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TGF-β induced miR-132 enhances the activation of TGF-β signaling through inhibiting SMAD7 expression in glioma cells

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ABSTRACT

Transforming growth factors β (TGF-β) pathway has been proven to play important roles in oncogenesis and angiogenesis of gliomas. MiR-132 might be related to TGF-β signaling pathway and high miR-132 expression was reported to be a biomarker of poor prognosis in patients diagnosed with glioma. However, the expression regulation way involved in TGF-β pathway and clinical significance of miR-132 have not been investigated in glioma cells. Here we reported that the mRNA level of miR-132 and TGF-β concentration were both increased in patients with brain glioma. Correlation analysis revealed that TGF-β concentration was positively correlated with mRNA level of miR-132. In addition, the mRNA level of miR-132 was up-regulated by TGF-β in a concentration-dependent and time-dependent manner. Furthermore, we found that miR-132 was involved in modulation of the TGF-β signaling pathway and down-regulation of SMAD7 expression by directly targeting the SMAD7 3′-UTR. MiR-132 was negatively correlated with SMAD7 in patients with brain glioma. Taken together, our results suggest that miR-132 could be stimulated by TGF-β and might enhance the activation of TGF-β signaling through inhibiting SMAD7 expression in glioma cells. These findings contribute to a better understanding of the mechanism of the activation of TGF-β signaling by miR-132.

1. Introduction

Gliomas represent the most common tumors in the central nervous system [1]. Because of their invasiveness and high recurrence rate, gliomas lead to a high mortality rate. Despite the advances in therapeutic intervention, such as glioma surgery, radiotherapy, chemotherapy, gene therapy, immunotherapy and other novel biological therapies, prognosis and treatment of this tumor type continue to be dismal [2]. Mounting evidence suggests that transforming growth factors β (TGF-β) play a critical role in oncogenesis and angiogenesis of gliomas, which attracts increasing attention as a potential target for the treatment of gliomas [3]. Therefore, understanding the key regulatory mechanisms mediating TGF-β signal pathway is important to develop novel and effective therapeutic strategies for gliomas.

TGF-β is a multifunctional polypeptide cytokine and activates a membrane receptor serine/threonine kinase complex [4]. TGF-β plays an important role in cancer development and cancer cell migration. Studies also showed that TGF-β was highly active in high-grade glioma and that elevated TGF-β activity confered poor prognosis in glioma patients [5]. When binding to TGF-β, the receptor complex phosphorylates the transcription factors SMAD2 and SMAD3, which then bind to SMAD4 and translocate into the nucleus, where they regulate transcription [6].

MicroRNAs (miRNAs) are approximately 22-nucleotide-long, single-stranded, noncoding RNA molecules that negatively regulate gene expression through complementarity to the 3′ untranslated region (3′-UTR) of mRNAs [7]. Many miRNAs are abnormally expressed in gliomas and involve in the origin and development of glioma [8–10]. As a member of the miRNAs family, miR-132 was found to be overexpressed in glioma and high miR-132 expression was a biomarker of poor prognosis in patients diagnosed with glioma [11]. Accumulating evidence indicate that miR-132 may be related to TGF-β signaling pathway [12,13]. Bo Yang et al. reported that miR-132 was significantly down-regulated in TGF-β3 induced

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mesenchymal stem cell chondrogenesis [13]. However, its expression regulation way and clinical significance have not been investigated in glioma cells.

The primary aim of this study was to investigate the relationship between miR-132 and TGF-β signal pathway in glioma cells. We found that TGF-β concentration was positively correlated with mRNA level of miR-132 and they were mutually improved in glioma cells. In addition, miR-132 enhanced the activation of TGF-β signaling through inhibiting SMAD7 expression in glioma cells.

2. Materials and methods

2.1. Brain tissues

This study was approved by the Huaihe Hospital of Henan University. Informed consents were obtained from all subjects. A total of 20 human glioma tissue samples and blood samples were obtained from patients who underwent surgery in the Department of Neurosurgery of Huaihe Hospital of Henan University. Samples resected during the operation were divided into two parts. One part was frozen and stored in liquid nitrogen until RNA isolation, and the other one was fixed in formalin for pathologic diagnosis by two pathologists according to the 2007 WHO classification. The characteristics of patients were showed in Table 1. None of the patients received preoperative treatment, such as radiation or chemotherapy. Twenty normal brain tissue samples used as controls were obtained from internal decompression of patients who underwent surgical operation for cerebral injury and cerebral hemorrhage.

