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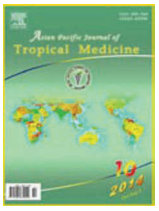

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Evaluation of TAZ expression and its effect on tumor invasion and metastasis in human glioma

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ABSTRACT

Objective: To evaluate the expression of TAZ and its role in tumor invasion and metastasis in human glioma. **Methods:** The expression of TAZ protein was measured in 48 samples of surgically resected human glioma and 13 samples of normal brain tissues using immunohistochemistry. TAZ was knocked down by a retrovirus-mediated TAZ shRNA in a glioma cell line, SNB19. Transwell cell migration and invasion assays were used to determine migration and invasion of SNB19 cells. **Results:** The positive expression rate of TAZ protein in glioma tissues was significantly higher than that in normal brain tissues (79.2% *vs.* 15.4%, $P<0.001$). Furthermore, clinical analysis suggested that the positive expression rate of TAZ protein in poorly differentiated tumor tissues was significantly higher as compared with that in well differentiated tissues (96.0% *vs.* 60.9%, $P<0.01$). TAZ was significantly knocked down by TAZ shRNA ($P<0.001$), and TAZ knockdown significantly reduced cell migration and invasion ($P<0.01$, respectively) in SNB19 cells. **Conclusions:** TAZ protein overexpression is observed in human glioma and its elevated expression is significantly correlated with poor differentiation. TAZ knockdown prominently reduces cell migration and invasion in SNB19 cells, suggesting that TAZ may play a key role in the initiation and progression of human glioma.

1. Introduction

Gliomas, which account for 40%-50% of tumors in the central nervous system, are initiated from various glial cells[1]. Glioblastoma multiform, the most common primary tumor in brain, is characterized by high invasion and tends to diffusely infiltrate into the adjacent normal brain parenchyma[1]. These features result in poor outcome for glioma patients. The mechanisms involved in invasion and metastasis of gliomas have been extensively investigated in various studies. Therefore, it is important to explore a novel biomarker for diagnosis and predicting prognosis of glioma.

Hippo signaling pathway plays an important role in cell proliferation, organ size control and tumorigenesis[2]. Transcriptional coactivator with PDZ-binding motif (TAZ), paralog of Yes-associated protein (YAP), is a downstream effector of Hippo signaling pathway[2]. Hippo pathway is a serial of kinase cascade, autophosphorylation of mammalian sterile 20-like-1/2 (MST1/2) and its regulatory protein, salvador-1, subsequently results in phosphorylation and activation of large tumor suppressor homolog-1/2 (LATS-1/2)[3-5]. Then, activation of LATS-1/2 leads to phosphorylation of four serine residues in TAZ[3-5]. Hippo pathway prevents TAZ from moving to nuclear by cytoplasmic sequestration[5]. Dysfunction of Hippo pathway results in TAZ nuclear accumulation and subsequently activation of its downstream target genes[6]. TAZ is a transcription coactivator and interacts with several transcription factors, such as TEA domain family members (TEAD), mothers against decapentaplegic homologs (SMADs), paired box-3, paired box-8, T-box 5, transcription termination factor-1, runt-related transcription factor 2, and MyoD[7-12]. TAZ controls mesenchymal stem cell differentiation through regulating runt-related transcription factor 2 and peroxisome proliferator activated receptor γ-dependent gene expression[7]. Moreover, TAZ regulates stem cell self-renew by controlling location of SMAD[13]. Increasing evidences indicate that TAZ plays a critical role in epithelial-mesenchymal transition (EMT), cell

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growth and organ development[14]. Aberrant expression of TAZ has been observed in various human malignant tumors including breast cancer, colorectal cancer, lung cancer, gastric cancer and hepatocellular carcinoma[15-19]. Otherwise, TAZ acts as a key factor in initiation and metastasis of breast cancer[15]. In non-small cell lung cancer, TAZ knockdown inhibits cell proliferation and decreases grade malignancy of tumor cells[16]. But the expression of TAZ and its role in human glioma remain poorly understood. In current study, we detected TAZ expression in human glioma and normal brain tissues. The correlations between TAZ protein expression and clinicopathologic parameters were systemically analyzed. Furthermore, we investigated the effect of TAZ on invasion and metastasis of glioma cell, in order to identify the function of Hippo-TAZ axis in the initiation and development of glioma.

