Retinol dehydrogenase 10 promotes metastasis of glioma cells via the transforming growth factor-β/SMAD signaling pathway

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Abstract

Background: Glioma is the most common primary malignant tumor in the central nervous system. Because of the resistance of glioma to chemoradiotherapy and its aggressive growth, the survival rate of patients with glioma has not improved. This study aimed to disclose the effect of retinol dehydrogenase 10 (RDH10) on the migration and invasion of glioma cells, and to explore the potential mechanism.

Methods: Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the expression levels of RDH10 in healthy glial cells and glioma cells. Human glioma cell strains, U87 and U251, were infected with negative control or RDH10-interfering lentiviruses. RT-PCR and Western blotting were performed to determine the knockdown efficiency. Scratch and transwell assays were used to assess cell migration and invasion after RDH10 knockdown. Finally, changes in transforming growth factor-β (TGF-β)/SMAD signaling pathway-related expression were examined by Western blotting. Differences between groups were analyzed by one-way analysis of variance.

Results: RDH10 was highly expressed in glioma cells. Compared with the control group, RDH10 knockdown significantly reduced RDH10 messenger RNA and protein expression levels in U87 and U251 glioma cells (U87: 1.00 ± 0.08 vs. 0.22 ± 0.02, t = 16.55, P < 0.001; U251: 1.00 ± 0.17 vs. 0.39 ± 0.01, t = 6.30, P < 0.001). The scratch assay indicated that compared with the control group, RDH10 knockdown significantly inhibited the migration of glioma cells (U87: 1.00% ± 0.04% vs. 2.00% ± 0.25%, t = 6.08, P < 0.01; U251: 1.00% ± 0.11% vs. 2.48% ± 0.31%, t = 5.79, P < 0.01). Furthermore, RDH10 knockdown significantly inhibited the invasive capacity of glioma cells (U87: 97.30 ± 7.01 vs. 13.70 ± 0.58, P < 0.001; U251: 96.20 ± 7.10 vs. 18.30 ± 2.08, t = 18.51, P < 0.001). Finally, Western blotting demonstrated that compared with the control group, downregulation of RDH10 significantly inhibited TGF-β expression, phosphorylated SMAD2, and phosphorylated SMAD3 (TGF-β: 1.00 ± 0.10 vs. 0.53 ± 0.06, t = 7.05, P < 0.01; phosphorylated SMAD2: 1.00 ± 0.20 vs. 0.42 ± 0.17, t = 4.01, P < 0.01; phosphorylated SMAD3: 1.00 ± 0.18 vs. 0.41 ± 0.12, t = 4.12, P < 0.01).

Conclusion: RDH10 knockdown might inhibit metastasis of glioma cells via the TGF-β/SMAD signaling pathway.

Keywords: Retinol dehydrogenase; Metastasis; Glioma; RNA; Lentivirus

Introduction

Glioma is the most common primary malignant tumor in the central nervous system and accounts for approximately 70% of primary malignant brain tumors, with an incidence rate of about 5/100,000, especially in people older than 65 years.1,2 Brain glioma mainly shows intracranial infiltrative growth with strong invasion capability. The high proliferation and invasion of glioma results in a 5-year survival rate of less than 5% in glioma patients.3 Treatment for glioma includes surgery combined with chemoradiotherapy. However, because of the resistance of glioma to chemoradiotherapy and its intense infiltration and invasive growth capability, the survival rate of patients with glioma has not improved.4,5 For glioblastoma of the highest degree of malignancy, the median survival time is only 12 to 15 months.4 Therefore, investigating the molecular mechanism of glioma cell proliferation and invasion, and identifying specific therapeutic targets is of great importance for the development of new and effective targeted drugs that will extend survival and improve the quality of life for glioma patients.