2.2. Cell culture and transfection

Human glioma cell lines U87MG used in this study were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone) supplemented with 10% FBS (TSB, Tianjin, China) in a humidified chamber with 5% CO2 at 37 °C. Oligonucleotides miR-132 mimics (hsa-miR-132 mimics), NC (miR-control), miR-132 inhibitor (anti-miR-132), inhibitor NC (anti-miR-control), were purchased from GenePharma (Shanghai, PR China). Cells were seeded into 6-well plates and transfected with 100 nM miR-132 mimic, miR-132 inhibitor or corresponding controls using Lipofectamine 2000 (Invitrogen, USA) according to the instructions provided by the manufacturer. Transfection efficiency was monitored by qRT-PCR.

Table 1
Characteristic of individuals.

Variable	Patients
Number of cases	20
Sex	
Male/Female	11/9
Age at diagnosis (mean)	48.2
Clinical pathology features	
Extent of resection	
Subtotal	6
Total	14
Radiographic pattern	
Solitary lesion	13
Invasive and multifocal lesions	7
WHO grading	
I–II	8
III–IV	12
KPS score	
≥80	13
<80	7

WHO: World Health Organization, *KPS*: Karnofsky performance score.

2.3. Quantitative real-time PCR

Total RNA was extracted with Trizol Reagent (Takara, Dalian, China) according to the manufacturer's instructions. NanoDrop ND-2000 Spectrophotometer (NanoDrop Technologies, Houston, TX, USA) was used to detected the concentration and purity of all RNA samples. cDNA was synthesized using M-MLV reverse transcriptase (Promega) according to the supplier's protocol. FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Indianapolis, IN, USA) was used to amplify SMAD7 and mature miR-132. The mRNA and miRNA expression levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 small nuclear RNA (snRNA), respectively. qRT-PCR was performed by using the ABI 7900 Fast Real-Time PCR system (ABI, CA, USA). Data were analyzed by using the $2^{-\Delta\Delta Ct}$ method.

2.4. Western blotting

The expression of phosphorylated SMAD2/3 and SMAD7 in U87MG cells was determined by western blot analyses. Cells were lysed using ice-cold RIPA buffer and centrifuged at 14,000 × g 4 °C for 5 min. Protein concentrations were determined using the BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). The antibodies against phosphorylated SMAD2 (1:500; Abcam), phosphorylated SMAD3 (1:500; Abcam), and SMAD7 (1:500; Abcam) were used in this experiment, along with horseradish peroxidase-conjugated secondary antibodies (Bioss, Beijing, China). The scanned images were semi-quantitated using Quantity One software (Bio-Rad Laboratories, Milan, Italy). The relative integrated density values were calculated by the FluorChem 2.0 software and normalized with GAPDH.

2.5. Luciferase activity assay

For pGL3-SMAD7-3′UTR plasmid construction, the SMAD7-3′UTR DNA fragment containing miR-132 binding sites was amplified and cloned into the dual-luciferase reporter vector pGL3 (Promega, Madison, WI, USA). U87MG cells were seeded in 24-well plates (1×10^5 cells/well) and cotransfected with pGL3-SMAD7-3′UTR and either miR-132 mimics (inhibitor) or a negative control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 12 h, cells were lysed and the luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. U87MG cells were transfected with miRNA mimics or inhibitor and collected 24 h later for analysis by quantitative RT-PCR and 48 h later for analysis by Western blotting.

2.6. Measurement of TGF-β concentration using ELISA

The peripheral blood from patients with brain glioma and non-cancer individuals were collected and centrifuged. TGF-β concentration was examined with an ELISA kit (R&D Systems, Shanghai, China) according to the manufacturer's instructions. Absorbance (A) was measured at 450 nm, and the TGF-β concentration of each sample was calculated based on a standard curve and was expressed in nanograms per milliliter.

