European Review for Medical and Pharmacological Sciences 2020; 24: 4382-4388

LncRNA FOXD2-AS1 stimulates glioma progression through inhibiting P53

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Abstract. – OBJECTIVE: The aim of this study was to elucidate whether FOXD2-AS1 stimulated glioma progression by inhibiting the P53 level.

PATIENTS AND METHODS: FOXD2-AS1 expression in glioma tissues and cell lines was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Meanwhile, FOXD2-AS1 expression in glioma patients with different tumor tissues and tumor staging was examined as well. The subcellular distribution of FOXD2-AS1 was analyzed. RNA Binding Protein Immunoprecipitation (RIP) and Chromatin immunoprecipitation (ChIP) assay were applied to explore the interaction between FOXD2-AS1 and P53. Furthermore, the influences of FOXD2-AS1 and P53 on the viability and colony formation abilities of LN229 and U87 cells were assessed.

RESULTS: FOXD2-AS1 was significantly upregulated in glioma tissues and cells. The expression level of FOXD2-AS1 was positively correlated with tumor size and staging of glioma. FOXD2-AS1 was mainly distributed in the nucleus, which could attenuate recruitment ability to P53 by bounding to EZH2. The silence of FOXD2-AS1 significantly decreased the viability and colony formation abilities of glioma cells. However, the attenuated proliferative ability was partially reversed by P53 knockdown.

CONCLUSIONS: FOXD2-AS1 stimulated the proliferation of glioma by inhibiting P53, thus aggravating the progression of glioma.

Key Words:
Glioma, FOXD2-AS1, P53.

Introduction

Currently, 10-20 years and 30-40 years are the age vulnerable to glioma. The invasive growth of glioma leads to an obscured boundary between tumor tissues and surrounding normal tissues. It has been found that the survival of low-grade glioma is 3-5 years. Meanwhile, this is only 1-2 years in high-grade glioma patients even after comprehensive treatment of surgery and postoperative chemotherapy[1]. Therefore, it is urgent to uncover reliable and accurate biomarkers to predict the prognosis of glioma[2,3].

In recent years, lncRNAs have been identified to participate in various life activities[4], such as gene expression regulation, genomic imprinting, intranuclear transport, chromatin modification, cell cycle and etc[5]. LncRNA FOXD2-AS1, acting as an oncogene, promotes cell proliferation and invasion in many types of malignancies[6]. Dong et al[7] have reported the involvement of FOXD2-AS1 in the progression of glioma.

The p53 gene is a vital tumor suppressor, whose inactivation is of great significance in tumorigenesis[8]. It is reported[9] that P53 mutation occurs in over 50% malignancies. P53-encoded proteins are a type of transcription factors responsible for cell cycle initiation. They can monitor or inhibit normal cell division under normal circumstance[10]. P53 determines the degree of DNA variation, which triggers P53 to promote cell self-repair. Otherwise, P53 induces cell apoptosis[11]. Doan et al[12] have pointed out that P53 can mediate the apoptosis of glioma cells, thus triggering tumorigenesis of glioma. In this study, we investigated the biological functions of FOXD2-AS1 and P53 in influencing the progression of glioma.

Patients and Methods

Patients

Glioma tissues were collected from 21 patients undergoing surgery in the Fuzong Clinical Medical College of the Fujian Medical University. During the same period, 21 normal brain tissues were collected from epilepsy or trauma patients undergoing surgery for intracranial decompression. Patients with preoperative anti-tumor treatment or the history of other malignancies were excluded. Collected tissue samples were pathologically confirmed and preserved in liquid ni-

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trogen for use. This investigation was approved by the Ethics Committee of the Fuzong Clinical Medical College of Fujian Medical University. Informed content was obtained from each patient before the study.

Cell Culture

Normal brain glial cell line (HEB) and glioma cell lines (U251, U87, and LN229) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C. Culture medium was replaced every three days.

