

Original Article

FOXK1 promotes cell growth through activating wnt/ β -catenin pathway and emerges as a novel target of miR-137 in glioma

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Abstract: Glioma is the most common primary malignant brain tumor in adults. Forkhead box k1 (FOXK1) was reported to be dysregulated and play important roles in multiple human cancers. However, the expression pattern and roles of FOXK1 in glioma has never been investigated. In this study, we firstly observed that the expression of FOXK1 was significantly increased in glioma tissue samples and cell lines. Functional assays demonstrated that overexpression of FOXK1 promoted proliferation, cell cycle transition and inhibited apoptosis in glioma cell lines. On the contrary, knockdown of FOXK1 exhibited an opposite effect on glioma cells proliferation, cell cycle and apoptosis. Data of western blot indicated that FOXK1 overexpression increased while FOXK1 knockdown decreased the levels of β -catenin, c-myc and cyclinD1 in glioma cells. Moreover, we demonstrated that FOXK1 was a novel target of miR-137 in glioma and FOXK1 restoration abolished the tumor suppressive effect of miR-137 in glioma cells. Statistical analysis showed that the mRNA level of FOXK1 was inversely correlated with miR-137 expression in glioma tissues. In conclusion, the present study demonstrated that FOXK1 promoted cell growth through activating wnt/ β -catenin pathway and is negatively regulated by miR-137 in glioma.

Keywords: FOXK1, wnt/ β -catenin, glioma, miR-137

Introduction

Glioma is the most common primary malignant brain tumor, and accounts for 50-60% of all brain malignancies in adults [1]. It is characterized by high mortality rate, recurrence and malignancy, due to difficult to completely remove by operation, and not sensitive to radiotherapy and chemotherapy [2, 3]. According to the histological characteristics, glioma can be divided into several subtypes including astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas [4]. Although great improvements have been made in the diagnosis and treatment of glioma over the past few decades, the prognosis of patients with glioma still remains poor. The 5-year survival rate of patients with low-grade gliomas (WHO grade I and II) is approximately 30 to 70%, while the median survival time of patients with glioblastoma multiforme (GBM) (grade IV) ranges from 9 to 12 month [5]. Therefore, identifying novel biomark-

ers involved in the development of glioma is needed to develop new therapeutic strategies and improve the clinical outcome for patients with glioma.

The forkhead box (Fox) gene family is a group of highly conserved transcription factors that are characterized by the presence of an evolutionary conserved "forkhead" or "winged-helix" DNA-binding domain [6, 7]. FOX proteins are reported to play important roles in embryonic development and organogenesis [8, 9]. Forkhead box k1 (FOXK1), a member of the FOX transcription factor family, has been documented to play pivotal roles in cell proliferation, cell growth, and metabolism [10]. Recently, increasing evidence indicated the significance of FOXK1 in tumorigenesis. FOXK1 is dysregulated in various human cancers, including colorectal cancer [11], esophageal cancer [12] and hepatocellular carcinoma [13]. However, the expression pattern and roles of FOXK1 in glioma has never been investigated.

Role of FOXK1 in glioma

MicroRNAs (miRNAs) are a group of small, endogenous and non-coding RNAs that directly bind to the 3'untranslated region (3'UTR) in target mRNAs to regulate gene expression [14, 15]. Increasing studies demonstrated that the aberrant expression of miRNAs is involved in the initiation and development of multiple human cancers including glioma [16, 17]. miR-137 was reported as a tumor suppressor and downregulated in different types of human cancers such as cervical cancer [18], melanoma [19] and pancreatic cancer [20]. However, the importance of miR-137 in the development of glioma is unclear.

In the present study, we investigated for the first time the expression level and biological function of FOXK1 in glioma. Firstly, we detected the expression of FOXK1 in glioma tissue samples and cell lines. Then, we assessed the effects of FOXK1 on glioma cell lines growth and apoptosis by FOXK1 overexpression or knockdown. In mechanism, we demonstrated that FOXK1 may exist its functions in glioma through activation wnt/ β -catenin pathway. Furthermore, we identified FOXK1 as a novel target of miR-137 in glioma and FOXK1 restoration abolished the tumor suppressive effect of miR-137 in glioma cells.

Materials and methods

Clinical samples and cell lines

A total of 30 human glioma specimens and 10 normal brain tissues were obtained from Department of Neurosurgery, Liaocheng People's Hospital. Tissue samples were immediately frozen in liquid nitrogen after surgical removal and stored at -80°C until use. This study was approved by the Ethics Committees of Liaocheng People's Hospital and written informed consent was obtained from each patient prior operation. Human glioma cell lines A172, U251, Ln229, U87 and normal human astrocytes (NHA) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle medium (DMEM) (Gibco, CA, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen). Cells were maintained in a humidified incubator containing 5% CO_2 at 37°C .

