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·*Original Article*·

Role of DJ-1-induced PTEN down-regulation in migration and invasion of human glioma cells

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[Abstract] Background and Objective: DJ-1, a suppressor of PTEN, promotes metastasis of different tumors, but its function and mechanisms in glioma metastasis remain unclear. This study aimed to investigate the effect of the DJ-1 protein on the migration and invasion of human glioma cells, and to explore potential mechanisms. **Methods:** The eukaryotic expression vector pEGFP/DJ-1 and small interfering RNA (siRNA) were constructed and transfected into human glioma SWO-38 cells. The expression of DJ-1 and PTEN in SWO-38 cells were detected by Western blot. Cell migration and invasion were detected by transwell assay. **Results:** After transfection of pEGFP/DJ-1, the expression of DJ-1 (1.28 ± 0.15 vs. 0.89 ± 0.04, $P < 0.05$) and focal adhesion kinase (FAK) phosphorylation (0.76 ± 0.12 vs. 0.51 ± 0.04, $P < 0.05$) were increased, whereas the expression of PTEN (0.74 ± 0.2 vs. 1.04 ± 0.14, $P < 0.05$) was suppressed. After transfection of DJ-1 siRNA, both DJ-1 (0.33 ± 0.04 vs. 0.88 ± 0.06, $P < 0.05$) and p-FAK levels (0.33 ± 0.01 vs. 0.44 ± 0.05, $P < 0.05$) were decreased, but PTEN expression (1.1 ± 0.06 vs. 0.81 ± 0.12, $P < 0.05$) was increased. Transwell assay data showed that pEGFP/DJ-1 transfection promoted SWO-38 cell migration (57.2 ± 6.50 vs. 40.4 ± 5.0, $P < 0.05$) and invasion (54.6 ± 4.9 vs. 27 ± 6.7, $P < 0.05$), whereas DJ-1 siRNA transfection inhibited SWO-38 cells migration (54.4 ± 6.9 vs. 73.4 ± 7.6, $P < 0.05$) and invasion (44.6 ± 5.8 vs. 69.2 ± 9.2, $P < 0.05$). **Conclusion:** Over-expression of DJ-1 promotes SWO-38 cell migration and invasion possibly through the DJ-1 and the PTEN/FAK pathway.

Key words: DJ-1, PTEN, migration, invasion, glioma

DJ-1, was firstly reported as a mitogen-dependent oncogene, was identified by Nagakubo *et al.* in 1997 [1]. Many studies have found that DJ-1 participates in cellular biological processes of various tumors, such as proliferation and differentiation, arrest of cell cycle[2], and autophagy[3]. In addition, DJ-1 also has functions of anti-oxidation and molecular chaperones[4]. DJ-1 gene is expressed widely in multiple human tissues. In normal human brain, DJ-1 is expressed mainly in glial cells in the cerebral cortex and in neurons in the substantia nigra and corpus striatum[5]. Previous researches focused mostly on DJ-1 mutations and abnormal expression regulation that lead to degeneration of the nervous system. Recent studies showed that DJ-1 was closely related with tumorigenesis, progression, metastasis, and prognosis. DJ-1 is highly expressed in multiple tumor tissues. In 2005, Kim *et al.*[6] found that in breast cancer cells, DJ-1 negatively regulated PTEN expression, promoted phosphorylation of PI3K/Akt, and activated cell proliferation and transformation. It has been confirmed that PTEN, the first tumor suppressor gene with phosphatase activity, plays important roles in tumor invasion and metastasis. In vivo, PTEN interacts directly with FAK and induces FAK dephosphorylation, which effectively inhibits integrin-mediated cell adhesion and migration[7]. In 2008, Yuan *et al.*[8] reported high nuclear DJ-1 expression in highly metastatic esophageal squamous cells; in 2009, Wu *et al.*[9] found that DJ-1 was involved in metastasis of liver cancer. Therefore, DJ-1 can be used as a marker of cancer metastasis and can be a potential therapeutic target.