2.7. Statistical analysis

All data were expressed as mean ± standard deviation (means ± SD) and were analyzed with SPSS software. All variables measured in this study were normally distributed, and the groups were compared with Student's t-test or one-way analysis of

variance (ANOVA), followed by Newman–Keuls post-hoc analysis. A P value less than 0.05 was considered to be statistically significant.

3. Results

3.1. TGF-β is positively correlated with miR-132 in patients with brain glioma

We determined the expression levels of miR-132 in glioma brain tissue and non-cancer brain tissue with qRT-PCR. Then we found the higher mRNA levels of miR-132 in glioma brain comparing to the controls (Fig. 1A). Furthermore, ELISA was performed to analyze the concentration of TGF-β in the peripheral blood of patients with brain glioma and non-cancer individuals. As shown in Fig. 1B, TGF-β concentration was enhanced in the peripheral blood of patients with brain glioma. The relationship between miR-132 and TGF-β was studied by correlation analysis. And the results showed that TGF-β concentration was positively correlated with mRNA level of miR-132 (Fig. 1C).

3.2. The relationship between TGF-β and miR-132

To further elucidate the relationship between TGF-β and miR-132, we detected the expression of miR-132 in U87MG cells treated with different concentration (2.5 ng/ml, 5 ng/ml, 10 ng/ml) of TGF-β. The mRNA level of miR-132 was up-regulated by TGF-β in a concentration-dependent manner (Fig. 2A). When we treated U87MG cells with 5 ng/ml TGF-β for 6 h, 12 h and 24 h, the mRNA level of miR-132 was detected. As shown in Fig. 2B, the mRNA level of miR-132 was also increased in a time-dependent manner. These results indicated that miR-132 could be stimulated by TGF-β. On the other hand, we examined the effect of miR-132 on the TGF-β signal pathway. U87MG cells were treated with miR-132 mimic, miR-132 inhibitor or corresponding controls, and western blotting was performed to detect the protein level of phosphorylated SMAD2 and phosphorylated SMAD3. As demonstrated in Fig. 2C and D, the protein level of phosphorylated SMAD2 and phosphorylated SMAD3 were enhanced by miR-132 mimic and suppressed by miR-132 inhibitor. These findings supposed that TGF-β signal pathway could be activated by miR-132. Taken together, these data indicated that TGF-β and miR-132 were mutually improved.

3.3. SMAD7 is a target of miR-132 in glioma cells

Bioinformatics methods was used to identify the target of miR-132 in glioma cells. SMAD7 3′UTRs was found to have binding sequences for miR-132 (Fig. 3A). To verify the effects of miR-132 on SMAD7, luciferase activity assay was performed. Here, we found that miR-132 significantly inhibited the luciferase activity of 3′-UTR of SMAD7 in U87MG cells (Fig. 3B). Moreover, overexpression of miR-132 significantly suppressed levels of both SMAD7 mRNA and protein, whereas the inhibition of miR-132 significantly increased these levels (Fig. 3C–E). These data suggested that miR-132 might induce the degradation of SMAD7 mRNA, leading to a reduction in levels of SMAD7 protein and implying that SMAD7 is a direct target of miR-132.

3.4. miR-132 is negatively correlated with SMAD7 in patients with brain glioma

Further experiments were performed to investigate the expression of SMAD7 in glioma brain tissue and normal brain tissue. qPCR showed that the mRNA expression of SMAD7 in glioma