2. Materials and methods

2.1. Clinical samples

Forty-eight formalin-fixed and paraffin-embedded samples were collected from patients including 31 males and 17 females, who underwent the resection of their primary glioma in the Department of Neurosurgery, the Fifth Affiliated Hospital of Zhengzhou University, during Jan 2008 to Dec 2012. The median age of the glioma patients was 47 years (range, 31-68 years). According to the nervous system tumor classification criteria (World Health Organization, 2000), 23 samples were recognized as histological grade Ⅰ and Ⅱ, while other samples were identified as grade Ⅲ and Ⅳ. All samples were pathologically approved after operation. Thirteen samples of normal brain tissue were collected from patients with brain trauma or encephalorrhagia including eight males and five females. The median age of these patients was 46.2 years (range, 35-68 years). All these samples located at nonfunctional areas of brain, which must be resected for operation. It is comparable between two groups in sex ratio and age. Samples were collected and used after obtaining informed consent. The Zhengzhou University Ethics Committee approved all protocols according to the Helsinki Declaration (as revised in Edinburgh 2000).

2.2. Cell line and transfection

The human glioma cell lines, SNB19 (ATCC, Manassas, VA, USA), were cultured in complete Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) with 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma, St-Louis, MO, USA) in a humidified containing of 5% CO_2 incubator at 37 ℃.

The shRNA against human TAZ (5′-GGA UAC AGG AGA AAA CGC A-3′) and non-targeting shRNA (NT shRNA) were obtained from QIAGEN Co. (QIAGEN, Valencia, CA, USA). Retrovirus packaging and transduction were proceeded using Effectene transfection reagent (QIAGEN) and polybrene (8 μg/mL) as described previously[20].

2.3. Immunohistochemical staining

Immunohistochemistry with streptavidin peroxidase conjugated method was performed on formalin-fixed paraffin sections. Sections that were underwent dewaxed, rehydration, antigen retrieval, endogenous peroxidase activity blocking and goat serum blocking were incubated with TAZ (ab118373, Hong Kong, China) (1:100) antibody at 4 ℃ overnight. Streptavidin peroxidase conjugated secondary antibody and diaminobenzidine were used for staining of sections. According to the percentage of positive tumor cells, TAZ expression was classified as negative expression (less than 10%) and positive expression (equal and more than 10%).

2.4. Western blot

TAZ (1:1 000) and reduced glyceraldehyde-phosphate dehydrogenase (G8140, US Biological, Salem, MA, USA) (1:5 000) antibodies were used for immunoblotting assay. Horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Bio-Rad, Hercules, CA, USA) were used at a dilution from 1:1 000-1:5 000 and detected by a western blotting luminol reagent (sc-2048, Santa Cruz, CA, USA)[20].

2.5. Transwell cell migration and invasion assays

Transwell cell migration assays were done in 12 well plates with 8-μm BioCoat control inserts (Becton Dickinson Labware, Bedford, MA). 1-2×10^5 TAZ shRNA or NT shRNA transfected SNB19 cells that were suspended in 500 μL serum free DMEM were seeded in the upper well and DMEM medium with 10% fetal bovine serum, as indicated, in the lower well. After completion, membranes were removed, wiped on the side facing the upper well, and stained with crystal violet. At least 6 representative images of each well were taken and cell numbers were counted using ImageJ. BioCoat matrigel invasion chamber (Becton Dickinson Labware) was used for transwell cell invasion assays and the following protocols were the same as transwell cell migration assays. The experiments were performed in triplicate.

2.6. Statistical analysis

Results are expressed as mean±SEM. Significance was established, with GraphPad Prism 5 software (GraphPad Software, Inc, San Diego, CA, USA) and SPSS statistical package for Windows version 13 (SPSS, Chicago, IL, USA), using a Pearson *Chi*-squared test and Student's *t*-test when appropriate. Differences were considered significant when $P<0.05$.

3. Results

3.1. Expression of TAZ in glioma and normal brain tissues

To determine the status of TAZ expression in glioma, the expression of TAZ protein in a retrospective cohort of 48 glioma and 13 normal brain samples was determined using immunohistochemical staining. According to the percentage of positive tumor cells, TAZ immunoreactivity was considered as either negative (less than 10%) or positive (equal and more than 10%). In these cases, it was found

that 79.2% (38/48) of glioma samples showed TAZ positive expression, while TAZ positive expression was observed in only 15.4% (2/13) of normal brain samples. The difference was statistical significant (χ^2=15.717, P<0.001, Figure 1). These data indicated that TAZ overexpression may act as an oncogene in human glioma.

