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Original Article

Overexpression of TEAD4 correlates with poor prognosis of glioma and promotes cell invasion

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Abstract: This study aimed to reveal the correlation of increased TEA domain transcription factor 4 (TEAD4) expression and disease prognosis in glioma. The expression data of TEAD4 mRNA in glioma were collected from GEO database (GSE4290), and the expression of TEAD4 protein in glioma was confirmed using western blot and Immunohistochemistry. Kaplan-Meier analysis with the log-rank test was used to reveal the correlation of TEAD4 expression level and patients' survival. The effects of TEAD4 on migration and invasion were separately examined by Transwell assay and Boyden assay. Gene set enrichment analysis (GSEA) was performed to predict the possible biological function of TEAD4 in glioma. The results showed that TEAD4 mRNA and protein expression were upregulated in glioma tissues compared to normal brain tissues. Furthermore, overexpression of TEAD4 correlated with poor prognosis in glioma patients. Knockdown of TEAD4 markedly inhibited glioma cells migration and invasion in vitro. Consistent with the result that TEAD4 was associated with epithelial-mesenchymal transition (EMT) closely by GESA, knockdown of TEAD4 resulted in N-cadherin, vimentin and Slug downregulated but E-cadherin upregulated. Our study indicated that overexpression of TEAD4 may represent as a potential unfavorable marker for poor survival and prognosis in glioma. Knockdown of TEAD4 led to suppressed glioma migration and invasion.

Keywords: TEAD4, glioma, prognosis, migration, invasion, EMT

Introduction

As the most common form of malignant primary brain tumor, glioma makes up 30% of central nervous system tumors and 80% of malignant brain tumors [1]. The average survival time of patients getting anaplastic glioma (WHO II) is approximately 3 years, and glioblastoma multiforme (WHO IV) has a poor survival time less than 15 months [2]. As of now, the prognosis is still very poor, despite using multiple managements involved with surgery resection, radiotherapy and chemotherapy of temozolomide [3]. Changed gene expression with altered molecular regulation is an important factor of glioma pathogenesis. Thus, it is urgent to understand the mechanism of glioma tumorigenesis and find new therapeutic targets [4].

The TEA domain transcription factor (TEAD) family members, TEAD1-4, have the same domain structure [5]. The TEADs are known to interact with co-activators to promote genes transcription, cell proliferation, and inhibit apoptosis [6]. By binding with co-activators, TEADs function as key mediators in tumorigenesis [7]. As an important co-activator, YAP/TAZ interacting with TEAD at the downstream process of Hippo pathway enhances cell proliferation, migration and invasion [8, 9].

As a TEAD family member, TEA domain transcription factor 4 (TEAD4) has been found to be a poor prognosis marker of several cancers, such asgastric [10], colorectal [11] and breast cancers [12] and so on, suggesting that it has the potential to be a key molecule in cancer therapy [4, 7, 11-13]. However, the critical roles of TEAD4 in glioma remain unclear.

In this study, analyzing data obtained from public dataset and specimen collected from glioma resection, overexpression of TEAD4 was found in glioma compared with normal brain tissue. Moreover, overexpression of TEAD4 correlating with poor prognosis of glioma was revealed by survival analysis. Knockdown of TEAD4 expression in glioma cells reduced cell migration and

Table 1. Expression of TEAD4 in glioma and normal brain tissue

Group	Cases	TEAD4 expression		*p* value
		High expression	Low expression	
Normal Brain	26	5 (19.2)	21 (80.8)	
Glioma	88	50 (56.8)	38 (43.2)	0.001*

*P<0.05 was considered significant.