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However, the detailed mechanism is not fully understood. Glioma is the most common malignancy in the central nervous system. As mostly taking an invasive growth pattern, glioma is treated with surgical resection, radiotherapy, chemotherapy, and gene therapy, but the treatment efficacy is unsatisfactory[10]. Therefore, determining the pathogenesis of glioma and finding key factors involved in tumor migration and invasion are essential for clinical treatment. PTEN is a key regulator for glioma development. However, its upstream regulators have not been delineated. DJ-1 suppresses PTEN expression, which is important for inhibiting tumorigenesis and metastasis. Whether DJ-1 regulates glioma metastasis through inhibiting the PTEN/FAK pathway is unclear. In this study, we aimed to determine the mechanism of DJ-1 in promoting glioma metastasis. Recombinant plasmid pEGFP/DJ-1 was transfected into human glioma cell line SWO-38 to induce DJ-1 overexpression. DJ-1 expression in SWO-38 cells was knocked down by small interfering RNA (siRNA) targeting DJ-1. In vitro migration and invasion was measured to test whether DJ-1 was involved in this process through the PTEN/FAK pathway.

Materials and Methods

Materials

Cell line and culture

Human glioma cell line SWO-38 was established in our department in 1985 [11]. SWO-38 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Hyclone) and incubated in a 37℃, 5% CO_2 humidified incubator.

Reagents

Antibodies to DJ-1 and PTEN were purchased from Cell Signaling Technology, Inc. Antibodies to FAK and phospho-FAK were purchased from Santa Cruz Biotechnology, Inc. Lipofectamine 2000™ was purchased from Invitrogen Inc. Transwell chamber was purchased from BD Inc.

Plasmids

Eukaryotic expression vector pEGFP/DJ-1 was constructed in our lab. The open reading frame (ORF) of human DJ-1 was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from HeLa cells.

The sequence of forward primer was 5'-GGCGAATT-CATGGCTTCCAAAAGAGCT-3' and that of reverse primer was 5'-GCGGGTACCGTCTTTAAGAACAAGTGGA-3'. *Eco*R I restriction sites was introduced at the 5' end and *Npn* I at the 3' end. DJ-1 cDNA was cloned into the vector pEGFP-C2 to prepare recombinant plasmid pEGFP/DJ-1, which was confirmed by restriction enzyme digestion and DNA sequencing.

Small interfering RNA

siRNAs targeting human DJ-1 were designed and synthesized by Shanghai GenePharma Co., Ltd. The sequence of sense strand was 5'-GCUCUGUUGGCUCAU-GAAATT-3'; and anti-sense strand was 5'-UUUCAUGAGCC-AACAGAGCAG-3'. Negative control siRNAs were non-specific random sequences gifted by Shanghai GenePharma Co., Ltd. The sequence of sense strand was 5'-UUCUCCGAACGUGUCACGUTT-3' and anti-sense strand was 5'-ACGUGACACGUUCGGAGAATT-3'.

Methods

Cell transfection with pEGFP/DJ-1

Fresh medium was replaced 24 h before transfection. Transfection was conducted according to the manufacturer's instructions. Briefly, pEGFP/DJ-1 was diluted with culture medium without fetal calf serum and mixed with Lipofectamine 2000™ for 5 min, then incubated at room temperature for 20 min and dropped into cells. Cells were collected 24 h later, and DJ-1 expression was detected by Western blot. The cells without transfection or transfected with empty plasmid pEGFP-C2 were used as controls.

Cells transfection with DJ-1 siRNA

Using the same methods in the aforementioned subsection, DJ-1 siRNA was diluted to 20 nmol/L, mixed with Lipofectamine 2000™, and transfected into SWO-38 cells. Cells were collected 24 h later, and DJ-1 expression was detected by Western blot. The cells transfected with 20 nmol/L scrambled siRNA were used as controls.

Western blot

The day before transfection, SWO-38 cells were seeded into 6-well plates to obtain 60% to 70% confluence. Total protein was extracted with the Protein Extraction solution from Beyotime Institute of Biotechnology. Protein concentration was quantified by the Bradford method with the Protein quantification kit from Shanghai Biocolor BioScience & Technology Company. Conventional SDS-PAGE gels (12% separating gel and 5% stacking gel) were used to resolve proteins. After electrophoretic transmembrane, polyclonal rabbit anti-human DJ-1 and anti-human PTEN antibodies (1:1000), rabbit anti-human FAK and phospho-FAK antibodies (1:500), rabbit anti-human GAPDH antibody (1:1000) were added into primary antibody solution (Beyotime Institute of Biotechnology). The membrane was incubated at 4℃ overnight. After washed with TTBS three times, the membrane was incubated with secondary antibody solution (1:4000) at room temperature for 1 h. After three times of TTBS wash, the membrane was incubated with Super Signal west PicoChemiluminescent Substrate (Thermo) and then exposed with X-ray film (FUJI).