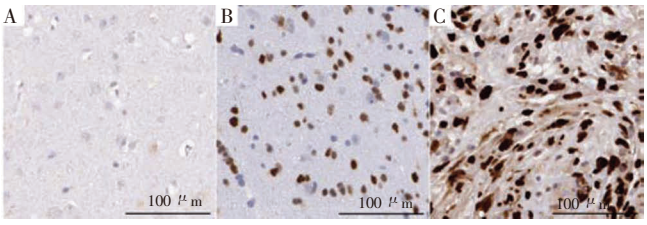

Figure 1. Immunohistochemical analysis of TAZ in glioma and normal brain samples.
A) Representative immunostaining showed negative expression of TAZ protein in normal brain tissues; B) Representative immunostaining showed positive expression of TAZ protein in well differentiated glioma tissues; C) Representative immunostaining showed positive expression of TAZ protein in poorly differentiated glioma tissues μ.

3.2. Correlation between TAZ protein expression and tumor differentiation in glioma

Elevated TAZ expression was correlated with malignant clinicopathologic features in human cancers[15-18]. To determine the clinical significance of TAZ in glioma, the correlation between TAZ protein expression and tumor differentiation was analyzed. The positive expression rate of TAZ was 96.0% (24/25) in poorly differentiated glioma tissues (grade Ⅲ+Ⅳ), while it was only 60.9% (14/23) in well differentiated glioma tissues (grade Ⅰ+Ⅱ). Clinical association analysis indicated that increased TAZ protein level in glioma tissues was prominently related to poor histological differentiation (χ^2=6.960, P=0.008). Taken together, our results indicated that the expression of TAZ was up-regulated in glioma and elevated TAZ protein level was associated with malignant clinicopathologic parameters.

3.3. Cell migration and invasion inhibited by TAZ knockdown in SNB19 cells

Previous studies demonstrated that TAZ acted as an oncogene by promoting cancer cell proliferation, transformation and invasion[15,16]. To identify the role of TAZ in glioma, glioma cell line and SNB19 were transduced with NT shRNA retroviruses or TAZ shRNA retroviruses. Transfected cells were collected and subjected to transwell cell migration and invasion assays for cell migration and invasion. As measured by western blot, the TAZ protein level was significantly knocked down by TAZ shRNA in SNB19 cells (t=8.004, P<0.001, Figure 2). Transwell cell migration assays were performed to test the effect of altering TAZ levels on tumor cell migration. It was found that TAZ knockdown led to a significant reduction of cell migration in SNB19 cells (t=5.942, P=0.004, Figure 3). Furthermore, as determined by transwell cell invasion assays, the number of invaded SNB19 cells was significantly decreased after TAZ knockdown (t=6.115, P=0.003, Figure 4). Thus, TAZ may exert a pro-metastatic effect by promoting cell migration and invasion in glioma.

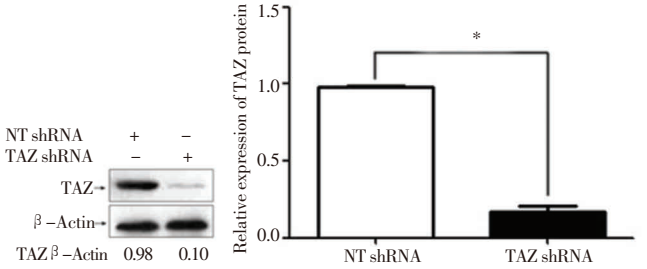

Figure 2. TAZ knocked down by a specific shRNA in SNB19 cells.
SNB19 cells that were transfected with NT shRNA or TAZ shRNA were subjected to western blot for TAZ. Representative western blot analysis and quantification of the data revealed that TAZ was significantly knocked down by retrovirus-mediated TAZ shRNA in SNB19 cells. * P<0.05 by t test; n=3 repeats with similar results.

