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miR-126-3p sensitizes glioblastoma cells to temozolomide by inactivating Wnt/β-catenin signaling via targeting SOX2

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ABSTRACT

Aims: The acquired drug resistance has been regarded as a main barrier for the effective treatment of temozolomide (TMZ) in glioblastoma (GBM). MiR-126-3p is commonly down-regulated and exerts tumor-suppressive roles in kinds of human cancers, including GBM. This study was designed to investigate the functions and mechanisms of miR-126-3p in regulating TMZ resistance in GBM.

Materials and methods: qRT-PCR analysis was used to measure the expressions of miR-126-3p and SOX2 mRNA in GBM tissues and cells. Cell viability, colony forming ability and apoptosis were detected to evaluate the effect of miR-126-3p or SOX2 on TMZ resistance. Luciferase reporter experiments were applied to identify the target genes of miR-126-3p. Western blot analysis was performed to determine the protein levels associated with Wnt/β-catenin signaling. TOP/FOP Flash assays were conducted to determine the effects of miR-126-3p or SOX2 on Wnt/β-catenin signaling.

Key findings: miR-126-3p expression was decreased in TMZ-resistant GBM tissues and cells. High levels of miR-126-3p enhanced TMZ sensitivity by inhibiting cell viability, reducing colony forming potential and inducing apoptosis. Additionally, SOX2 was identified as a downstream target of miR-126-3p. On the contrary, SOX2 overexpression conferred TMZ resistance of GBM cells. Moreover, miR-126-3p-mediated TMZ sensitivity was reversed following increased expression of SOX2. Furthermore, miR-126-3p-induced inactivation of Wnt/β-catenin signaling was greatly abrogated by SOX2 up-regulation.

Significance: MiR-126-3p sensitizes GBM cells to TMZ possibly by repressing SOX2 expression and blocking Wnt/β-catenin signaling. This study provides novel targets to overcome TMZ resistance in GBM chemotherapy.

1. Introduction

Glioblastoma (GBM) is the most common and aggressive intracranial tumor in central nervous system [1]. Surgery combined with chemotherapy and/or radiotherapy is currently the standard regimen for GBM therapy [2]. However, the prognosis of GBM patients is still poor, with the median survival rates of only 12–15 months [3]. Temozolomide (TMZ), a DNA alkylating antineoplastic drug, has been applied as a first-line chemotherapeutic reagent for GBM treatment [4]. The acquired resistance to TMZ is a serious impediment for its efficiency and clinical application [5]. Therefore, understanding the possible molecular mechanisms of TMZ resistance is important for identifying the novel targets and enhancing the responsiveness of patients with GBM.

MicroRNAs (MiRNAs) are a serial of small endogenous noncoding RNAs that could degrade mRNA or inhibit translation by base-pairing to the 3′-untranslated region (UTR) of their target genes. MiRNAs have been demonstrated as oncogenes or tumor suppressors to affect cell proliferation, cell cycle, apoptosis, invasion, and angiogenesis in a variety of human cancers [6,7]. Increasing evidence reveals that miRNAs are involved in the regulation of chemoresistance through different mechanisms, such as drug metabolism, drug uptake, drug target, DNA repair, cell cycle, and apoptosis [8,9]. For instance, up-regulation of miR-634 decreased TMZ resistance in glioma by targeting CYR61 and inactivating Raf-ERK signaling [10]. Overexpression of miR-1268a enhanced the sensitivity of GBM cells to TMZ via suppressing ABCC1 expression [11]. These documents unravel the significance of miRNAs in modulating chemoresistance, and provide direction for

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exploring the molecular basis of TMZ resistance.

MiR-126-3p was previously found to be commonly down-regulated, and exert antitumor effect in multiple malignancies through repressing a range of critical gene targets [12]. In glioma, Li et al. disclosed that miR-126-3p expression was decreased, and restoration of miR-126-3p inhibited cell proliferation and invasion by blocking ERK signaling via targeting KRAS [13]. Rouigari et al. illustrated that miR-126-3p promoted the formation of glioma cancer stem cell possibly by down-regulating IRS-1 in neurotrophin signaling pathway [14]. Nevertheless, no reports have focused on the biological functions of miR-126-3p in regulating TMZ resistance in GBM.