In vitro cell migration and invasion assay

Cells at logarithmic growth phase were cultured in serum-free RPMI-1640 medium for 24 h. For invasion assay, transwell chambers were treated with 20 μL culture medium containing 6.7% Matrigel™ so that the inner surface of the filter membrane (diameter, 6.5 mm; pore diameter, 4 mm) was coated with extracellular matrix. Cells were treated with 2.5% trypsin and suspended in serum-free RPMI-1640 medium at a concentration of 1×10^6/L. Cell suspension (200 μL) was added into each upper chamber and 600 μL RPMI-1640 medium containing 10% FBS was added into each lower chamber. Then the transwell chambers were incubated in a 37℃, 5% CO_2, humidified incubator for 24 h. For cell migration assay, 100 μL cell suspension (2×10^5/L) was added directly into the transwell chamber (not coated with extracellular matrix) and incubated for 16 h. The cells on the inner surface of the filter membrane were removed. The cells on the lower surface of the membrane were stained with crystal violet, and counted in five random fields under 200-fold light microscope.

Statistical analysis

All data are expressed as mean ± standard deviation. Analysis of variance was conducted by SPSS13.0 software package. The difference was considered statistically significant when $P < 0.05$.

Results

Amplification of DJ-1 cDNA

RT-PCR products were resolved on 1% agarose gel. A single 600 bp band was observed (Figure 1A), which corresponded to human DJ-1 cDNA.

Confirmation of recombinant plasmid

The validity of pEGFP/DJ-1 was confirmed by *Eco*R I/*Npn* I restriction enzyme digestion. A 600 bp band was observed (Figure 1B), which was consistent with the RT-PCR result. The reading frame was confirmed by DNA sequencing.

Detection of DJ-1, PTEN, FAK, and phospho-FAK by Western blot after pEGFP/DJ-1 transfection

SWO-38 cells was transfected with pEGFP/DJ-1 for 24 h. Compared with those in the cells transfected with empty plasmid pEGFP-C2, DJ-1 expression was up-regulated in SWO-38 cells transfected with pEGFP/DJ-1 ($P < 0.05$); PTEN expression was down-regulated ($P < 0.05$), and phospho-FAK was up-regulated ($P < 0.05$) (Figure 2).

In vitro migration and invasion assay of SWO-38 cells after pEGFP/DJ-1 transfection

Migration and invasion of SWO-38 cells overexpressing DJ-1 were promoted. The mean numbers of migrated cells and invaded cells were significantly higher in pEGFP/DJ-1 group than in non-transfection group and pEGFP-C2 group ($F = 19.215$, $P < 0.01$; $F = 17.419$, $P < 0.01$) (Figure 3).

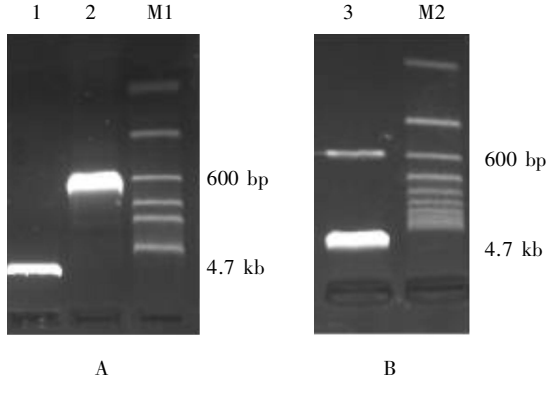

Figure 1 The construction and identification of pEGFP/DJ-1 recombinant vector

A, RT-PCR amplification of DJ-1 cDNA (lane 1: empty vector pEGFP-C2; lane 2: DJ-1 cDNA; lane M1: DNA marker). B, *Eco*R I/*Npn* I restriction enzyme digestion of recombinant vector pEGFP/DJ-1 (lane 3: recombinant vector pEGFP/DJ-1 digested by *Eco*R I/*Npn* I; M2: DNA marker). RT-PCR amplification and enzyme digestion confirmed that pEGFP/DJ-1 had been constructed successfully.