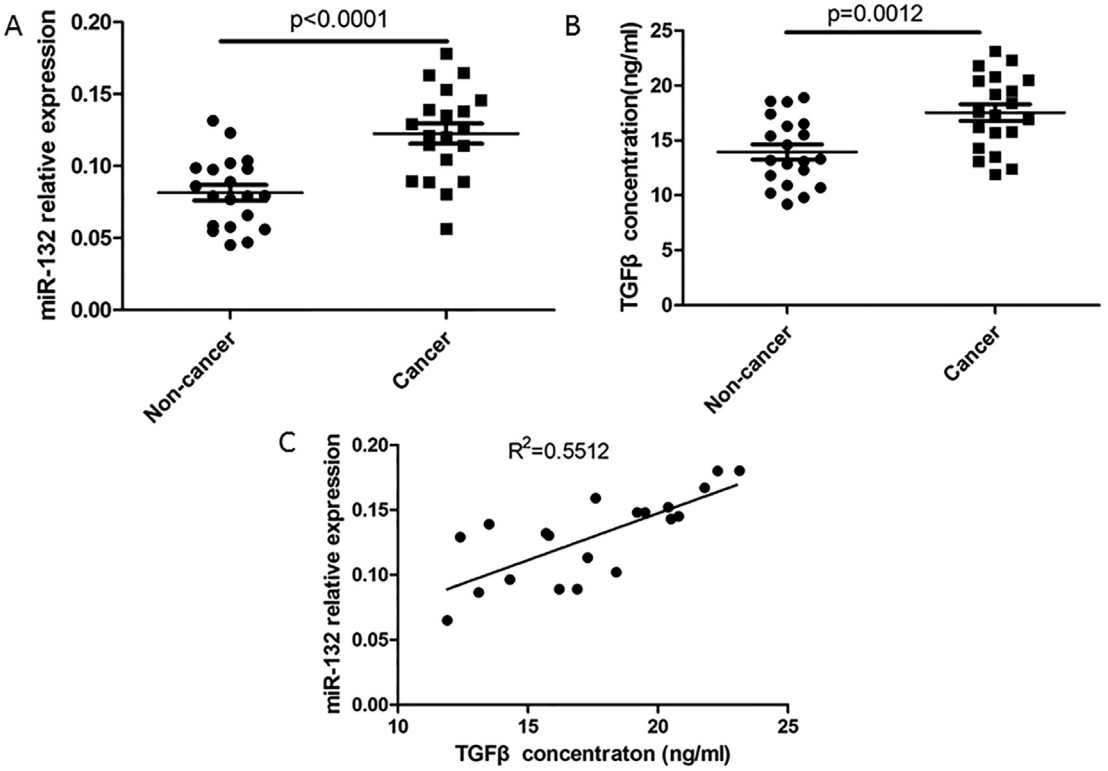

Fig. 1. Expression of miR-132 and TGF-β in patients with brain glioma and non-cancer individuals. A. qRT-PCR was performed to detect the expression of miR-132 in glioma brain tissue and non-cancer brain tissue. **B**. ELISA analyzed the expression of TGF-β in the peripheral blood of patients with brain glioma and non-cancer individuals. **C**. Association of miR-132 mRNA levels and TGF-β concentration. Spearman's correlation coefficient [rs] = $r^2 = 0.5512$, $P < 0.001$.