In the present study, we found that miR-126-3p expression was lowered in TMZ-resistant GBM tissues and cells. Furthermore, miR-126-3p directly targeted SOX2 and inactivated Wnt/β-catenin signaling, thereby increasing the sensitivity of GBM cells to TMZ. Our findings offer support for employing miR-126-3p as a potential target to reduce TMZ resistance in GBM.

2. Materials and methods

2.1. Patients and tissues

Tumor tissues were collected from GBM patients who were receiving surgery at the First Affiliated Hospital of Zhengzhou University from January 2013 to December 2014. All samples were promptly frozen in liquid nitrogen and stored at −80 °C until use. After surgery, all patients were treated by TMZ. According to the criteria of Response Evaluation Criteria in Solid Tumors (RECIST), patients were allocated into Response group (n = 36) and Non-response group (n = 44). This study was performed with approval of the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All participants provide informed written consents prior to enrolling in this study.

2.2. Cell lines

Human GBM cells (U87 and U251) were acquired from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). TMZ-resistant GBM cells (U87/TR and U251/TR) were generated by culturing U87 and U251 cells (normally sensitive to TMZ) in incremental concentrations of TMZ up to 400 μM over 6 months in our laboratory with stepwise selection and the subculture of resistant clones, as reported by Zhang et al. [15] To maintain resistant phenotype, the culture medium with 50 μM of TMZ (Sigma-Aldrich, St. Louis, MI, USA) was used for the incubation of U87/TR and U251/TR cells. All cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) at 37 °C in an incubator with 5% CO_2.

2.3. Oligonucleotides, plasmid construction, and transfection

MiR-126-3p mimic (MiR-126-3p), miR-126-3p inhibitor (anti-miR-126-3p), and corresponding scrambled control (miR-NC, anti-miR-NC) were purchased from GenePharma (Shanghai, China). To up-regulate sex-determining region Y (SRY)-box 2 (SOX2) expression, SOX2 cDNA sequence was cloned into pcDNA3.1 vector (Invitrogen) to construct SOX2-overexpressing plasmid (SOX2). Cells were seeded into 6-well plates, and transfected with indicated concentration of oligonucleotides (50 nM) or plasmids (100 ng) using Lipofectamine 2000 reagent (Invitrogen) at the ratio Lipo/DNA of 2:1 according to the manufacture's manual.

2.4. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was separated from GBM tissues and cells using TRIzol reagent (Takara, Dalian, China). To detect the level of miR-126-3p and SOX2 mRNA, qRT-PCR reactions were performed with THUNDERBIRD SYBR® qPCRMix kit (Toyobo Co., Ltd., Tokyo, Japan) on an ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The relative expressions of miR-126-3p and SOX2 mRNA were calculated by the $2^{-\Delta\Delta CT}$ method, with U6 snRNA and GAPDH as respective internal reference. The primer sequences used were: miR-126-3p, 5′-CGCGCCGTACCGTGAGTAA-3′ (forward) and 5′-GTGCAGGGTCCGAGGT-3′ (reverse); SOX2, 5′-GGGCTCTGTGGTCAAGTC-3′ (forward) and 5′-TAGTCGGCATCACGGTTT-3′ (reverse). U6, 5′-CTCGCTTCGGCAGCACA-3′ (forward) and 5′-AACGCTTCACGAATTTGCGT-3′ (reverse); GAPDH, 5′-AGAAGGCTGGGGCTCATTTG-3′ (forward) and 5′- AGGGGCCATCCACAGTCTTC-3′ (reverse).

2.5. Drug resistance assay

Transfected U87/TR and U251/TR cells were plated in 96-well plates and treated with indicated concentrations of TMZ (7.5, 15, 30, 60, 120, 240, 480 μM) for 48 h. Then, cell viability was evaluated by Cell-Counting Kit 8 (CCK-8, Dojindo Laboratories, Japan) according to the manufacture's instruction. Half maximal inhibitory concentration (IC50) values were calculated through nonlinear regression, and the data were fitted to a sigmoidal dose-response relation using GraphPad Prism 5.0 software (GraphPad Software Inc. La Jolla, CA, USA): Y = 100/(1 + 10^((LogIC50-X) ∗ HillSlope)). X: log of dose or concentration. Y: Normalized response, 100% down to 0%, decreasing as X increases. logIC50: Same log units as X. HillSlope: Slope factor or Hill slope, unitless.