RNA isolation and qRT-PCR

Total RNA was isolated from tissues or cultured cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed on an ABI 7500HT fast real-time PCR System (Applied Biosystems, CA, USA). mRNA levels were detected by using the SYBR Green PCR Master Mix and miR-137 levels were detected by using the TaqMan microRNA assay kit (Applied Biosystems). The relative expression of FOXK1 and miR-137 was normalized to the internal control GAPDH and U6, respectively, by using the $2^{-\Delta\Delta\text{Ct}}$ method. The sequences of PCR primers were as follows: FOXK1 forward, 5'-ACACGTCTGGAGGAGACAGC-3' and reverse, 5'-GAGAGGTTGTGCCGATAGA-3'. miR-137 forward, 5'-GCGCTTATTGCTTAAGAATAC-3' and reverse, 5'-CAGTGCAGGGTCCGAGGT-3'. GAPDH forward, 5'-AACGGATTTGGTCGTATTG-3' and reverse, 5'-GGAAGATGGTGATGGGATT-3'. U6 forward, 5'-CTCGCTTCGGCAGCAC-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. Primers were purchased from Ribobio (GuangZhou, China). The experiment was independently repeated three times.

Western blot

Tissue samples and cultured cells were lysed with radio immune precipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Proteins were separated by SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocked in 5% nonfat milk in TBST for 1 h, membranes were incubated with primary antibodies overnight at 4°C . GAPDH was used as internal control. All the primary antibodies were purchased from Abcam (Cambridge, UK). Following three times of wash, the membranes were incubated with horseradish peroxidase-coupled secondary antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Finally, the signals were detected with the ECL analysis system (Amersham; GE Healthcare, Chicago, IL, USA).

Cell transfection

Cell transfections were all performed using Lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. For FOXK1 overexpression, the FOXK1 cDNA was obtained by

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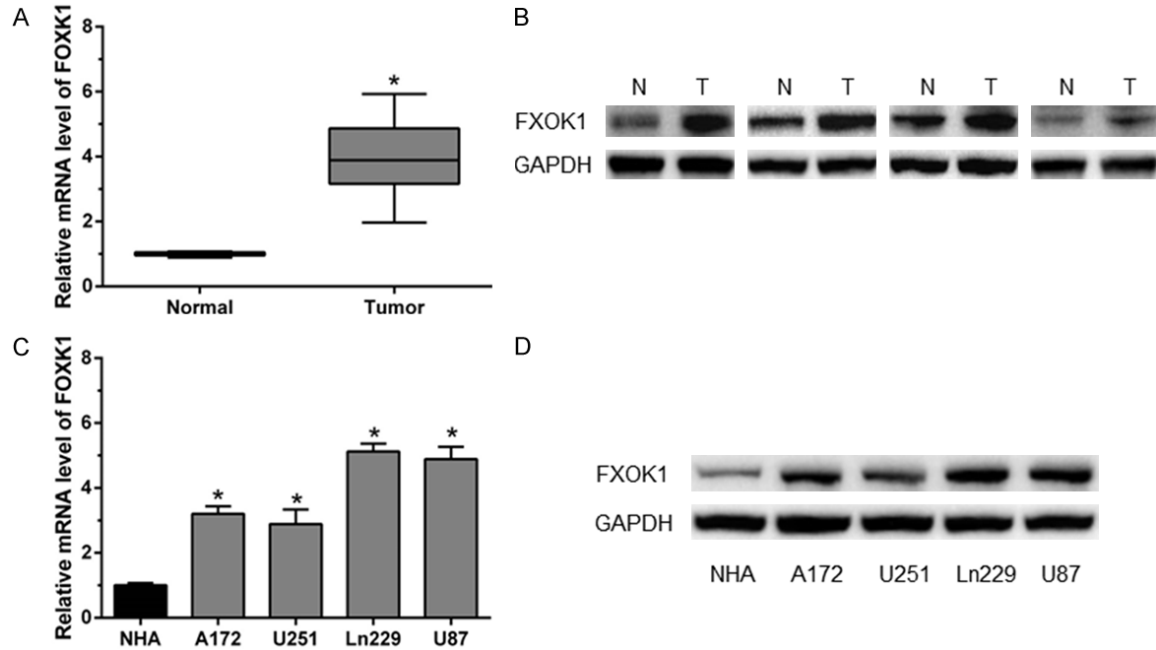


Figure 1. FOXK1 is upregulated in glioma tissues and cell lines. A. The mRNA levels of FOXK1 in glioma tissues (Tumor) and normal brain tissues (Normal) as determined by qRT-PCR. B. Representative images of FOXK1 protein expression in glioma tissues (T) and normal brain tissues (N) as detected by western blot. C. The mRNA levels of FOXK1 in glioma cell lines A172, U251, Ln229, U87 and normal human astrocytes (NHA) as determined by qRT-PCR. D. The FOXK1 protein expression in four glioma cell lines and normal human astrocytes (NHA) as detected by western blot. Data were mean \pm SD. *P < 0.05.