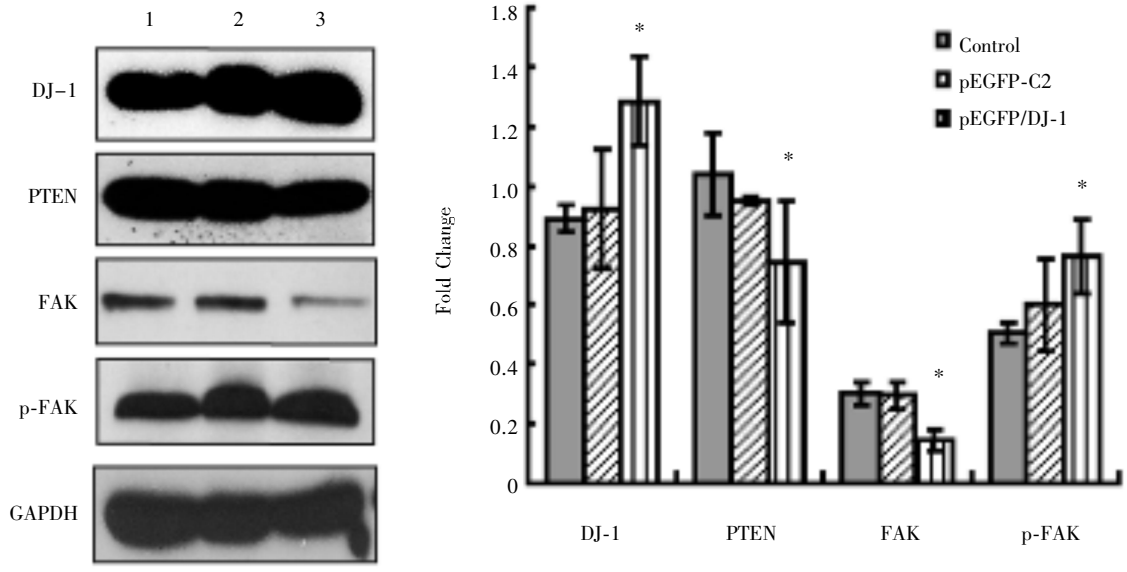

Figure 2 DJ-1, PTEN, FAK, and p-FAK expression in SWO-38 cells after transfecting pEGFP/DJ -1 detected by Western blot

Lane 1, untransfected SWO-38 cells; lane 2, SWO-38 cells transfected with empty vector pEGFP-C2; lane 3, SWO-38 cells transfected with recombination vector pEGFP/DJ-1. Each bar in the left panel represents relevant expression (mean ± standard deviation) of protein in cells. After pEGFP/DJ-1 transfection, DJ-1 expression was up-regulated, PTEN expression was down-regulated, and phospho-FAK was up-regulated. *P < 0.05, vs. control and pEGFP-C2-transfected cells.

Detection of DJ-1, PTEN, FAK, and phospho-FAK expression in SWO-38 cells by Western blot after DJ-1 siRNA transfection

SWO-38 cells were transfected with DJ-1 siRNA for 24 h. Compared with the scrambled siRNA transfected control cells, DJ-1 expression in siRNA-transfected cells was down-regulated (P < 0.01), PTEN expression was up-regulated (P < 0.05), and FAK phosphorylation was down-regulated (P < 0.05) (Figure 4).

Migration and invasion of SWO-38 cells after DJ-1 siRNA transfection

After DJ-1 siRNA transfection, DJ-1 expression was suppressed, the migration and invasion of SWO-38 cells were inhibited. The mean numbers of migrated cells and invaded cells were significantly lower in DJ-1 siRNA group than in scrambled siRNA group (F = 12.459, P < 0.05; F = 23.516, P < 0.05) (Figure 5).

Discussion

Glioma cells are characterized by migration and invasion, and can invade from primary lesion to normal brain tissue. Because of the invasive growth pattern, glioma is resistant to treatment and hard to be resected completed [10]. With the development of molecular biology, gene therapy has attracted much attention. Identifying appropriate targets is essential for successful gene therapy of glioma. The most common therapeutic target of multiforme glioblastoma is PTEN, which localizes in human chromosome 10 and shows abnormal expression. Recently, many studies confirmed that PTEN can inhibit the invasion of glioma cells[12-14]. Tamura *et al.*[7] found that the inhibition of migration and invasion of glioma by PTEN was significantly correlated with the decreased phosphorylation of FAK and p130Crk substrates, that is, PTEN can promote FAK dephosphorylation and then inhibit the migration and invasion of glioma.