2.6. Apoptosis assay

Transfected U87/TR and U251/TR cells were placed into 6-well plates and treated with indicated dose of TMZ for 48 h. Then, cells were stained with FITC-Annexin V and propidium iodide (PI) (BD Biosciences, San Jose, CA, USA), and analyzed using a flow cytometry (FACScan; BD Bioscience). Cells were discriminated into necrotic cells (Annexin V-/PI+), viable cells (Annexin V-/PI-), late apoptotic cells (Annexin V+/PI+), and early apoptotic cells (Annexin V+/PI-). The apoptotic rate was displayed as the sum percentage of early and late apoptotic cells.

2.7. Colony formation assay

Transfected U87/TR and U251/TR cells were plated onto 6-well plates, and treated with indicated concentration of TMZ for 48 h. Then, the drug was removed and the culture medium was changed every 4 days. Following incubation for 12 days, cells were fixed with 96% ethanol and stained with 0.1% crystal violet. The number of colonies containing > 50 cells were captured and counted.

2.8. Luciferase reporter assay

The 3′UTR region of SOX2 containing the predicted miR-126-3p binding sites (wild type, WT) and corresponding mutant sites (MUT) were amplified by PCR and subcloned into the *Sac I* and *Hind III* sites of the pmiRNA-report firefly luciferase vector (Genechem, Shanghai, China). U87/TR and U251/TR cells were co-transfected with reporter constructs (SOX2-WT or SOX2-MUT) and miR-NC or miR-126-3p using Lipofectamine 2000 (Invitrogen). At 48 h after transfection, Firefly and Renilla luciferase activities were determined with a dual-luciferase assay kit (Promega, Madison, WI, USA). The relative luciferase activity (fold) was calculated as the ratio of Firefly luciferase intensity/Renilla luciferase intensity.

2.9. TOP/FOP flash reporter assay

The reporter plasmids containing TOPflash or mutated FOPflash TCF/LEF DNA binding sites were purchased from Upstate Biotechnology Lake Placid, NY, USA. Briefly, U87/TR and U251/TR cells were seeded in 24-well plates and then transfected with TOP Flash or FOP Flash construct, and 10 ng of internal control pRL-TK Renilla luciferase vector (Promega) using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, the luciferase activities were measured using a dual-luciferase assay kit (Promega). The luciferase activity of each sample was normalized with respective Renilla luciferase activity.

2.10. Western blot assay

The collected cells were washed by PBS and lysed with RIPA Buffer (Cell Signaling Technology, Danvers, MA, USA). Protein concentration was detected by a BCA Protein Assay kit (Beyotime, Shanghai, China). Equal amount of protein extracts (30 μg/lane) were separated by 10% SDS-PAGE, electrotranferred to polyvinylidene fluoride membranes (Thermo Scientific, Pittsburgh, PA, USA), and then incubated with primary antibodies including anti-SOX2 (1:500, Cell Signaling Technology Cat# 3579S, RRID:AB_2195767), anti-β-catenin (1:500, Santa Cruz Biotechnology Cat# sc-7963, RRID:AB_626807), anti-c-myc (1:500, Santa Cruz Biotechnology Cat# sc-40, RRID:AB_627268), anti-cyclin D1 (1:1000, Cell Signaling Technology Cat# 2922S, RRID:AB_10695719), anti-histone H3 (1:1000, Abcam Cat#Ab1791, RRID:AB_302613), and anti-β-actin (1:2000, Santa Cruz Biotechnology Cat# lsc-8432, RRID:AB_626630) overnight at 4 °C. Following washed with TBST for 3 times, the membranes were further probed with HRP-conjugated goat anti-mouse secondary antibody (1:2000, Thermo Fisher Scientific Cat# 62-6520, RRID:AB_2533947) at room temperature for 1 h. Finally, the membranes were scanned with electro-chemiluminescence (ECL) and photographed using a gel imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The isolation of nuclei and cytosol proteins of U87/TR and U251/TR was performed using a Nuclear and Cytoplasmic Protein Extraction Kit (KeyGEN Biotech, Jiangsu, China) according to the manufacturer's manual. Histone H3 was used as a loading control of nuclear protein fraction and β-actin was used as a loading control of cytoplasmic protein fraction.