Cell Transfection

Cells were first inoculated into 6-well plates, with 4×10^5 cells per well. At 80% of confluence, the cells were cultured in 1.5 mL of serum-free medium and 500 μL of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) containing transfection vectors. At 4-6 h, complete medium was replaced. Sequences of FOXD2-AS1 siRNA were as follows: si-FOXD2-AS1-1*, sense: 5′-GGGCAAAGUUCGAGAGUGATT-3′ and anti-sense: 5′-UCACUCUCGAACUUUGCCCTT-3′; si-FOXD2-AS1-2*, sense: 5′-GGACUGGUUCUGAGACAAATT-3′ and anti-sense: 5′-UUUGUCUCAGAACCAGUCCTT-3′; si-FOXD2-AS1-3*, sense: 5′-GCAAAGUU CGAGAGUGAAUTT-3′ and anti-sense: 5′-AUUCACUCUCGAACUUUGCTT-3′.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA in cells and tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was detected using a spectrometer. Reverse transcription was performed according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was performed in accordance with SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Relative level of genes was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: FOXD2-AS1, forward: 5′-TGTTCGTGGGAAGAGGGTTG-3′ and reverse: 5′-TACCACTCCGGGAACTCTGT-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5′-ACTGCCACCCAGAAGACT-3′ and reverse: 5′-GCTCAGTG-TAGCCCAGGAT-3′; P53, forward: 5'-GATCGTACGTACGTACGTAGCAT-3' and reverse: 5'-CGTACGATCGTACGATCGATCGG-3'.

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were seeded into 96-well plates at a density of 5.0×10^3 cells per well. At appointed time points, 10 μL of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added in each well, followed by incubation for 2 h in the dark. Absorbance at 450 nm was finally measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Transfected cells were seeded into 6-well plates, with 1000 cells per well. Then, the cells were cultured for 14 days. After washing with phosphate-buffered saline (PBS) twice, formed colonies were fixed with 4% paraformaldehyde and dyed with Giemsa solution for 30 min. Finally, the colonies were captured under a microscope.

Determination of Subcellular Distribution

Cytoplasmic and nuclear RNAs were extracted using the PARIS kit (Invitrogen, Carlsbad, CA, USA), followed by qRT-PCR analysis. U6 and GAPDH were used internal references for nucleus and cytoplasm, respectively.

RNA Binding Protein Immunoprecipitation (RIP) Assay

2×10^7 cells were collected and processed according to the procedures of Millipore Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Then, the cells were incubated with anti-EZH2 or anti-IgG at 4°C overnight. A protein-RNA complex was obtained when intracellular specific proteins were captured by the antibody. Subsequently, proteins were digested with proteinase K and RNA molecules were extracted. During the research, magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. Immunoprecipitant RNA was finally subjected to qRT-PCR for determining the relative level.

Chromatin Immunoprecipitation (ChIP)

The cells were cross-linked with 1% formaldehyde for 10 min. Subsequently, the cross-linked cells were lysed, and incubated with ChIP Dilution Buffer, PIC, and Protein A Agarose/Salmon Sperm DNA. After centrifugation, 20 μL of the

supernatant was collected as input. The supernatant was incubated with 1 μL of antibody overnight at 4°C, followed by culture in 60 μL of Protein A Agarose/Salmon Sperm DNA on the other day. Immunoprecipitation complex was washed, eluted, and cross-linked overnight at 65°C. DNA fragments were finally subjected to PCR.

Western Blot

The total protein in tissues and cells was extracted, and the protein concentration was determined. Protein sample was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skimmed milk for 1 h, the membranes were incubated with primary antibodies overnight at 4°C. On the next day, the membranes were incubated with the corresponding secondary antibody for 2 h at room temperature. Next, the membranes were washed with 1×Tris-Buffered Saline and Tween-20 (TBST) for 1 min. Finally, immuno-reactive bands were exposed by the chemiluminescent substrate kit.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Experimental data were expressed as mean ± standard deviation ($\overline{x}\pm s$). *t*-test was applied to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