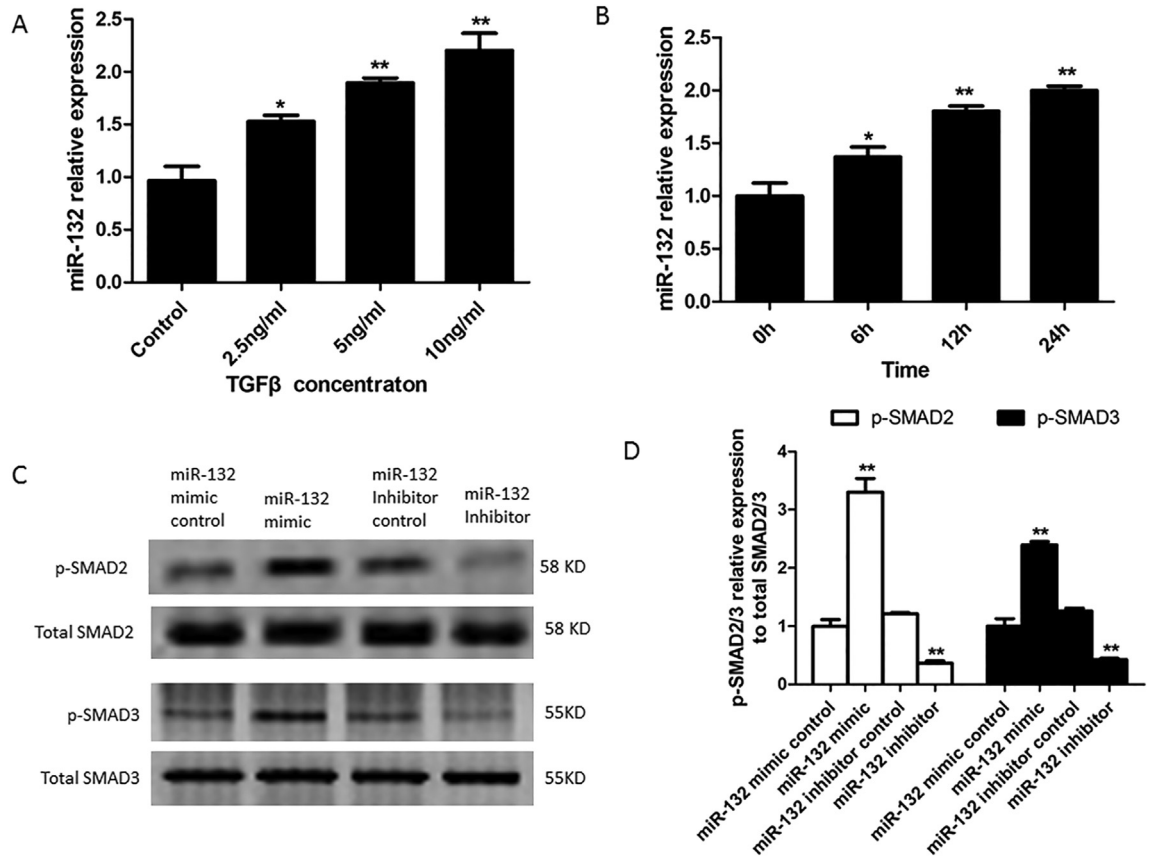

Fig. 2. The relationship between TGF-β and miR-132. A. qRT-PCR was performed to detect the expression of miR-132 in U87MG cells treated with different concentration of TGF-β. **B**. qRT-PCR was performed to detect the expression of miR-132 in U87MG cells treated with TGF-β (5 ng/ml) for different time. **C**. Western blotting analyzed TGF-β signal pathway in U87MG cells treated with miR-132 mimic, miR-132 inhibitor or control. **D**. phosphorylated SMAD2/3 relative expression to total SMAD2/3 according to the results of Western blotting. The results are shown as mean ± S.D. from three representative independent experiments $^{*}p < 0.05$; $^{**}p < 0.01$.

brain tissue was lower than that in control (Fig. 4A). Moreover, we detected the relationship between miR-132 and SMAD7 expression in patients with brain glioma. The results of correlation analysis revealed that miR-132 is negatively correlated with SMAD7 in patients with brain glioma (Fig. 4B).

4. Discussion

In the present study, we found that the mRNA level of miR-132 and TGF-β concentration were both increased in patients with brain glioma. Correlation analysis revealed that TGF-β concentration was positively correlated with mRNA level of miR-132. In addition, the mRNA level of miR-132 was up-regulated by TGF-β in a concentration-dependent and time-dependent manner. Furthermore, we found that miR-132 was involved in modulation of the TGF-β signaling pathway and down-regulation of SMAD7 expression by directly targeting the SMAD7 3′-UTR. Taken together, our results suggest that miR-132 plays an important role in the activation of TGF-β signaling pathway in gliomas.

The release of immune-inhibitory molecules such as TGF-β is commonly observed in glioblastoma [14]. TGF-β is a superfamily of multifunctional growth factors that plays an important role in the formation of gliomas and the incidence of the overall tumor development process. Studies have shown that TGF-β plays a dual role in the occurrence and development of tumor [15]. TGF-β can be used as an inhibitor of tumor growth in the early stage of tumor growth and then function as an oncogenic factor inducing cell proliferation, invasion, angiogenesis, and immune suppression with tumor progression [1]. Jing Sun et al. showed that TGF-β promoted glioma cell growth via activating Nodal expression through SMAD and ERK1/2 pathways [5]. Alejandra Bruna found that high TGFβ-SMAD activity was present in aggressive, highly proliferative gliomas and conferred poor prognosis in patients with glioma [15]. Here, we also found the higher TGF-β concentration in the peripheral blood of patients with brain glioma.