Table 2. Correlation between clinicopathologic characteristics and expression of TEAD4 in glioma

Characteristics	n	TEAD4 expression (%)		*p* value
		High expression	Low expression	
Gender				0.888
Male	54	31 (57.4)	23 (42.6)	
Female	34	19 (55.9)	15 (44.1)	
Age				0.765
<50	73	42 (57.5)	31 (42.5)	
≥50	15	8 (53.3)	7 (46.7)	
Histologic Type				0.522
Astrocytic tumors	51	30 (58.8)	21 (41.2)	
Oligodendroglial tumors	10	4 (40.0)	6 (60.0)	
Other	27	16 (59.3)	11 (40.7)	
Tumor Location				0.418
Frontal	43	25 (58.1)	18 (41.9)	
Temporal	12	8 (66.7)	4 (33.3)	
Parietal	5	4 (80.0)	1 (20.0)	
Other	28	13 (46.4)	15 (53.6)	
WHO Grade				0.017*
I+II	36	15 (41.7)	21 (58.3)	
III+IV	52	35 (67.3)	17 (32.7)	

*P<0.05 was considered significant.

invasion *in vitro*. To investigate the molecular mechanism of TEAD4 in glioma, we used gene set enrichment analysis (GSEA) to predict the possible biological functions of TEAD4 in glioma with public dataset. We found that TEAD4 might be involved in glioma epithelial-mesenchymal transition (EMT). This result was confirmed by detecting the expression of EMT-related proteins in TEAD4 silenced glioma cells.

Materials and methods

Bioinformatic analysis

Data used in this study for bioinformatic analysis was obtained from public datasets, including GEO Datasets (https://www.ncbi.nlm.nih.gov/gds/), TCGA (https://cancergenome.nih.gov/) and CGGA (http://www.cgga.org.cn/).

Clinical tissue sample collection

A total of 88 paraffin-embedded glioma and 26 normal brain samples were obtained from the Nanfang Hospital of Southern Medical University, Guangzhou, China. All the gliomas had confirmed pathologic diagnosis and classification according to the World Health Organization (WHO) criteria. These glioma cases were 54 males and 34 females with ages ranging from 3 to 77 years (median age, 38 years) (**Table 2**). Moreover, the Ethics Committees of Nanfang Hospital of Guangdong Province approved this research, and each human tissue was obtained with prior consent from patients or their guardians before participation in the study.

Western blot analysis

Western blot was carried out according to our previous studies [14, 15] with rabbit polyclonal TEAD4 (1:1000; Abcam), N-Cadherin (1:1000; Proteintech), E-cadherin (1:1000; Proteintech), vimentin (1:1000; Cell Signaling Technology), slug (1:1000; Cell Signaling Technology), GAPDH (1:1000; Cell Signaling Technology) antibodies. An HRP-conjugated anti-rabbit or anti-mouse IgG antibody was used as the secondary antibody (1:2000; CoWin Bioscience, Beijing, China). Signals were detected using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA). All experiments were independently performed in triplicate.

Immunohistochemistry

The details of immunohistochemistry methods were used as described previously [14]. Paraffin sections were deparaffinized in 100% xylene and rehydrated in descending ethanol series and PBS. Heat-induced antigen retrieval was performed in 10 mM citrate buffer for 15 min in a microwave oven. Endogenous peroxidase

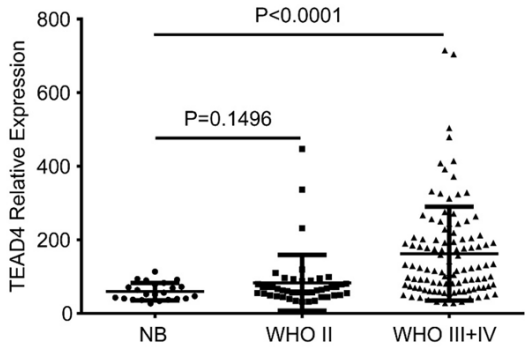

Figure 1. TEAD4 mRNA expression in glioma and normal brain tissues. Scatter plots showed that expression of TEAD4 mRNA was significantly increased in high-grade gliomas (WHO grade III+IV, grade IV, n=76; grade III, n=32) compared with normal brain (NB) tissues (n=23) (P<0.0001), but the expression in low-grade gliomas (WHO grade I+II, n=45) compared with NB tissues was not increased (P=0.1496).

activity and non-specific antigens were blocked with peroxidase blocking reagent containing 3% hydrogen peroxide and serum, followed by incubation with rabbit anti-human TEAD4 antibody (1:150) overnight at 4°C. After washing, the sections were incubated with biotin-labeled rabbit anti-goat antibody for 40 min at room temperature, and subsequently the peroxidase reaction was developed using 3,3-diami nobenzidine (DAB) chromogen solution in DAB buffer substrate. Sections were counter stained with hematoxylin, mounted in neutral gum, and analyzed with a bright field microscope.