RT-PCR amplification and subcloned into the vector pcDNA3.1 (Invitrogen, Carlsbad, CA) to construct the FOXK1 expression plasmid. The empty vector was used as a control. Small interfering RNA targeting FOXK1 (si-FOXK1) and the negative control (si-NC) were synthesized by Genepharma Company (Shanghai, China). The sequence of si-FOXK1 is as follow: 5'-GAGACAGCCCCAAGGAUGA-3' (sense) and 5'-UCAUCCUUGGGCUGUCUC-3' (antisense). miR-137 mimics (miR-137) and its non-specific control miRNA (miR-NC) were purchased from RiboBio (Guangzhou, China). The sequences of miR-137 mimics were: 5'-UUAUUGCUAAGAAUACGCGUAG-3' (sense) and 5'-ACGCGUUAUCUUAAGCAAUAAUU-3' (antisense). The efficiency of transfection was confirmed 48 hours later.

Cell proliferation assay

Cell proliferation was determined by using a Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) following the manufacturer's guideline. In brief, cells were seeded in 96-well plates and cultured for 24, 48, 72, and 96 h, respectively. Then, 10 μ l CCK-8 reagent was added to each well and cultured for further 2 hours. OD450

was measured with the microplate reader. This experiment was performed three times.

Apoptosis and cell cycle analysis

Cell apoptosis was analyzed by using an Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA). Briefly, cells were collected and resuspended in 200 μ l of binding buffer. After incubated with Annexin V-FITC and propidium iodide (PI) in darkness at room temperature for 15 min, apoptosis rate was detected using a Calibur Flow Cytometer (Becton, Dickinson and Company, CA, USA). For cell cycle analysis, cells were collected and fixed with 70% ethanol at 4°C overnight. After washed twice with cold PBS, cells were stained with propidium iodide (PI, sigma) at room temperature for 30 min and the proportion of cells in each phase was counted by Calibur Flow Cytometer. Each experiment was conducted triplicated.

Luciferase reporter assay

The wild-type (wt) and mutant (mut) 3'UTR of FOXK1 were created and cloned from human