According to the experimentally validated interactions between TGF-β signaling and miRNAs, it is shown that miRNAs influence the TGF-β pathway at multiple levels. In addition, TGF-β signaling itself enhances the maturation of miRNAs, resulting in a bidirectional functional link [16]. Interestingly, we found this bidirectional functional link between TGF-β pathway and miR-132. When U87MG cells were treated with different concentration of TGF-β or 5 ng/ml TGF-β for different time, we found that mRNA level of miR-132 was up-regulated in a concentration-dependent and time-dependent manner. Conversely, TGF-β/SMAD2/3 signal pathway could be activated in U87MG cells treated with miR-132 mimic. These data indicated that TGF-β pathway and miR-132 were mutually improved. Recently, some studies showed that the abnormal expression of miR-132 was related to a variety of human tumors, such as osteosarcoma, breast cancer, hepatocellular carcinoma (HCC), prostate cancer, gastric cancer [17–21]. Qiang Liu found that miR-132 was overexpressed in glioma and might be an independent biomarker for predicting the prognosis of glioma [11].

Study evidence indicated that miRNAs regulated the TGF-β signaling via direct interaction with downstream members of canonical signaling pathways. In this study, bioinformatics methods

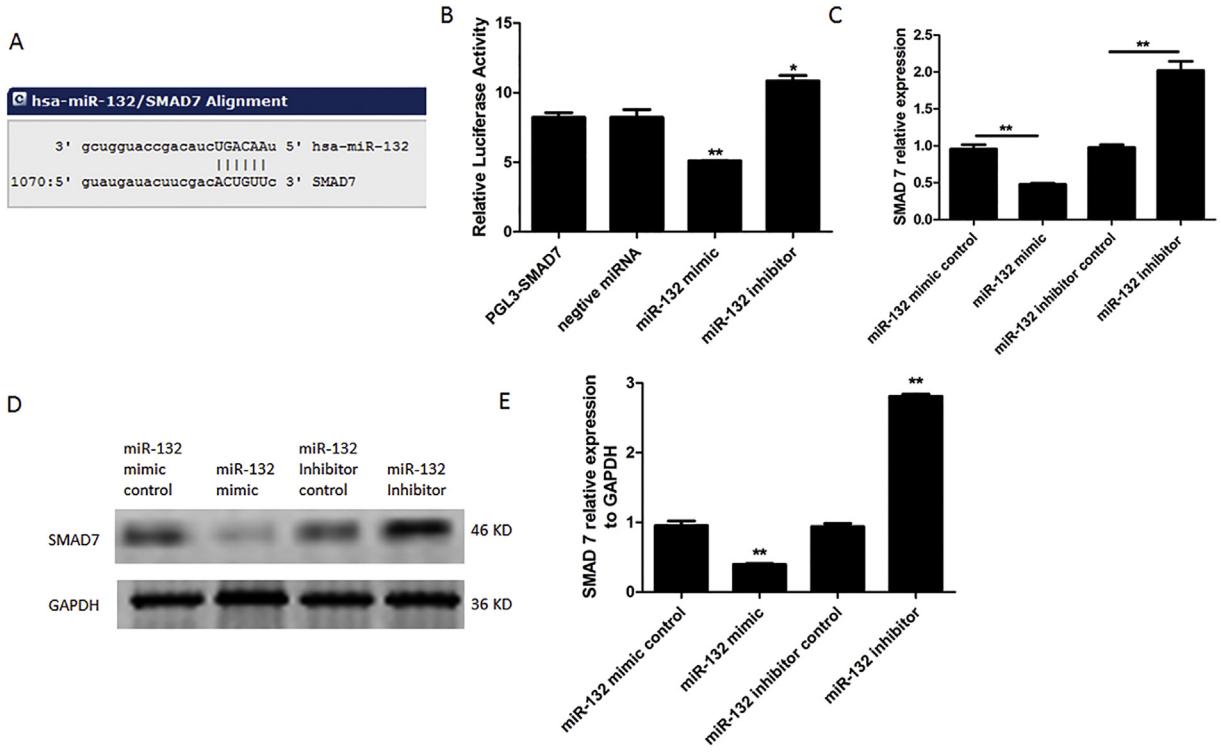

Fig. 3. miR-132 targets SMAD7. **A**. SMAD7 3′UTRs have binding sequences for miR-132. **B**. The luciferase activity of U87MG cells was measured after co-transfection with the indicated SMAD7 3′UTR constructs and miR-132 mimic, miR-132 inhibitor or control for 12 h. **C**. qRT-PCR was performed to detect the expression of SMAD7 in U87MG cells transfected with miR-132 mimic, miR-132 inhibitor or control for 24 h. **D**. Western