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Retinol dehydrogenase 10 (RDH10) was initially identified in retinal pigment epithelial cells and is a member of the short-chain dehydrogenase/reductase family. It possesses retinoid oxidoreductase activity and plays a vital role in the retinoid A visual cycle. The RDH10 amino acid sequence has extremely high homology among different species; the homology between human and rat RDH10 is 99%. RDH10 mediates oxidation of retinol (vitamin A) into retinal, which is an essential substance in the synthesis of retinoic acid. A previous study has found that RDH10 participates in multiple critical physiological development processes and is highly expressed during differentiation of forelimbs and hindlimbs. Mice expressing a missense mutation of RDH10 died during the embryonic development period. However, retinoic acid supplementation during pregnancy prevented the lethal embryo phenotype, indicating that RDH10 has a vital role in development by regulating retinoic acid metabolism. Recently, it has been demonstrated that RDH10 also has a vital role in the occurrence and development of tumors, and participates in the initiation and development of liver cancer and prostate cancer. Our previous study demonstrated that RDH10 promoted the proliferation of glioma cells in vitro and in vivo. However, the effect of RDH10 on glioma cell metastasis and invasion is mostly unclear.

Transforming growth factor β (TGF-β) is a multifunctional cytokine that promotes epithelial differentiation and inhibits cell proliferation. Abnormality of the TGF-β signaling pathway correlates with the occurrence and development of various tumors, including glioma. The TGF-β/SMAD signaling pathway is highly activated in high-grade glioma, promoting the proliferation, migration, and invasion of glioma cells and leading to poor prognosis. It has been reported that the TGF-β signaling pathway promotes the proliferation of glioma cells by inducing the expression of platelet-derived growth factor subunit B. Moreover, TGF-β supports glioma invasion by promoting the expression of matrix metalloproteinase 2. Liu et al. reported that TGF-β-induced microRNA (miRNA)-10a/miRNA-10b expression promoted the invasion of glioma cells by targeting phosphatase and tensin homolog expression. All the above studies indicated that the TGF-β signaling pathway possibly supports the invasion of glioma cells. Thus, elucidating the mechanism of this highly activated pathway in glioma cells is of great importance for identifying drug targets.

Therefore, in this study, we investigated the effect of RDH10 and TGF-β/SMAD on metastasis of glioma to understand the occurrence and development mechanism of glioma, and to provide a reference for screening drug targets for the treatment of glioma.

**Methods**

**Ethical approval**

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Beijing Shijitan Hospital, Capital Medical University.

**Cell culture**

Glioma cell lines U87, U251, U373, and A172, and normal human astrocytes (NHA) were obtained from American Type Culture Collection (ATCC) (https://www.atcc.org/). The cells were cultured in F12/Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics in a 37°C incubator with 5% CO₂. The medium was replaced every 1 to 2 days. When the cells were 90% confluent, the medium was discarded and the cells were digested with 0.25% trypsin for 5 min and then observed using an inverted microscope. When the shape of cells became round, the digestion was terminated by adding culture medium containing serum and cells were sub-cultured at a ratio of 1:3 after being suspended in a single-cell suspension.

**Lentiviral packaging and transfection**

HEK293T cells were used as lentiviral packaging cells. Twenty-four hours before transfection, 5 x 10⁶ cells were seeded in a 10-cm culture dish. Two hours before transfection, the culture medium was replaced with medium without serum. After the lentiviral packaging plasmid and the target gene-interfering plasmid were mixed at a specific ratio, transfection was performed with Lipofectamine 2000 (Invitrogen Thermo Fisher, Carlsbad, CA, USA). After 6 h transfection, the culture medium was replaced with fresh medium. The lentiviruses were collected after 48 to 72 h. The lentivirus titer was measured after concentration and purification. RDH10-interfering (shRDH10) and control (shCtrl) lentiviruses were used to infect the target cells. The following experiment was performed 48 h after lentivirus infection. The RDH10-interfering sequence was: 5'-TAGATGCTGGAGATTTAAT-3'. The control sequence was: 5'-TTCTCCGAAACGTCAGTCTGACGT-3'.

**RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted using the TRIZol method. Complementary DNA was synthesized by reverse transcription. The messenger RNA (mRNA) levels of the target gene were measured by RT-PCR amplification, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The experiment was performed in triplicate to quantify relative mRNA expression and primers were as follows: RDH10 forward primer: 5'-TGGGACATCACAGCAGAAACG-3', RDH10 reverse primer: 5'-TGCAAGTTACAGTGCGAGAGA-3'; GAPDH forward primer: 5'-TGACTTACAACCCGGACACCCAA-3', GAPDH reverse primer: 5'-CACCCGTGGTGCTGCTAGC-3'.