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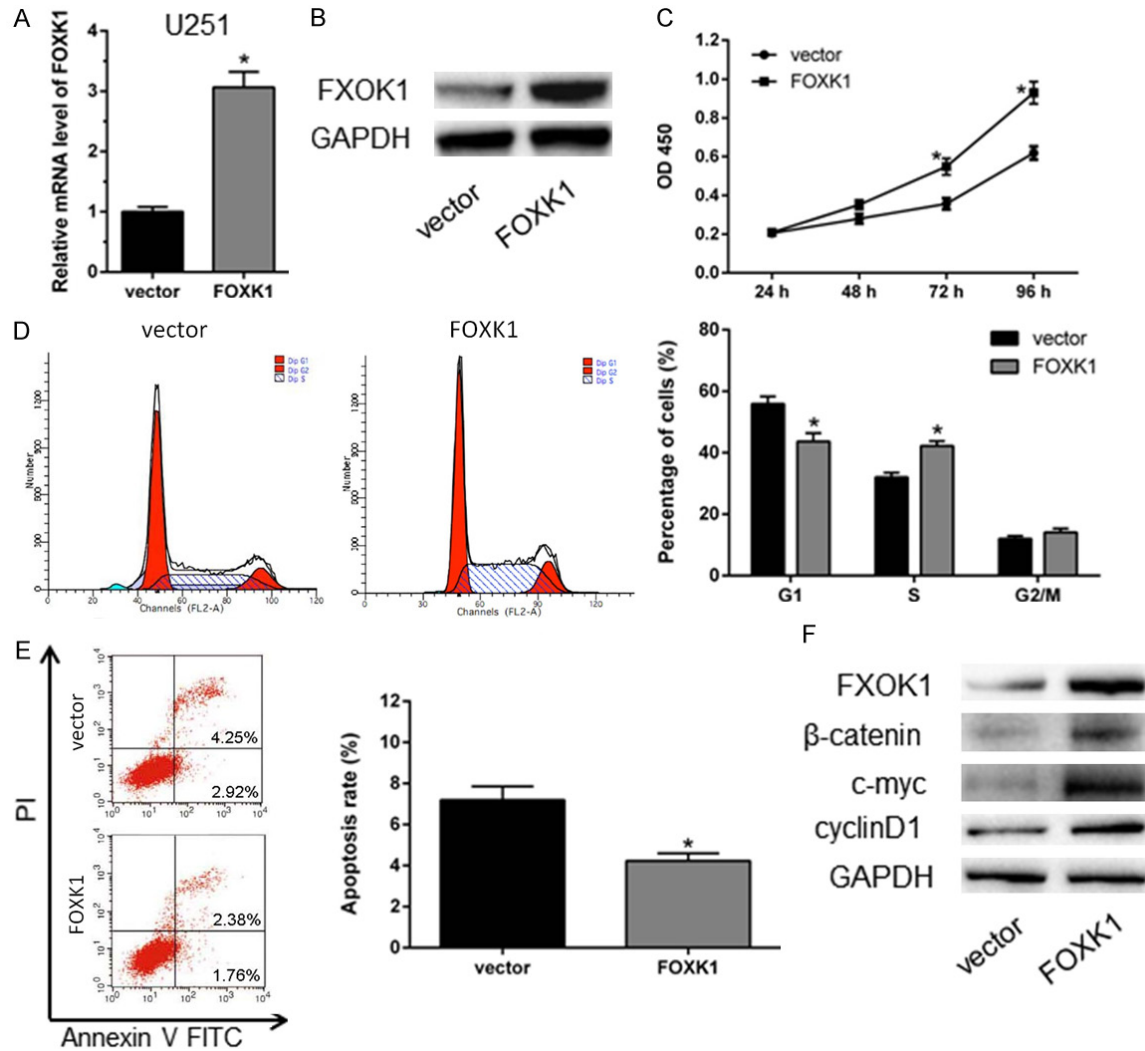


Figure 2. Overexpression of FOXK1 promoted cell growth by activating wnt/ β -catenin pathway in glioma. A and B. The mRNA and protein levels of FOXK1 in glioma cell line U251 following FOXK1 overexpression. C. Cell proliferation of U251 cells as analyzed by CCK-8 assay after FOXK1 overexpression. D and E. Cell cycle distribution and apoptosis rate of U251 cells as assessed by flow cytometry after FOXK1 overexpression. F. Protein expression of β -catenin, c-myc and cyclinD1 in U251 cells following FOXK1 overexpression. Data were mean \pm SD. * $P < 0.05$.

genomic DNA. For luciferase reporter assay, cells were seeded and cultured on 24-well plates. Then, cells were co-transfected with miR-137 mimic or miR-NC plus wt or mut 3'UTR of FOXK1. After 48 h, cells were collected and the luciferase activity was detected by using Dual Luciferase Reporter Assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Firefly luciferase signal was normalized to Renilla luciferase signal.

Statistical analysis

Statistical analyses were performed by using the SPSS 17.0 statistical software (Chicago, IL) and GraphPad Prism 5.0 (San Diego, CA). Data

were expressed as mean \pm SD. Statistical significance between groups were compared by using the t-test or one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

FOXK1 is upregulated in glioma tissues and cell lines

To elucidate the role of FOXK1 in glioma, the expression pattern of FOXK1 was examined in glioma tissue samples and cell lines by using qRT-PCR and western blot. Data showed that the mRNA and protein levels of FOXK1 are both significantly upregulated in glioma tissues com-