DJ-1 was initially defined as an oncogene candidate. DJ-1 is expressed highly in several tumors, and can also be detected in the serum of some patients. DJ-1 gene localizes on human chromosome 1p36 and spans 24 kb comprising of 8 exons, of which exon 1A and 1B are non-coding. DJ-1 protein is coded by exons 2–7, contains 189 amino acids, and weights about 20 kD. DJ-1 protein is highly conserved and belongs to the ThiJ/Pfpl protein superfamily[15]. In 2009, researchers found that DJ-1 reduced the phosphatase activity of PTEN and interfered with cell cycle and inhibited apoptosis by inhibiting the PTEN/Akt pathway [16]. We hypothesized that DJ-1 down-regulates the expression of

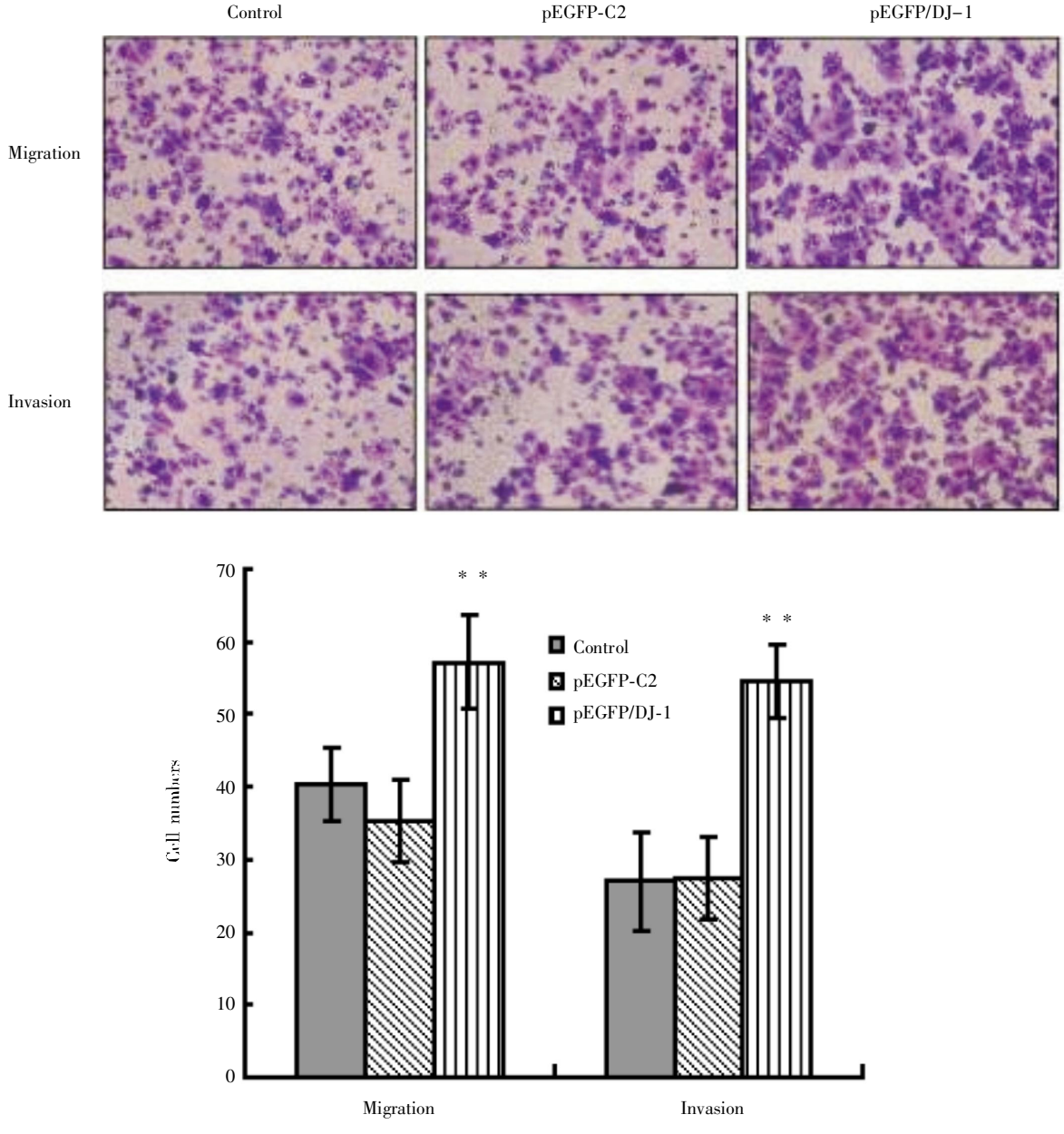

Figure 3 Migration and invasion of SWO-38 cells at 24 h after pEGFP/DJ-1 transfection detected with transwell chambers

The migrated and invaded cells were stained in purple with crystal violet. Both migration and invasion of SWO-38 cells were enhanced. Each bar in the lower panel represents the number (mean ± standard deviation) of migrated or invaded cells. After pEGFP/DJ-1 transfection, both migration and invasion of SWO-38 cells were enhanced. ** $P < 0.01$, vs. control and pEGFP-C2-transfected cells.

PTEN and increases the phosphorylation level of FAK to promote migration and invasion of glioma.