Staining evaluation of immunohistochemistry

The anti-human TEAD4 antibody (1:150; SAB) was used. The immunohistochemically stained tissue sections were reviewed and scored separately by two pathologists blinded to the clinical parameters. The expression of TEAD4 in the nucleus and in the cytoplasm was independently evaluated. For cytoplasmic staining, the score was evaluated according to the sum of cytoplasm staining intensity and the percentage of positive staining areas in cells. The staining intensity was scored as previously described (0-3) and the percentage of positive staining areas of cells was defined as a scale of 0-3 (0: <10%, 1: 10-25%, 2: 26-75%, and 3: >76%). For nuclear staining, the staining score was defined based on the sum of nuclear staining intensity and the percentage of positive nuclear staining numbers. Nuclear staining intensity score was the same as cytoplasm (0-3). The positive nuclear staining scores were defined 0-3 (0: <20%, 1: 20-49%, 2:50-79%, and 3: >80%). The sum of the cytoplasm and nuclear staining scores was used as the final staining score for TEAD4 (0-12). For statistical analysis, a final staining score of 0-6 was considered to be low expression, and a final staining score of 7-12 was considered to be high expression.

Cell culture

The human glioma cell lines U87 and U251 were purchased from the Chinese Academy of Sciences (Shanghai, China). In the laboratory, all cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and incubated in a humidified atmosphere of 5% CO_2 at 37°C.

Transient transfection with siRNAs

Small-interfering RNA (siRNA) for TEAD4 was designed and synthesized by Guangzhou RiboBio (RiboBio Inc, China). The target sequence forTEAD4 was 5'-AGACAGAGTATGCTCGCTAT-3'. The efficiency of siRNA (siTEAD4) identified by western blot, and the siTEAD4 was applied for the further experiments. Glioma cells were plated onto a 6-well plate at 30-50% confluence. After 6 hours, siTEAD4 was then transfected into cells with the help of lipo2000 according to the manufacturer's protocol. Cells were collected after 24 hours for Functional experiment and 48 hours for western blot.

Cell migration and invasion assay

In vitro, cell migration and invasion assays were examined according to our previous study [15]. The cell migration assays were carried out with Transwell assays. About 5×10^4 cells in 100 μL DMEM medium without FBS were seeded on a fibronectin-coated polycarbonate membrane inserted in a Transwell apparatus (Costar, MA). In the lower chamber, 500 μL DMEM with 10% FBS was added as a chemoattractant. After the cells were incubated for appropriate time according to specific cell lines in a 5% CO_2 atmosphere at 37°C, the insert was washed with PBS, and cells on the top surface of the insert were removed with a cotton swab. Cells adhering to the lower surface were fixed with methanol for 30 minutes, and stained with 1% crystal violet solution for 1 min and counted under a microscope in three predetermined fields. All assays were independently performed in triplicate.