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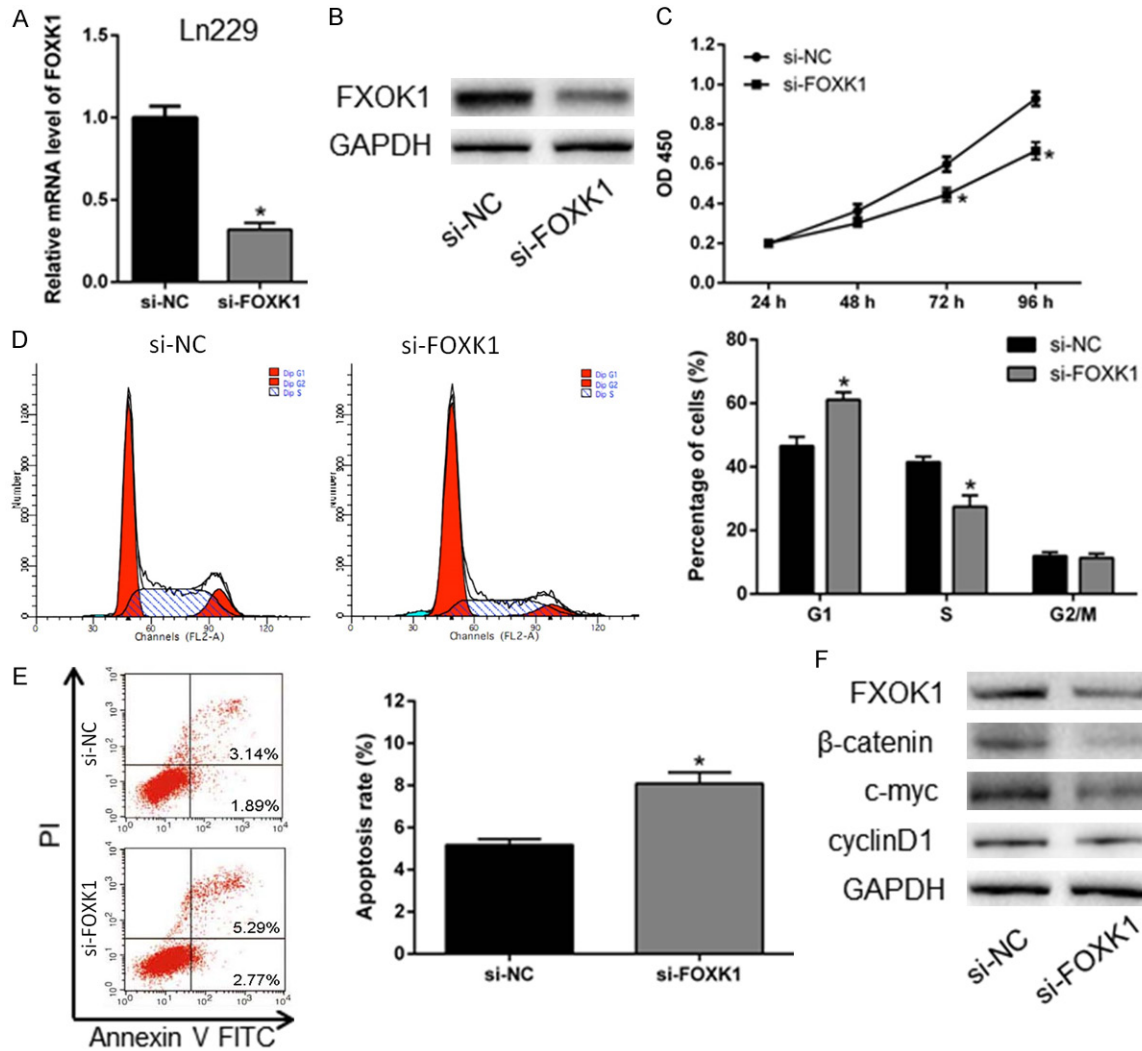


Figure 3. Knockdown of FOXX1 suppressed cell growth by inhibiting wnt/ β -catenin pathway in glioma. A and B. The mRNA and protein levels of FOXX1 in glioma cell line Ln229 after knockdown of FOXX1. C. Effect of FOXX1 knockdown on Ln229 cells proliferation as analyzed by CCK-8 assay. D and E. Effect of FOXX1 knockdown on Ln229 cells cycle distribution and apoptosis rate as assessed by flow cytometry. F. Protein expression of β -catenin, c-myc and cyclinD1 in Ln229 cells following FOXX1 knockdown. Data were mean \pm SD. *P < 0.05.

pared to normal brain tissues (Figure 1A, 1B). Additionally, the level of FOXX1 is increased in four glioma cell lines in comparison with normal human astrocytes (NHA) (Figure 1C, 1D). These results suggest that upregulation of FOXX1 may be involved in the progression of glioma.

Overexpression of FOXX1 promoted cell growth by activating wnt/ β -catenin pathway in glioma

Given that FOXX1 is upregulated in glioma, we investigated the effect of FOXX1 on glioma cells proliferation, cell cycle distribution and apoptosis. The glioma cell line U251 was transfected with pcDNA3.1-FOXX1 plasmid (FOXX1)

to overexpress FOXX1, and the empty vector (vector) was used as a control. qRT-PCR and western blot were used to confirm the overexpression of FOXX1 in U251 cells (Figure 2A, 2B). Then, CCK-8 assay and flow cytometry were performed on treated cells to determine cell proliferation, cell cycle and apoptosis. Our results showed that overexpression of FOXX1 significantly promoted cell proliferation and cell cycle transition in U251 cells (Figure 2C, 2D). Data from apoptosis analysis showed that the apoptosis rate of U251 cells was markedly decreased after FOXX1 overexpression (Figure 2E). In addition, we assessed the effect of FOXX1 overexpression on wnt/ β -catenin path-