Results

FOXD2-AS1 Was Upregulated in Glioma

FOXD2-AS1 expression was significantly upregulated in glioma tissues relative to adjacent normal tissues (n=21) (Figure 1A). FOXD2-AS1 was highly expressed in glioma patients with larger tumor size (Figure 1B) and advanced staging (Figure 1C). These results suggested that FOXD2-AS1 was closely correlated with tumor size and staging of glioma. Compared with normal glial cells, FOXD2-AS1 expression was significantly upregulated in glioma cells, especially in U87 and LN229 cells (Figure 1D). Therefore, these two glioma cell lines were chosen for the following experiments.

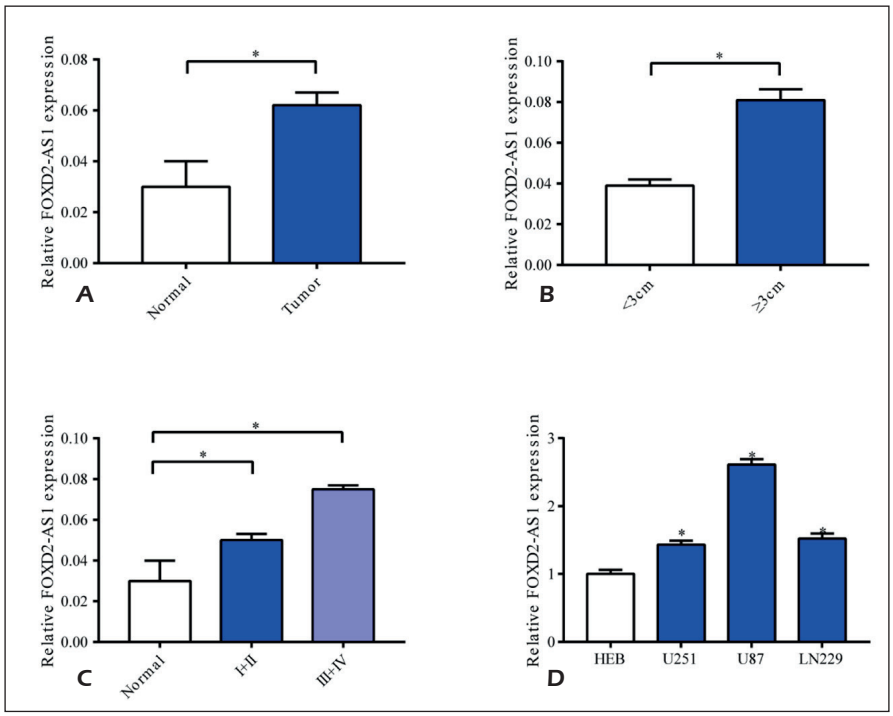

Figure 1. FOXD2-AS1 was upregulated in glioma. **A**, FOXD2-AS1 level in glioma tissues and adjacent normal tissues (n=21). **B**, FOXD2-AS1 level in glioma tissues with <3 cm and ≥3 cm of tumor size. **C**, FOXD2-AS1 level in adjacent normal tissues, and glioma tissues with stage I+II and stage III+IV. **D**, FOXD2-AS1 level in normal brain glial cell line (HEB) and glioma cell lines (U251, U87 and LN229).

FOXD2-AS1 Stimulated Proliferative Ability of Glioma

Transfection of si-FOXD2-AS1-1*, si-FOXD2-AS1-2* or si-FOXD2-AS1-3* markedly downregulated FOXD2-AS1 expression in U87 and LN229 cells. The former one showed the best transfection efficacy (Figure 2A). The subsequent results demonstrated that the viabilities in U87 and LN229 cells markedly decreased after transfection of si-FOXD2-AS1-1* (Figure 2B, 2C). Moreover, the colony formation ability was attenuated by the knockdown of FOXD2-AS1 in glioma cells (Figure 2D, 2E). Hence, FOXD2-AS1 was able to stimulate the proliferation of glioma cells.