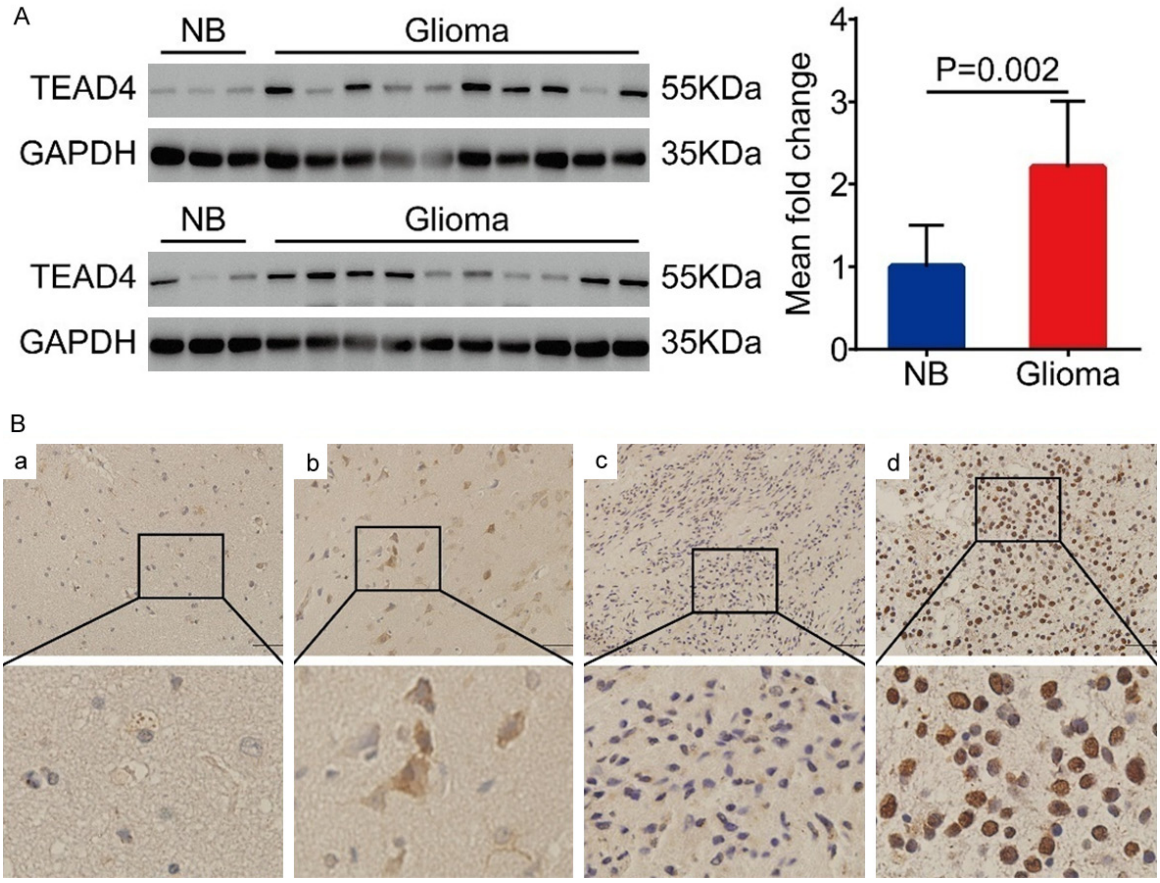

Figure 2. TEAD4 protein expression in glioma and normal brain tissues. A. The expression of TEAD4 was evaluated in 20 glioma tissues compared with 6 normal brain tissues by western blot. The unpaired t test was used for this assay (P=0.002). B. The expression and location of TEAD4 were examined by immunohistochemical staining. a. Weak staining of TEAD4 in normal tissues. b. Strong staining of TEAD4 in normal tissues. c. Weak staining of TEAD4 in glioma tissues. d. Strong staining of TEAD4 in glioma tissues. Original magnification 400×.

Table 3. Correlation of TEAD4 expression with IDH1 mutation

IDH1 mutation	Cases	TEAD4 expression		*p* value
		High expression	Low expression	
Positive	162	71 (43.8)	91 (56.2)	
Negative	163	92 (56.4)	71 (43.6)	0.023*

*P<0.05 was considered significant.

Cell invasion assays were carried out with Boyden assays, and the procedure was similar with the cell migration assay, except that Transwell membranes were precoated with 24 mg/ml Matrigel (R&D Systems, USA). All assays were independently performed in triplicate.

Statistical analysis

All quantified data represented an average of at least triplicate samples or as indicated. Statistical analysis used SPSS 13.0 and Graph Pad Prism 5.0 software. Survival analysis was performed using Kaplan-Meier method. Differences were considered statistically significant when P<0.05. Data were represented as mean ± SD. Two-tailed Student's t-test was used for comparisons of measurement data between control and experimental groups. Chi-square test was used to identify the differences of enumeration data between categorical variables.