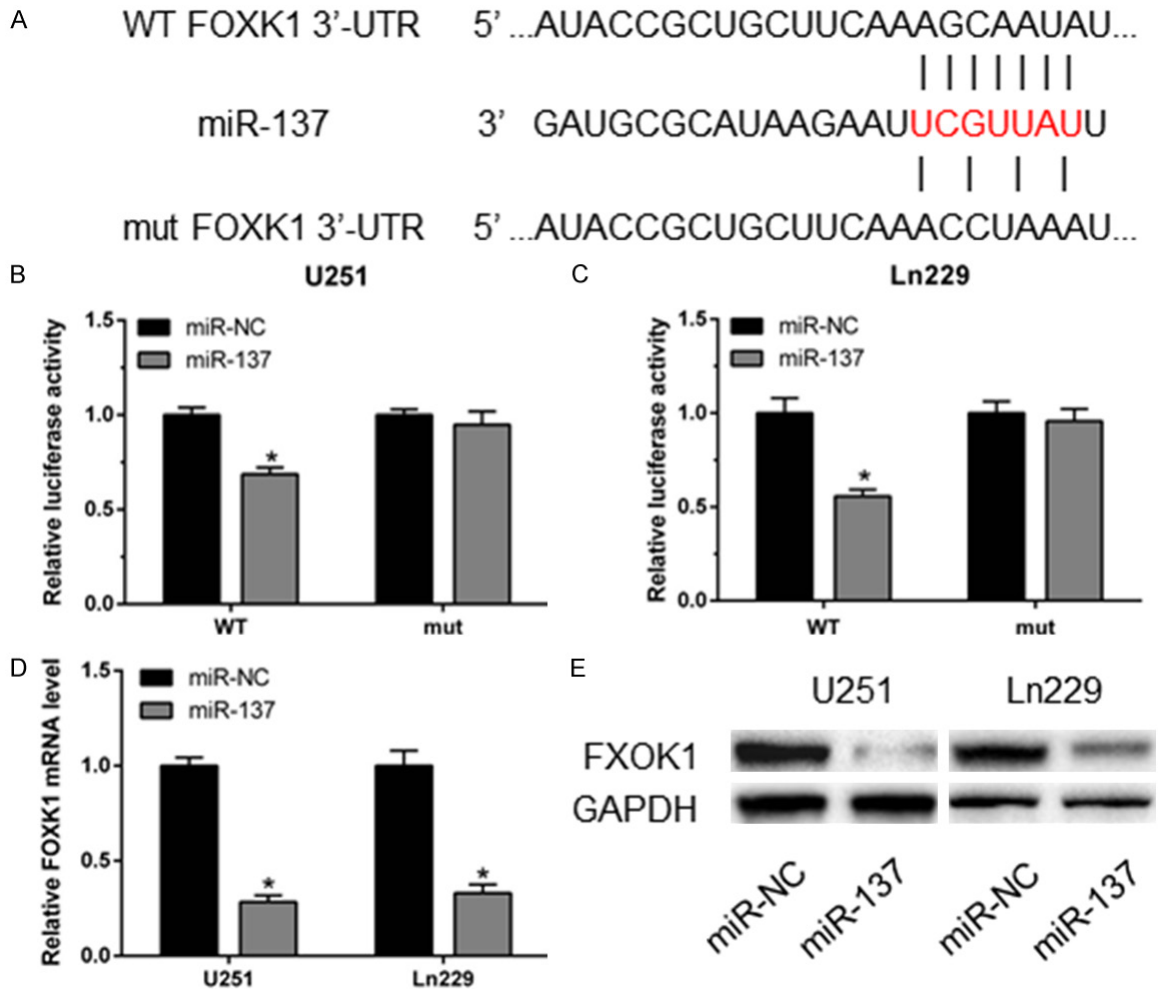


Figure 4. FOXK1 is a direct target of miR-137 in glioma. **A.** The sequences of miR-137 against the wild type (WT) or mutant (Mut) 3'-UTR of FOXK1 predicted by TargetScan. **B** and **C.** Glioma cell lines U251 or Ln229 was co-transfected with miR-137 mimics (miR-137) or its negative control (miR-NC) and wild-type (WT) or mutated (Mut) 3'-UTR of FOXK1. The luciferase activities were examined 48 h after transfection. **D.** Relative FOXK1 mRNA levels in U251 and Ln229 cells after miR-137 overexpression. **E.** The protein expression of FOXK1 in U251 and Ln229 cells examined by western blot following miR-137 overexpression. Data were mean \pm SD. * $P < 0.05$.

way in glioma cells. Data showed that the expression of β -catenin, c-myc and cyclinD1 were obviously increased after FOXK1 overexpression in U251 (**Figure 2F**).