Our results showed that overexpressing DJ-1 protein in glioma cells inhibited PTEN expression and indirectly enhanced the phospho-FAK level by reducing FAK dephosphorylation. On the other hand, we knocked down DJ-1 by siRNA and found that DJ-1 knockdown promoted PTEN expression and indirectly lowered phospho-FAK level by increasing FAK dephosphorylation. We also found migration and invasion of glioma cells corresponded to DJ-1 expression: more pEGFP/DJ-1-transfected SWO-38 cells migrated through filter membrane than non-transfected cells; fewer DJ-1-knockdown SWO-38 cells penetrated through filter membrane than control cells. Thus our results proved that DJ-1 negatively regulated PTEN expression and further promoted migration and invasion of glioma cells by

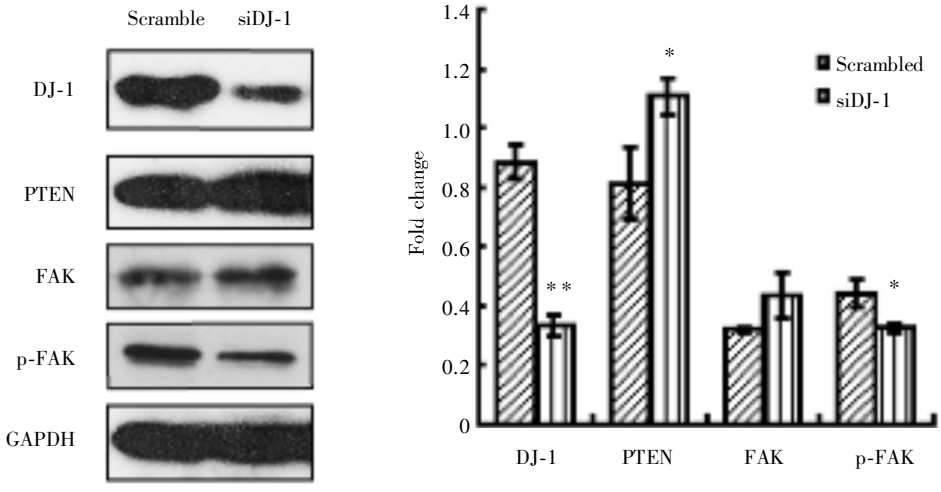

Figure 4 DJ-1, PTEN, FAK, p-FAK expression in SWO-38 cells after transfecting DJ-1 small interfering RNA (siRNA) detected by Western blot

Lane 1: SWO-38 cells transfected with scrambled siRNA; lane 2: SWO-38 cells transfected with DJ-1 siRNA. Each bar in the left panel represents relevant expression (mean ± standard deviation) of protein in cells. After DJ-1 siRNA transfection, DJ-1 expression was down-regulated, PTEN expression was up-regulated, and phospho-FAK was down-regulated. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. SWO-38 cells transfected with scrambled siRNA.