Results

TEAD4 mRNA expression increases in high-grade glioma

To clarify the role of TEAD4 in human glioma, TEAD4 mRNA expression was measured in 153

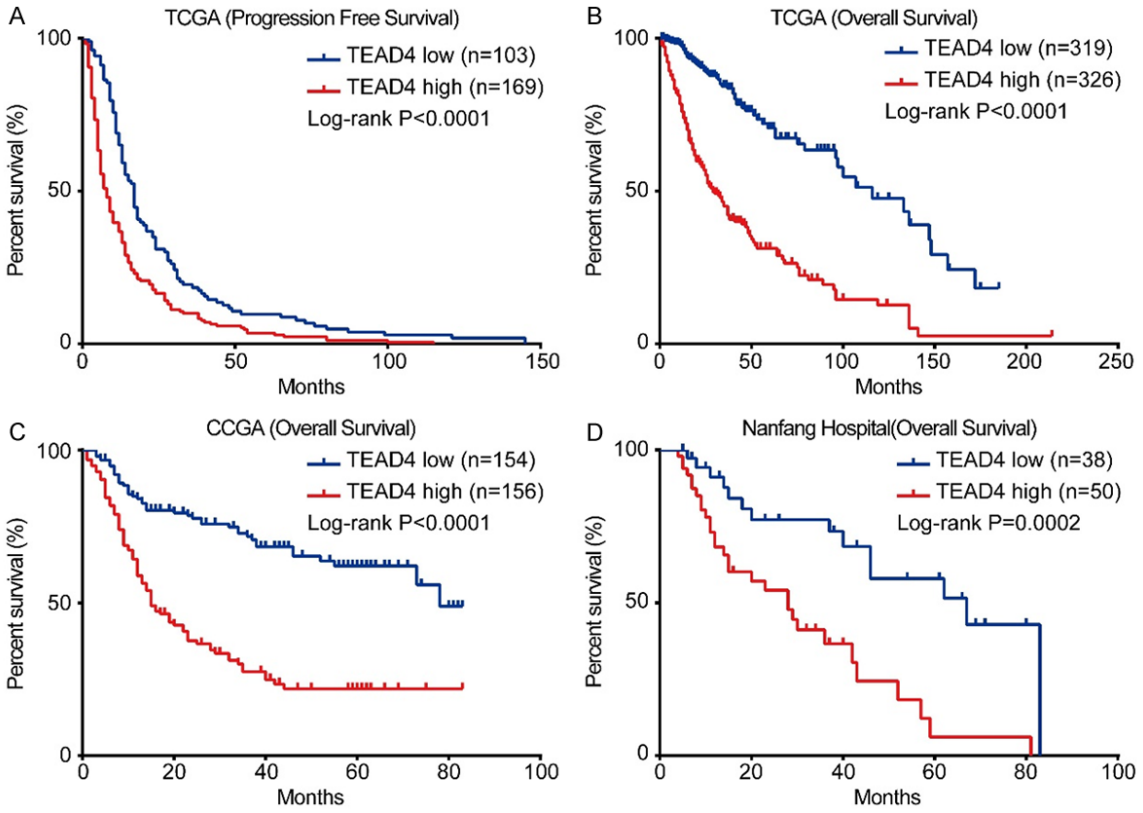

Figure 3. Overall survival and progression-free survival analysis for glioma patients according to TEAD4 expression levels (low and high). A. Comparison of progression free survival (PFS) of glioma patients with higher (n=169) and lower (n=103) expression of TEAD4 in TCGA databases (P<0.0001). B. Comparison of overall survival (OS) of glioma patients with higher (n=326) and lower (n=319) expression of TEAD4 in TCGA databases (P<0.0001). C. Comparison of overall survival (OS) of glioma patients with higher (n=156) and lower (n=154) expression of TEAD4 in CCGA databases. D. Comparison of overall survival (OS) of glioma patients with higher (n=50) and lower (n=38) expression of TEAD4 with collected samples from Nanfang Hospital (P=0.0002).

collected glioma tissues and 23 collected NB tissues using public database online (GSE42-90). TEAD4 mRNA was significantly increased in high-grade glioma (WHO grade III+IV; grade IV, n=76; grade III, n=32) compared with normal brain (NB) tissues (n=23) (P<0.0001) and low-grade glioma (WHO grade I+II, n=45). Furthermore, TEAD4 mRNA expression between normal brain (NB) tissues and low-grade glioma (WHO grade I+II) did not have statistical significance (P=0.1496) (**Figure 1**).