Knockdown of FOXK1 suppressed cell growth by inhibiting wnt/ β -catenin pathway in glioma

The glioma cell line Ln229 were transfected with siRNA targeting FOXK1 to downregulate its expression and the knockdown efficiency was confirmed 48 hours later by qRT-PCR and western blot (**Figure 3A, 3B**). Results of CCK-8 assay showed that the proliferation of Ln229 was significantly suppressed following FOXK1 knockdown (**Figure 3C**). Data from flow cytometry showed that knockdown of FOXK1 inhibited cell

cycle progression while promoted apoptosis in glioma cell line Ln229 (**Figure 3D, 3E**). Results of western blot indicated that knockdown of FOXK1 significantly suppressed the expression of β -catenin, c-myc and cyclinD1 in Ln229 (**Figure 3F**). These results collectively suggest that FOXK1 may promote cell growth by activating wnt/ β -catenin pathway in glioma.

FOXK1 is a novel target of miR-137 in glioma

TargetScan (<http://www.targetscan.org/>) was used to explore miRNAs that directly bind to the 3'-UTR of FOXK1 mRNA and miR-137 was predicted to directly target FOXK1 in glioma (**Figure 4A**). To confirm the prediction, a luciferase reporter assay was performed in glioma

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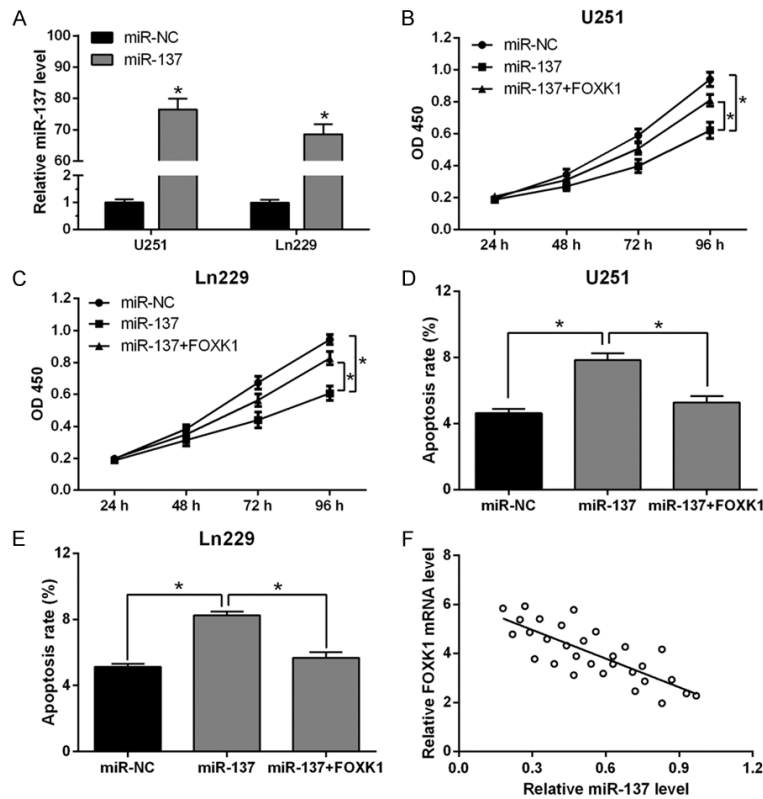


Figure 5. FOXC1 overexpression abolished the suppressive effect of miR-137 in glioma. A. Glioma cell lines U251 and Ln229 were transfected with miR-137 mimics or miR-NC. The relative expression levels of miR-137 were detected by qRT-PCR. B and C. U251 or Ln229 was transfected with miR-137 or/and FOXC1 plasmid to restore the expression of FOXC1 as indicated. Cell proliferation was determined by CCK-8 assay. D and E. Apoptosis rates of transfected U251 or Ln229 cells as detected by flow cytometry. F. The inverse correlation between relative FOXC1 mRNA levels and relative miR-137 expression in 30 glioma tissue samples. Data were mean \pm SD. *P < 0.05.

cell line U251 and Ln229. Results showed that miR-137 significantly suppressed the luciferase activity of wild type (WT) but not the mutant (Mut) 3'-UTR of FOXC1 in glioma cells (Figure 4B, 4C). Furthermore, miR-137 mimics or negative controls were transfected to glioma cell lines. Following that, the mRNA and protein levels of FOXC1 were examined. Data showed that the mRNA levels of FOXC1 were significantly decreased after miR-137 overexpression (Figure 4D). Data from western blot analysis showed that overexpression of miR-137 suppressed the expression of FOXC1 in glioma cells (Figure 4E). These results suggest that FOXC1 is a novel target of miR-137 in glioma.