blotting analyzed the protein level of SMAD7 in U87MG cells transfected with miR-132 mimic, miR-132 inhibitor or control for 48 h. **E**. SMAD7 relative expression to GAPDH according to the results of Western blotting. The results are shown as mean ± S.D. from three representative independent experiments. *$p < 0.05$; **$p < 0.01$.

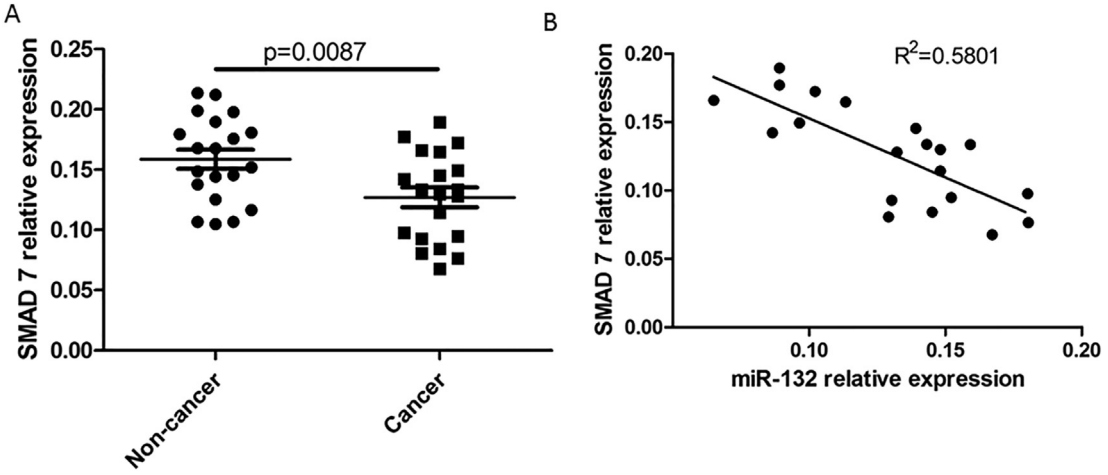

Fig. 4. Expression of SMAD7 in patients with brain glioma and non-cancer individuals. A. qRT-PCR was performed to detect the expression of SMAD7 in glioma brain tissue and normal brain tissue. **B**. Association of miR-132 and SMAD7mRNA levels. Spearman's correlation coefficient [rs] = $r^2 = 0.5801$, $P < 0.001$.

and luciferase activity assay indicated that miR-132 might induce the degradation of SMAD7 mRNA and lead to a reduction in levels of SMAD7 protein, which implied that SMAD7 was a direct target of miR-132. SMAD6 and 7 were reported to be inhibitory proteins that acted to negatively regulate TGF-family signaling [22]. In the liver and lung, miR-21 can also enhance TGF-β signaling through targeting the negative regulator SMAD7 [23]. In addition, many signaling pathways were shown to activate the expression of SMAD7 and thus led to attenuation of TGF-β signaling, such as the activation of ERK, JNK or p38 MAPK pathway [24].

In summary, we conclude that miR-132 could be stimulated by TGF-β and might enhance the activation of TGF-β signaling through inhibiting SMAD7 expression in glioma cells. These findings contribute to a better understanding of the mechanism of the activation of TGF-β signaling by miR-132.

Conflict of interest

No conflicts of interest were declared.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.001.

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