Analysis of TEAD4 protein expression in glioma and normal brain tissue

TEAD4 protein was found to be upregulated in 20 cases of glioma (WHO grade III+IV) compared with 6 cases normal brain (NB) tissues by western blot (P<0.0001) (**Figure 2A**). Furthermore, we also measured the expression levels and subcellular localization of TEAD4 protein in 88 archived paraffin-embedded gliomas and 26 NB samples using immunohistochemical staining (**Figure 2B**). TEAD4 protein was highly expressed in 56.8% (50/88) of glioma cells of glioma samples, while only 19.2% (5/26) of NB tissues, which was a significantly higher expression in glioma (**Table 1**).

The association between clinicopathological characteristics and TEAD4 expression in individuals was summarized. No significant association was found between TEAD4 expression levels and patient's age, sex, histologic type or tumor location. However, the expression of TEAD4 was positively corrected with the status of pathological classification (WHO I+II vs. WHO III+IV, P=0.017) (**Table 2**). The expression of TEAD4 was negatively correlated with IDH1 mutation (**Table 3**).

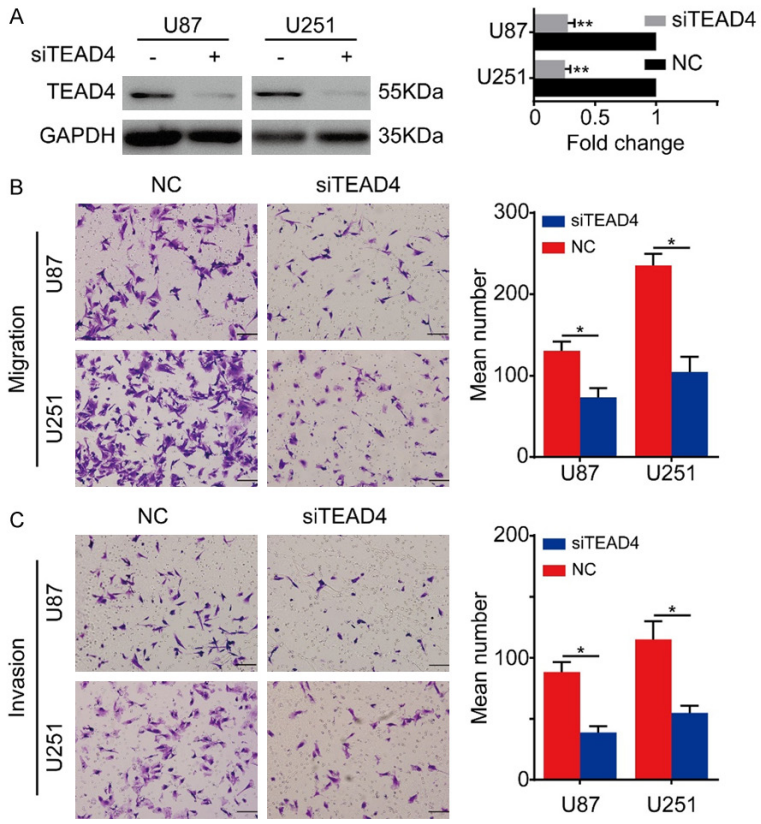

Figure 4. Knockdown of TEAD4 reduces cell migration and invasion *in vitro*. A. Western blot showed protein expression levels in NC and siTEAD4 treated U87 and U251; GAPDH served as a loading control. Bar graph shows the relative expression of protein among the groups. Data were presented as mean ± SD for three independent experiments. B. Downregulation of TEAD4 reduced U87 and U251 cell migration in vitro. Data are presented as mean ± SD for three independent experiments. C. Less expression of TEAD4 reduced U87 and U251 cell invasion *in vitro*. Data are presented as mean ± SD for three independent experiments. *P<0.05, significant difference. Scale bars.