FOXC1 overexpression abolished the suppressive effect of miR-137 in glioma

Our results showed that FOXC1 is an oncogene in glioma and miR-137 can directly target

FOXC1. We supposed that miR-137 may act as a tumor suppressor in the development of glioma and conducted functional assays to verify that. miR-137 was overexpressed in glioma cell lines U251 and Ln229 (Figure 5A). Meanwhile, FOXC1 plasmid was transfected to restore its expression following miR-137 overexpression in glioma cells. Data showed that miR-137 overexpression significantly suppressed proliferation yet induced apoptosis in glioma cells, and the suppressive effect was abolished following FOXC1 restoration (Figure 5B-E). In addition, the correlation between miR-137 expression and FOXC1 in glioma tissues was examined. We observed that the expression of FOXC1 is inversely correlated with miR-137 in glioma tissues (Figure 5F).

Discussion

Growing evidence indicated that FOXC1 is overexpressed and functions as an oncogene in many human cancers. However, its role in the carcinogenesis and development of glioma is unknown. In the present study, we investigated the expression level and biological role of FOXC1 in glioma for the first time. By using qRT-PCR and western blot, we observed that the expression of FOXC1 is significantly increased in glioma tissue samples and cell lines at both mRNA and protein levels. These indicated the potential oncogenic functions of FOXC1 in the development of glioma. Then, a series of functional assays showed that overexpression of FOXC1 promoted proliferation, cell cycle progression and inhibited apoptosis in glioma cells. Knockdown of FOXC1 exhibited an opposite effect on glioma cells. These results suggest that FOXC1 is upregulated and act as an oncogene in glioma.

Wnt signaling is one of the key cascades regulating the development especially cell proliferation in human cancers [21]. Studies suggested that the Wnt/ β -catenin signaling pathway is

associated with the tumorigenesis and can potentially regulate the growth of gliomas [22]. Recently, several documents reported that FOXK1 may regulate Wnt/ β -catenin pathway in the development of human cancers. Wang et al. demonstrated that FOXK1 can positively regulate Wnt/ β -catenin signaling by translocating DVL into the nucleus [23]. Li et al. found that knockdown of FOXK1 significantly inhibited cell proliferation, migration and invasion through the inactivation of Wnt/ β -catenin signaling pathway in hepatocellular carcinoma [13]. Chen et al. showed that knockdown of FOXK1 efficiently downregulated the expression levels of β -catenin, c-myc, and cyclin D1 in prostate cancer cells [24]. Consistent with previous studies, we demonstrated that overexpression of FOXK1 activated Wnt/ β -catenin pathway while knockdown of FOXK1 suppressed the Wnt/ β -catenin pathway in glioma cells.

Accumulating evidence suggests that miRNAs are deregulated and play important roles by regulating its target genes expression in human cancers. Recently, FOXK1 was reported to be regulated by microRNAs in cancer development. For example, Zhang et al. showed that miR-646 inhibited cell proliferation by directly targeting FOXK1 and regulating Akt/mTOR signaling in gastric cancer [25]. In our study, we found that miR-137 can directly bind to the 3'-UTR of FOXK1 after bioinformatics analysis by TargetScan. Then, results of luciferase reporter assay, qRT-PCR and western blot showed that miR-137 can directly target FOXK1 and suppress its expression in glioma. In addition, we demonstrated that miR-137 suppressed tumor progression in glioma cells, and the suppressive effect was abolished following FOXK1 restoration. And, the expression of FOXK1 is inversely correlated with miR-137 in glioma tissues. These results suggest that the oncogenic role of FOXK1 is negatively regulated by miR-137 in glioma.

Taken together, this study demonstrated that FOXK1 is upregulated in glioma tissues and cell lines. Overexpression of FOXK1 promoted cell growth by activating wnt/ β -catenin pathway in glioma and knockdown of FOXK1 exhibited a reverse effect. FOXK1 is a novel target of miR-137 and its oncogenic role is negatively regulated by miR-137 in glioma. Our results suggest that FOXK1 is a novel molecular involved in the development of glioma and may help us to

develop new therapeutic method for glioma patients.

Disclosure of conflict of interest

None.

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