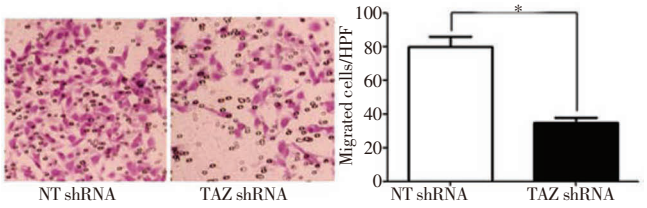

Figure 3. Reduced cell migration in SNB19 cells due to TAZ knockdown.
SNB19 cells that were transfected with NT shRNA or TAZ shRNA were subjected to transwell cell migration assays for cell migration. Quantification of the data revealed that TAZ knockdown significantly reduced cell migration in SNB19 cells. HPF: High power field; * P<0.05 by t test; n=3 repeats with similar results.

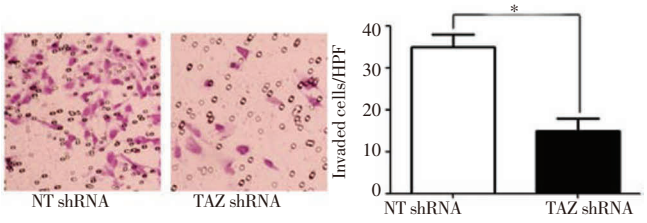

Figure 4. Reduction of cell invasion in SNB19 cells due to TAZ knockdown.
SNB19 cells that were transfected with NT shRNA or TAZ shRNA were subjected to transwell cell invasion assays for cell invasion. Quantification of the data revealed that TAZ knockdown prominently decreased cell migration in SNB19 cells. HPF, High power field; *P<0.05 by t test; n=3 repeats with similar results.

4. Discussion

YAP/TAZ promotes tumor growth by regulating genes involved in cell proliferation and anti-apoptosis, including Gli2, connective tissue growth factor, amphiregulin, transforming growth factor-beta target genes and bone morphogenetic protein target genes[21]. Increasing studies indicate that aberrant high-expression of TAZ is correlated with initiation and development of human cancer[15-17]. TAZ plays an important role in the progression of breast cancer and non-small cell lung cancer[15,16]. Importantly, TAZ negative expression confers a better survival for colorectal patients, suggesting that TAZ may be a potential therapeutic target for colorectal cancer[17]. We initially detected TAZ expression status in 48 samples of surgically resected glioma and 13 samples of normal brain tissues using immunohistochemistry. Our data indicated that most of glioma cases showed positive TAZ expression and nuclear accumulation. The positive expression rate of TAZ protein in glioma tissues was significantly higher than that in normal brain tissues. Furthermore, TAZ protein was expressed at significantly higher levels in glioma patients with poor

histological differentiation. Altogether, these results suggest that TAZ expression may be critical for prognosis determination in glioma patients.

EMT, a dynamic and reversible cellular process, is characterized by loss of cell polarity and intracellular junctions and acquirement of mesenchymal features, resulting in increased cell migration and invasion[22]. Cancer cells that were underwent EMT lead to tumor metastasis and poor survivals for patients[23]. Several studies have reported that glioma cells with EMT exhibit enhanced invasion and metastatic potential[24,25]. Furthermore, tumor tissues that were collected from glioma patients were used for molecular subtyping. The data indicated that tumors with mesenchymal gene characteristics conferred a worse overall survival and treatment-resistant in patients, suggesting that EMT plays a key role in the progression of glioma[22]. It has been reported that TAZ interacts with TEAD and promotes cell proliferation, EMT, invasion and cell transformation[12]. Otherwise, studies have reported that TAZ/YAP function as coregulators in SMAD activation induced by transforming growth factor-beta, which is an important EMT promoter[10,23]. Recent studies found that TAZ and TEAD directly promoted mesenchymal transition in glioblastoma multiform[22]. In our study, we found that TAZ knockdown significantly decreased cell migration in SNB19 cells. Furthermore, impaired TAZ expression by retroviruses-mediated shRNA conferred an inhibitory effect on cell invasion in SNB19 cells. Thus, TAZ may enhance cell migration and invasion by promoting EMT in glioma.

In conclusion, we find that TAZ is up-regulated in glioma tissues and its positive expression is related to malignant clinicopathologic characteristics. Moreover, we demonstrate that TAZ knockdown reduces migration and invasion of glioma cells, suggesting TAZ may facilitate tumour progression by promoting tumor invasion and metastasis.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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