**Protein extraction and Western blotting assay**

Cells expressing shCtrl or shRDH10 were cultured for 48 h, and then harvested for protein extraction. In brief, the culture medium was removed, and the cells were washed with phosphate-buffered saline (PBS). Lysis buffer (100 mmol/L Tris-HCl, pH 7.4, 0.15 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, pH 8.0, 1% Triton X-100, 5 mmol/L DL-dithiothreitol, 0.1 mmol/L...
phenylmethylsulfonyl fluoride) was added to 6-cm plates to extract total protein. Bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA) was used for protein quantification. Protein lysates (30 μg) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequently transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% skimmed milk for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. After washing the membranes with phosphate-buffered saline with Tween 20, they were incubated with horseradish peroxidase-conjugated secondary antibodies. Enhanced chemiluminescence (ECL)-Plus kit (Amersham Biosciences, Pollards Wood, UK) was used to determine the immunoreactivity. Primary antibodies against RDH10 (ab174340, dilution rate: 1:1000), TGF-β (ab64715, dilution rate: 1:1000), SMAD2 (ab40855, dilution rate: 1:1000), SMAD3 (ab40854, dilution rate: 1:1000), phosphorylated SMAD2 (ab53100, dilution rate: 1:1000), and phosphorylated SMAD3 (ab52903, dilution rate: 1:1000) were purchased from Abcam (Cambridge, UK). GAPDH (SC-32233) primary antibody and secondary antibodies (rabbit immunoglobulin G [IgG], sc-2004, dilution rate: 1:5000; mouse IgG, sc-2004, dilution rate: 1:5000) were obtained from Santa Cruz Biotechnology, Inc (Los Angeles, CA, USA).

Scratch assay
The cells were seeded in six-well plates and starved with serum-free DMEM for 6 h. A vertical line was made using a 200 μmol/L tip along the middle of the well. The medium was discarded, and then the cells were washed twice with PBS, incubated with fresh DMEM containing 1% FBS, and observed after 24 h.

Cell invasion
The transwell invasion kit was obtained from Corning (NY, USA). Cells were suspended with DMEM containing 1% FBS and then 1.0 × 10^5 cells were seeded in the upper chamber of the transwell (100 μL of suspension). DMEM containing 10% FBS (500 μL) was added to the lower chamber of the transwell for culturing at 37°C and 5% CO2. The cells in the upper chamber were removed after 24 h, and those on the bottom of the upper chamber were fixed with 4% paraformaldehyde. After 10 min of fixation, the cells were stained with crystal violet for 20 min. A light microscope equipped with a camera was used to take images of each well. Five visual fields were randomly chosen to calculate the average count of cells in each group.

Statistical analysis
The SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Measurement data were expressed as the mean ± standard deviation. Differences between groups were analyzed using one-way analysis of variance. A statistically significant difference was defined as P < 0.05.

Results
High expression of RDH10 in glioma cells
The mRNA expression levels of RDH10 in glioma cells were determined by RT-PCR. Compared with NHA cells in the control group, RDH10 was highly expressed in A172, U373, U87, and U251 cells [Figure 1]. The expression levels of RDH10 in A172 and U373 cells were higher than those in U87 and U251 cells.

RNA interference technology mediated by lentiviruses effectively downregulates RDH10 expression in glioma cells
To investigate the effect of RDH10 in glioma cells, RDH10-interfering lentiviruses (shRDH10) were established and packaged. RDH10 expression in U87 and U251 cells was downregulated by lentivirus-mediated RNA interference technology.
interference technology. The intervention efficiency of shRDH10 in U87 and U251 glioma cells was examined by RT-PCR and Western blotting assay. The RT-PCR results indicated that compared with the control, shRDH10 significantly downregulated RDH10 expression in both cell lines. The inhibition efficiencies were 86.2% (U87: 1.00 ± 0.08 vs. 0.22 ± 0.02, \( t = 16.35, P < 0.001 \)) and 69.2% (U251: 1.00 ± 0.17 vs. 0.39 ± 0.01, \( t = 6.30, P < 0.001 \)), respectively [Figure 2A and 2C]. Furthermore, Western blotting demonstrated that compared with the control group, shRDH10 significantly downregulated RDH10 protein levels in U87 and U251 cells [Figure 2B and 2D].