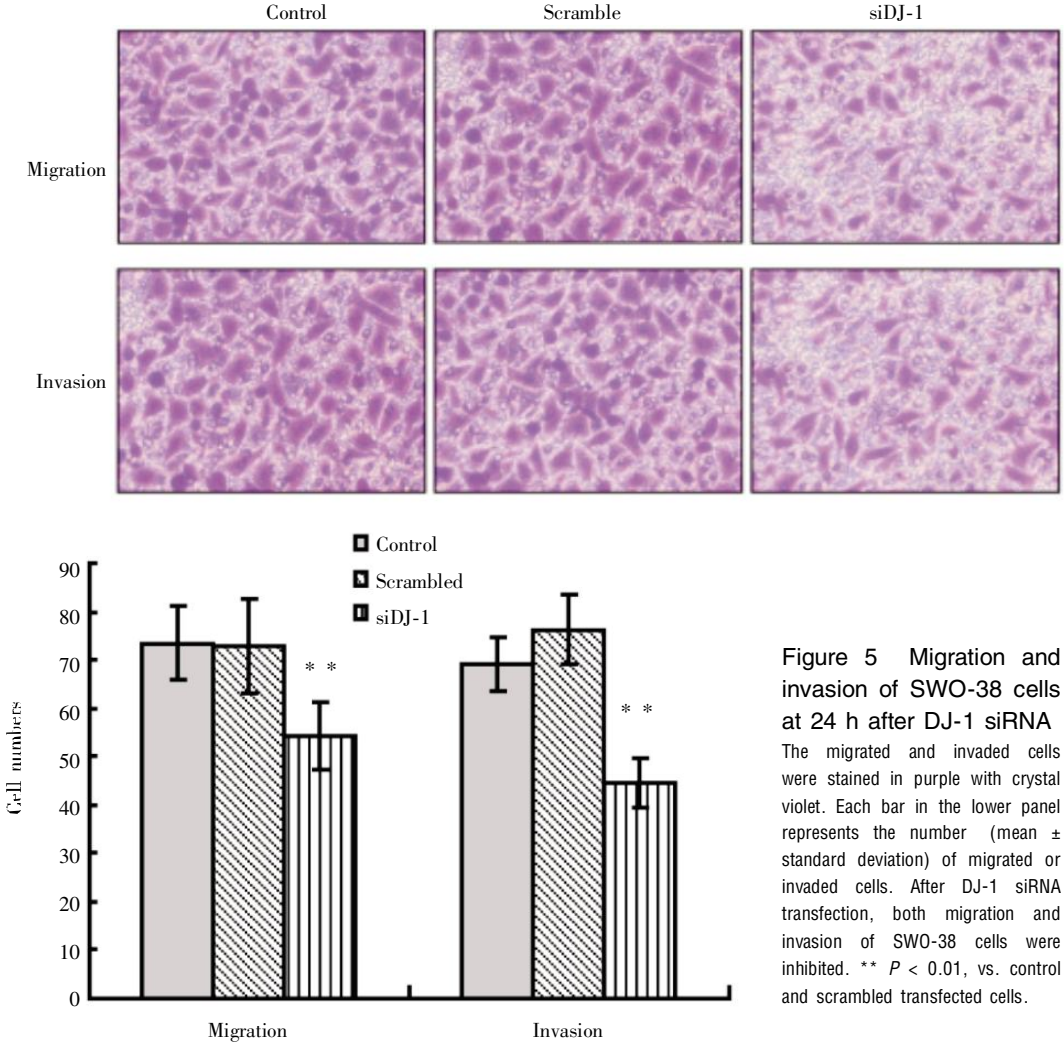

Figure 5 Migration and invasion of SWO-38 cells at 24 h after DJ-1 siRNA

The migrated and invaded cells were stained in purple with crystal violet. Each bar in the lower panel represents the number (mean ± standard deviation) of migrated or invaded cells. After DJ-1 siRNA transfection, both migration and invasion of SWO-38 cells were inhibited. $^{**}P < 0.01$, vs. control and scrambled transfected cells.

increasing FAK phosphorylation.

As a negative regulator of PTEN, DJ-1 has drawn more and more attention. DJ-1 plays an important role in neural tumorigenesis, which makes it a new target for gene therapy. PTEN regulates tumor invasion and metastasis by multiple pathways. Whether DJ-1 regulates migration and invasion of glioma via several pathways, such as the FAK/P130 pathway, needs to be investigated.

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