The correlation between TEAD4 expression levels and patient survival

To investigate the prognostic value of TEAD4 expression for glioma, Kaplan-Meier analysis with the log-rank test was used to examine the relationship between the expression of TEAD4 and patient survival. With survival data obtained from TCGA and CGGA databases, we assessed that higher TEAD4 expression had worse progression free survival (PFS) and overall survival (OS). The median PFS of glioma patients with higher and lower expression of TEAD4 was 8 months and 17 months, respectively (**Figure 3A**, P<0.0001). The median OS among patients with higher TEAD4 expression was 29 months compared to 116 months among those with lower expression in TCGA databases (**Figure 3B**, P<0.0001). The median OS among patients with higher TEAD4 expression was 15 months compared to 78 months among those with lower expression in CGGA databases (**Figure 3C**, P<0.0001). Finally, we confirmed that higher TEAD4 expression had worse overall survival (OS) in 88 collected glioma cases with survival data (**Figure 3D**, median OS, high: 28 months, low: 67 months, P<0.0001).

Knockdown of TEAD4 suppresses glioma migration and invasion in vitro

To examine the effect of TEAD4 in glioma biofunction, siRNA was used to specifically knockdown the expression of TEAD4 in U87 and U251 glioma cell lines. The efficiency of the siRNA was confirmed by western blot (**Figure 4A**, P<0.05). To examine the effect of TEAD4 on glioma biofunction, we used siTEAD4-transfected U87 and U251 cells and negative control (NC) cells for migration and invasion assays. In Transwell assay, cells were cultured on Transwell apparatus. After incubation for the same hours, the percentage of migrated cells in siTEAD4-treated groups was significantly less, compared with NC groups (P<0.05) (**Figure 4B**). consistent with the result of Transwell assay, the siTEAD4-treated U251 and U87 cells both exhibited decreased invasiveness compared with NC cells in Boyden assay (P<0.05) (**Figure 4C**).

TEAD4 involved in glioma epithelial-mesenchymal transition

To obtain further molecular mechanisms of TEAD4 in glioma cell migration and invasion, gene set enrichment analysis (GSEA) was performed to predict the possible biological functional of TEAD4 in glioma. The data showed that there was a clearly correlation between TEAD4 mRNA expression and EMT (**Figure 5A**).

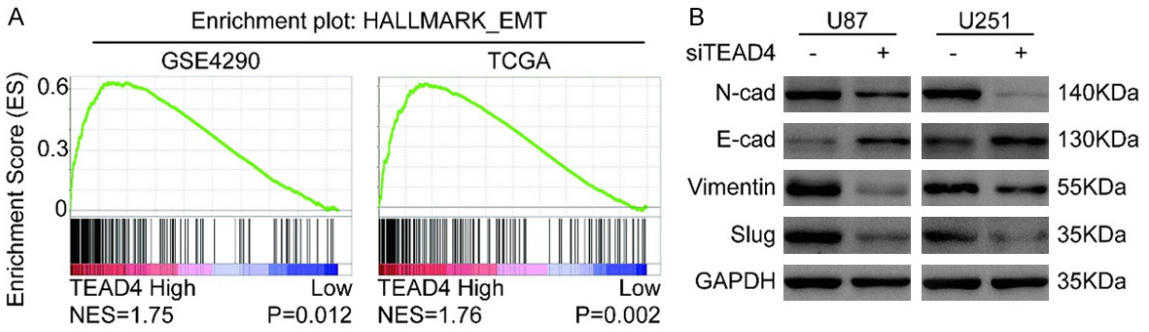

Figure 5. TEAD4 is involved in glioma epithelial-mesenchymal transition. A. Gene set enrichment analysis (GSEA) showed that there was a clear correlation between TEAD4 mRNA expression and the epithelial-mesenchymal transition (EMT) signature. B. Knockdown of TEAD4 enhanced the expression of E-cadherin and weakened the expression level of N-cadherin, vimentin and Slug in U87 and U251 cells. GAPDH was used as a loading control.