**RDH10 knockdown effectively inhibits the migration of glioma cells**

The effect of RDH10 downregulation on the migration capability of U87 and U251 cells were examined by scratch assay. As illustrated in Figure 3A and 3C, compared with the control group, downregulation of RDH10 expression significantly inhibited the migration capability of U87 and U251 cells. The inhibition rate of U87 cell migration area after RDH10 downregulation was about two-fold higher than that of the control cells (1.00% ± 0.04% vs. 2.00% ± 0.25%, \( t = 6.08, P < 0.01 \)) [Figure 3B]. Similar results were obtained in the U251 cells (1.00% ± 0.11% vs. 2.48% ± 0.31%, \( t = 5.79, P < 0.01 \)) [Figure 3D].

**RDH10 knockdown effectively inhibits the invasion of glioma cells**

Transwell assays was performed on U87 and U251 glioma cells infected with shRDH10 viruses. As illustrated in Figure 4, compared with the control group, RDH10 downregulation significantly inhibited the invasion capability of these glioma cells (U87: 97.30 ± 7.01 vs. 13.70 ± 0.58, \( t = 20.36, P < 0.001 \); U251: 96.20 ± 7.10 vs. 18.30 ± 2.08, \( t = 18.51, P < 0.001 \)).

**RDH10 silencing inhibits the TGF-β/SMAD signaling pathway**

The TGF-β/SMAD signaling pathway has a critical role in tumor metastasis by regulating the epithelial-mesenchymal transition (EMT). Thus, the Western blotting was performed to determine whether downregulation of RDH10 silencing inhibits the TGF-β/SMAD signaling pathway.
RDH10 expression affects glioma cell metastasis via the TGF-β/SMAD signaling pathway [Figure 5A]. The RT-PCR results showed the quantification of TGF-β (1.00 ± 0.10 vs. 0.53 ± 0.06, \( t = 7.05, P < 0.01 \)), SMAD2 (1.00 ± 0.20 vs. 1.03 ± 0.16, \( t = 0.23, P > 0.05 \)), phosphorylated SMAD2 (1.00 ± 0.20 vs. 0.42 ± 0.17, \( t = 4.01, P < 0.01 \)), SMAD3 (1.00 ± 0.15 vs. 0.98 ± 0.19, \( t = 0.09, P > 0.05 \)), and phosphorylated SMAD3 (1.00 ± 0.18 vs. 0.41 ± 0.12, \( t = 4.12, P < 0.01 \)) expression compared with GAPDH as the control group [Figure 5B]. Downregulation of RDH10 significantly inhibited the expression levels of TGF-β, phosphorylated SMAD2, and phosphorylated SMAD3. These results indicated that knockdown of RDH10 might inhibit metastasis of glioma cells via the TGF-β/SMAD signaling pathway.

**Discussion**

The incidence of glioma is the highest among intracranial tumors. Owing to its high degree of malignancy, strong invasion, and lack of specific diagnosis markers and targeted therapeutic drugs, the survival time of glioma patients is short, and prognosis is poor. Currently, surgical treatment together with chemoradiotherapy is the primary therapy. Therefore, elucidation of the molecular mechanism of proliferation and invasion of brain glioma, and identification of potential and specific therapeutic targets are vital for improving the therapeutic strategy, extending survival time, and improving quality of life.