FOXD2-AS1 Downregulated P53 by Recruiting EZH2

Subcellular distribution analysis uncovered that FOXD2-AS1 was mainly enriched in nucleus (Figure 3A, 3B). P53 expression level was significantly downregulated in glioma tissues when compared with normal tissues (Figure 3C). Meanwhile, P53 was downregulated in glioma cells transfected with si-FOXD2-AS1-1*, suggesting a potential relationship between FOXD2-AS1 and P53 (Figure 3D). RIP assay showed remarkably higher enrichment of FOXD2-AS1 in anti-EZH2 relative to anti-IgG. This indicated a direct interaction between FOXD2-AS1 and EZH2 (Figure 3E, 3F). Furthermore, transfection of si-FOXD2-AS1-1* decreased immunoprecipitant of P53 in anti-EZH2 and anti-H3K27me3 (Figure 3G). All these findings suggested that the silence of FOXD2-AS1 attenuated the recruitment ability of EZH2 to P53.

Knockdown of P53 Reversed the Effect of FOXD2-AS1 on Glioma Progression

Subsequently, the biological functions of FOXD2-AS1/P53 in glioma cells were explored. Transfection

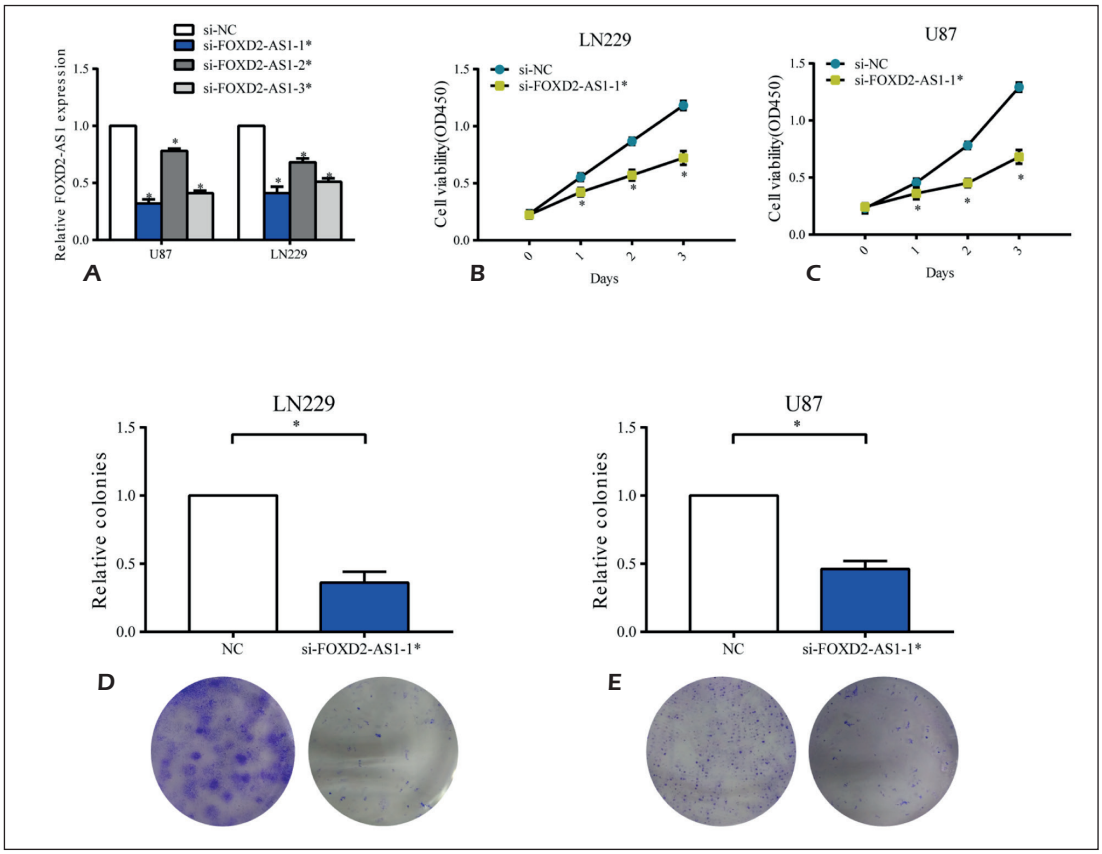

Figure 2. FOXD2-AS1 stimulated proliferative ability of glioma. **A**, Transfection efficacy of si-FOXD2-AS1-1*, si-FOXD2-AS1-2*, and si-FOXD2-AS1-3* in U87 and LN229 cells. **B**-**C**, Viability of LN229 (**B**) and U87 (**C**) cells transfected with si-NC or si-FOXD2-AS1-1*. **D**-**E**, Colony formation ability of LN229 (**D**) and U87 (**E**) cells transfected with si-NC or si-FOXD2-AS1-1* (magnification: 40x).