To confirm this finding, the expressions of several EMT-associated proteins were examined in U87 and U251 cells. After TEAD4 knockdown in U87 and U251 cells, the expression of E-cadherin was upregulated, but N-cadherin, vimentin, and slug were downregulated (**Figure 5B**).

Discussion

Overexpression of TEAD4 has been observed in some other types of tumors such as breast cancer, colorectal cancer, and gastric cancer. However, the role of TEAD4 in human glioma has not been revealed. In our study, we confirmed that TEAD4 was upregulated in glioma not only in mRNA level but also in protein level confirmed by western blot and immunohistochemistry analyses. In our data, higher level of TEAD4 was correlated to shorter overall survival (OS) time and progression free survival (PFS) time of patients. Moreover, levels of TEAD4 expression were positively associated with pathology classification in human glioma. All these suggested that TEAD4 may serve as a new important prognosis marker of glioma.

In agreement with previous studies, we further explored the biological functions of TEAD4 in glioma and found that knockdown of TEAD4 significantly downregulated glioma cells migration as well as invasion. GESA analysis revealed the TEAD4 was related to EMT closely in glioma. Expression of the EMT-related signature in tumors is correlated with poor prognosis, which involved in glioma cell migration and invasion [16]. Knockdown of TEAD4 with siRNA suppressed the EMT progression, as the western blot showed. The upregulated expression of E-cadherin and the downregulated expression of mesenchymal cell markers, including N-cadherin, Vimentin, Slug, and Snail were examined. The biological functions of TEAD4 in our study provided a mechanistic basis for the pathological and clinical observations.

In the past mechanical studies of TEADs, TEAD4 was one of the most effective members of the TEAD family which includes TEAD1-4 [5, 9]. TEADs contain a deep hydrophobic cavity like a pocket to accommodate low molecular weight compounds [17]. For example, in the hippo signaling pathway, YAP/TAZ is a transcription co-activator that regulated gene expression primarily through interacting with TEADs [7, 8, 11, 18]. TEAD4 acylation significantly enhanced YAP/TAZ stability. TEAD4 is one of most distal elements of Hippo pathway, which is essential to control organ size, tissue regeneration, stem cell self-renewal and so on [9, 19]. In addition to YAP/TAZ, TEAD4 can interact with other co-actors such as VGLL family members in mammalian cells [20]. The previous of TEAD4-knockout mice showed that TEAD4 played an important part in trophectoderm formation, regulating the trophectoderm (TE)-specific transcriptional program [21].

TEAD4 is not only crucial for the development process, but also involved in several cancer types. Recent oncology research revealed that TEAD4 was overexpressed in breast cancer [13, 22, 23], colorectal cancer [11, 24], gastric cancer (GC) [10, 25], and oral squamous cell carcinomas (OSCCs) [26]. Moreover, several findings suggested that TEAD4 overexpression is associated with aggressive tumor behavior,

such as proliferation, metastasis, migration, invasion, and so on [26, 27]. United with our research in glioma, these studies suggested that TEAD4 may be a poor prognostic factor and a potential therapeutic target for human malignant cancers. Thus, it is very interesting to further explore the potential of using TEAD4 in some more cancers or diseases as an individual prognosis biomarker or in combination with other biomarkers.

In summary, TEAD4 expression may be a valuable prognosis marker not only in glioma but also in some kinds of other cancers. Of course, further studies are required. We also have provided convincing evidence that downregulation of TEAD4 inhibits cell migration and invasion.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

TEAD4, TEA domain transcription factor 4 (TEAD4); EMT, Epithelial-Mesenchymal Transition; TCGA, The Cancer Genome Atlas; CGGA, Chinese Glioma Genome Atlas; WB, Western blot; IHC, Immunohistochemistry.

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