are required to elucidate the underlying mechanisms of the involvement of FOXP1 in glioma carcinoma.

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