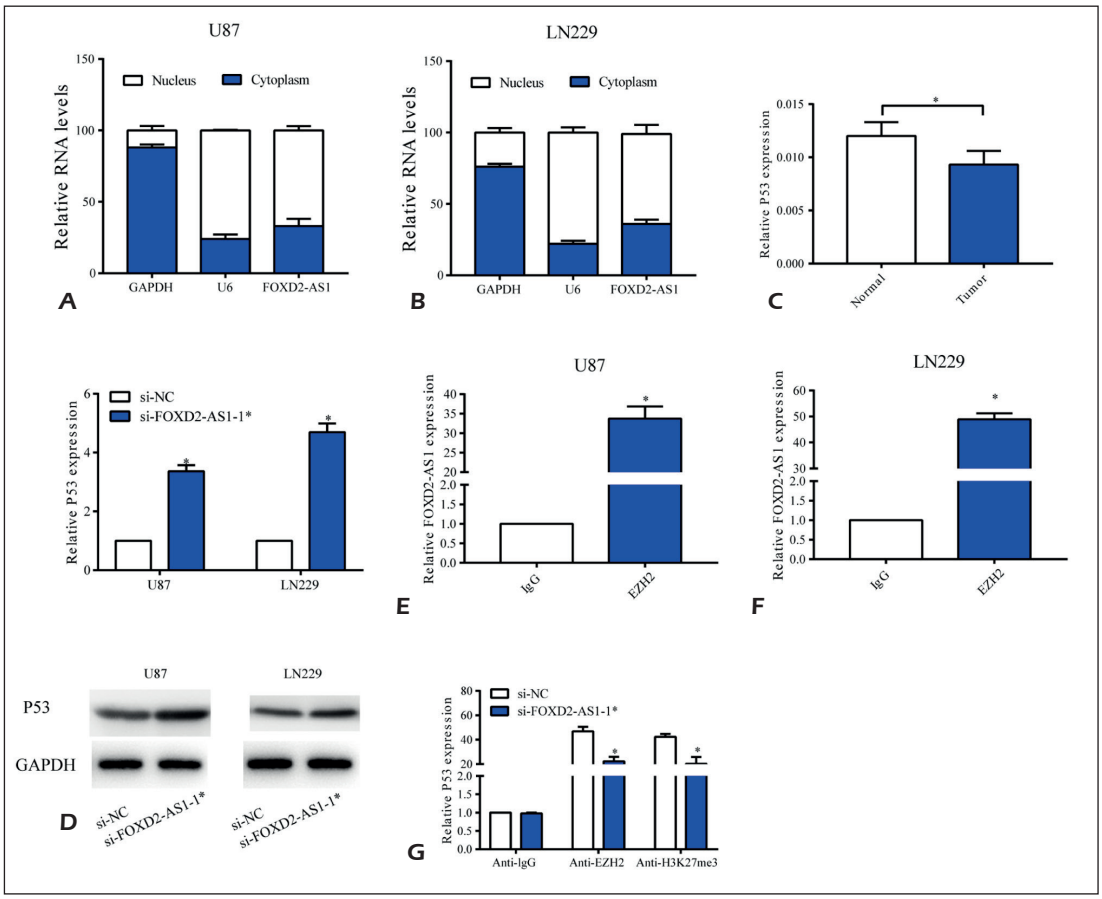

Figure 3. FOXD2-AS1 downregulated P53 by recruiting EZH2. **A**-**B**, Subcellular distribution of FOXD2-AS1 in U87 (**A**) and LN229 (**B**) cells. GAPDH and U6 were internal references for cytoplasm and nucleus, respectively. **C**, P53 level in glioma tissues and adjacent normal tissues. **D**, P53 level in U87 and LN229 cells transfected with si-NC or si-FOXD2-AS1-1*. **E**-**F**, FOXD2-AS1 level enriched in anti-IgG and anti-EZH2 in U87 (**E**) and LN229 (**F**) cells. **G**, Immunoprecipitant of P53 in anti-IgG, anti-EZH2, and anti-H3K27me3 in U87 cells transfected with si-NC or si-FOXD2-AS1-1*.

efficacy of si-P53 in U87 and LN229 cells was verified (Figure 4A). In glioma cells with FOXD2-AS1 knockdown, reduced viability was partially elevated after P53 knockdown (Figure 4B, 4C). Identically, attenuated colony formation ability by silence of FOXD2-AS1 was reversed by P53 knockdown (Figure 4D, 4E). Hence, P53 was responsible for glioma progression influenced by FOXD2-AS1.

Discussion

Glioma is one of the most common fatal malignancies in humans, with over 400,000 deaths each year[13]. It has been found that the 5-year survival of glioma is below 15%[14]. Currently, classic tumor markers of glioma include SCCA (serum squamous cell carcinoma antigen), CA19-9 (carbohydrate antigen 19-9), and CEA (carcinoembryonic antigen). However, the sensitivity and specificity of these tumor markers are insufficient in early-stage diagnosis of glioma[15]. Therefore, the development of effective hallmarks for early-stage glioma is of clinical significance.

LncRNAs do not have an open reading frame and cannot encode proteins. Abnormally expressed lncRNAs in tumor tissues have been discovered in recent years, which influence many aspects of the tumor cells[2,3]. Moreover, lncRNA expression has been confirmed associated with the survival and prognosis of tumor patients[16]. FOXD2-AS1, located on chromosome 1, spans 2509 nucleotides in length. FOXD2-AS1 serves as a carcinogenic role in tumors. Rong et al[17] have illustrated that FOXD2-AS1 is upregulated in non-small cell lung cancer and promotes the proliferative rate of tumor cells by activating the Wnt/β-catenin signaling. Upregulated lncRNA-FOXD2-AS1 in esophageal