In this study, we found that RDH10 regulated glioma cell metastasis. In 2002, Wu et al.[8] cloned RDH10, which converts trans-retinol to all-trans-retinol and regulates the synthesis of retinoic acid. It has been reported that RDH10 regulates physiological development during the embryonic period, such as forelimb and hindlimb differentiation. Knockout of RDH10 leads to death of mice during the embryonic development period, indicating that RDH10 has a critical function in embryonic development by regulating retinoic acid metabolism.[9] Retinoic acid inhibits the proliferation of liver cancer, gastric cancer, esophageal cancer, and colorectal cancer cells by inducing cell cycle arrest and apoptosis.[19-22] Retinoic acid also affects the proliferation and apoptosis of glioma cells. All-trans-retinoic acid has been used to treat glioma cells as it has been shown to significantly inhibit the proliferation of glioma cells and induce apoptosis.[23] Moreover, retinoic acid improves the pro-apoptosis effect of temozolomide on...
U251 cells via the Kelch-like ECH-associated protein 1-nuclear factor erythroid 2-related factor 2-antioxidant response elements signaling pathway,[24] which suggests that the retinoic acid signaling pathway possesses a strong inhibitory effect on proliferation of glioma at the cellular level. Recently, it has been shown that RDH10 is expressed in non-small cell lung cancer, mainly grade III disease, suggesting that it has a very important role in the
development of this cancer.\(^{11}\) Overexpression of RDH10 in liver cancer upregulated the expression of retinoic acid receptor \(\beta/p21\text{(Cip)}\), and inhibited the proliferation of the liver cancer cell line HepG-2.\(^{10}\) In our previous study, RDH10 was found to be highly expressed in glioma tissues. RDH10 silencing significantly inhibited the proliferation of glioma cells U87 and U251 \(\text{in vitro and in vitro}\). Additionally, we found that RDH10 knockdown suppressed the invasion of glioma cells.\(^{13}\) However, the role of RDH10 in the migration and invasion in glioma cells requires further investigation. In this study, we confirmed that RDH10 was highly expressed in glioma cells, and downregulation of RDH10 inhibited the invasion of glioma cells. Wound healing assay suggesting that RDH10 also promoted glioma cell migration. The results indicated that RDH10 is a proto-oncogene in glioma and maybe a potential target for the treatment of glioma.

Our previous study showed that the nuclear factor kappa-light-chain-enhancer of activated B cells signaling pathway in RDH10 regulated cell proliferation and apoptosis of glioma cells. Interestingly, we found that RDH10 silencing significantly inhibited the TGF-\(\beta/\text{SMAD}\) signaling pathway, indicating that RDH10 may regulate the migration and invasion of glioma cells by promoting TGF-\(\beta/\text{SMAD}\) signaling. TGF-\(\beta\) has a very important role in the regulation of migration and invasion of cancer,\(^{25}\) as well as other diseases such as endometriosis.\(^{26,27}\) As important downstream molecules of TGF-\(\beta\), SMAD2/SMAD3 have critical roles in TGF-\(\beta\) signal transduction. The TGF-\(\beta/\text{SMAD}\) signaling pathway participates in tumor invasion and metastasis of various cancers, such as colorectal cancer, breast cancer, bladder cancer, and glioma.\(^{12}\) However, the TGF-\(\beta\) signaling pathway has different effects on metastasis and invasion under different conditions.\(^{29,30}\) In the metastasis of prostate cancer, TGF-\(\beta\) inhibits proliferation and metastasis of tumor cells by inducing SMADs.\(^{31}\) In other tumors such as liver cancer, overexpression of TGF-\(\beta\) promotes the metastasis and invasion of liver cancer cells.\(^{52,53}\) It has been found that TGF-\(\beta\) is highly expressed in glioma tissues, and the expression level positively correlates with the degree of glioma malignancy.\(^{34,35}\) TGF-\(\beta\) can significantly reduce epithelial cadherin expression in glioma cells, simultaneously increase neuroadherin and vimentin expression, and promote the transition of E/N cadherin.\(^{36}\) Furthermore, the TGF-\(\beta/\text{SMAD}\) signaling pathway promotes the expression of \(\alpha\)\(V\beta\)3 integrin and matrix metalloproteinase-2 (MMP-2) and further promotes the migration of glioma cells.\(^{37}\) We found that downregulation of RDH10 significantly inhibited the TGF-\(\beta/\text{SMAD}\) signaling pathway, and inhibited metastasis and invasion of glioma cells. However, whether RDH10 affects glioma migration by regulating \(\alpha\)\(V\beta\)3 integrin, EMT, MMP-2, or other downstream regulatory factors requires further exploration.

In conclusion, glioma severely threatens the physical and psychological health of humans. Currently, surgery combined with chemoradiotherapy is still the primary therapeutic strategy. We found that RDH10 regulated the migration and invasion of glioma cells via the TGF-\(\beta/\text{SMAD}\) signaling pathway. Nevertheless, the detailed mechanism of RDH10 regulation of TGF-\(\beta/\text{SMAD}\)-induced cell metastasis requires further exploration. Because of the recurrence of disease after chemotherapy causing reduced progression,\(^{38,39}\) the role of RDH10 in glioma chemical response requires investigation in future studies. This study provides an essential theoretical basis to identify new and potential therapeutic targets for the treatment of glioma.

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Conflicts of interest
None.

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