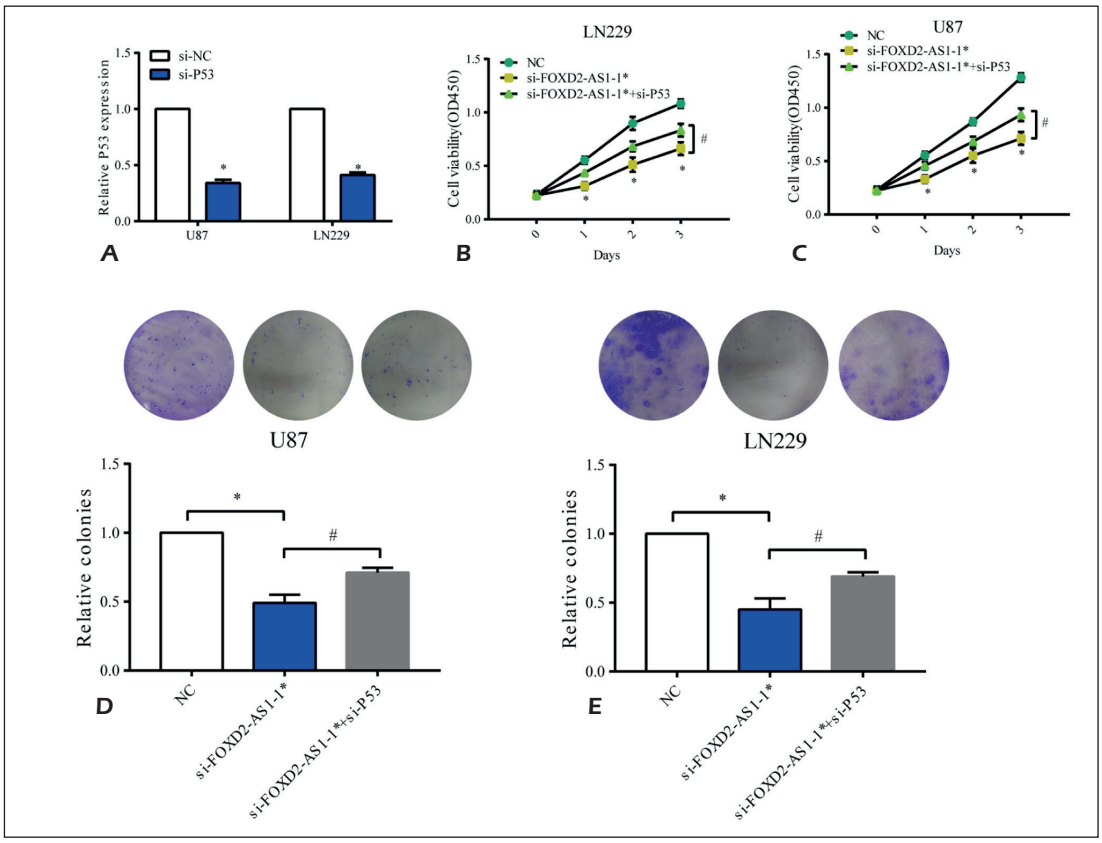

Figure 4. Knockdown of P53 reversed the effect of FOXD2-AS1 on glioma progression. **A**, Transfection efficacy of si-P53 in U87 and LN229 cells. **B**-**C**, Viability of LN229 (B) and U87 (**C**) cells transfected with si-NC, si-FOXD2-AS1-1* or si-FOXD2-AS1-1* + si-P53. **D**-**E**, Colony formation ability of LN229 (**D**) and U87 (**E**) cells transfected with si-NC, si-FOXD2-AS1-1* or si-FOXD2-AS1-1* + si-P53 (magnification: 40x).

squamous cell carcinoma predicts poor prognosis of patients[18]. In this study, FOXD2-AS1 was highly expressed in glioma tissues and was positively correlated with tumor size and staging.

P53 is involved in a variety of signal transduction processes[19]. Long-term silence of P53 may lead to tumorigenesis, while the reactivation of P53 may inhibit tumor growth. Abundant evidences have confirmed a strong correlation between P53 and lncRNAs. These lncRNAs serve as P53 regulators or effectors, thereby regulating tumor growth[20]. P53 induces the activation of lncRNAs. Conversely, activated lncRNAs regulate tumor progression by mediating P53 level. Previously, Jin et al[21] have demonstrated that P53 is a biomarker for glioma. Consistently, our results showed that P53 was highly expressed in glioma. P53 is upregulated after overexpression of MEG3 in some types of tumors[22-24], further suppressing proliferation and inducing apoptosis. In this paper, P53 level was negatively regulated by FOXD2-AS1. Notably, P53 knockdown reversed the attenuated viability and colony formation ability of glioma cells with FOXD2-AS1 knockdown. As a result, FOXD2-AS1 was able to aggravate glioma by inhibiting the P53 level. Our findings provided potential targets for early intervention of glioma.

Conclusions

Shortly, FOXD2-AS1 stimulated the proliferation of glioma by inhibiting the P53 level, thus aggravating the progression of glioma.

Funding Acknowledgements

Natural Science Foundation of Fujian (Project No. 2017J01318).

Conflict of Interests

The authors declare that they have no conflict of interests.

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