2.11. Statistical analysis

All quantitative data were shown as means ± SD from at least three independent experiments. For comparison between two groups, homoscedasticity was tested with an *F*-test, followed by either a Student's *t*-test for homoscedastic data, or a Welch's *t*-test for heteroscedastic data. For comparisons between 3 or more groups, homoscedasticity was tested with Bartlett's test, followed by either a Dunnett's test for homoscedastic data, or a Steel test for heteroscedastic data. The statistical significance levels were set at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. miR-126-3p expression is decreased in TMZ-resistant GBM tissues and cells

In order to investigate the influence of miR-126-3p on TMZ resistance in GBM, we firstly examined the expression patterns of miR-126-3p in patients treated with TMZ. As presented in Fig. 1A, miR-126-3p expression was significantly down-regulated in patients insensitive to TMZ compared with that in patients sensitive to TMZ. Also, we established TMZ-resistant GBM cells by exposing U87 and U251 cells (normally sensitive to TMZ) to gradually increasing concentrations of TMZ. Results showed that for U87 cells, the TMZ concentration to decrease cell viability to 50% was about 53.28 μM (IC50 = 53.28); for U87/TR cells this value was 271.3 μM (IC50 = 271.3) (Fig. 1B). For U251 cells, the TMZ concentration to reduce cell viability to 50% was about 58.47 μM (IC50 = 58.47), for U251/TR cells 278.1 μM (IC50 = 278.1) (Fig. 1C). Then, we detected the expression levels of miR-126-3p in TMZ-sensitive and TMZ-resistant GBM cells. As presented in Fig. 1D, miR-126-3p expression was decreased in U87/TR and U251/TR cells when compared to their matched parental cells (U87 and U251). Moreover, GBM patients with high miR-126-3p expression displayed a longer survival time than that with low miR-126-3p expression ($P = 0.0184$, Fig. 1E). These results showed the decrease of miR-126-3p expression in TMZ-resistant GBM tissues and cells, and low miR-126-3p expression may be correlated to a poor prognosis.

3.2. Overexpression of miR-126-3p increased TMZ sensitivity in TMZ-resistant GBM cells

To explore the roles of miR-126-3p in regulating the chemotherapy response of TMZ in GBM, we performed gain-of-function experiments in TMZ-resistant GBM cells (U87/TR and U251/TR). The results showed that miR-126-3p expression was greatly elevated in U87/TR and U251/TR after transfection with miR-126-3p mimic (Fig. 2A). CCK-8 assay revealed that up-regulation of miR-126-3p led to a dramatic decrease of cell viability and IC_{50} values in U87/TR and U251/TR cells compared to miR-NC group (Fig. 2B and C). Colony formation assay manifested that enforced expression of miR-126-3p reduced the colony number of U87/TR and U251/TR cells upon TMZ treatment (Fig. 2D and E). Then, we further surveyed whether miR-126-3p-mediated TMZ susceptibility was related to apoptosis. Flow cytometry data manifested that TMZ-induced apoptosis was enhanced in miR-126-3p-transfected U87/TR and U251/TR cells when compared to miR-NC-transfected cells (Fig. 2F and G). In summary, restoration of miR-126-3p sensitized GBM cells to TMZ.

3.3. MiR-126-3p directly targets SOX2 in TMZ-resistant GBM cells

As is well known, miRNAs exerts their functions mainly by binding to the 3′UTR of downstream targets through partial sequence homology. To better understand the mechanism of miR-126-3p in TMZ resistance in GBM, we used the bioinformatic algorithm miRcode (http://www.mircode.org/) to identify the possible target genes of miR-126-3p. SOX2 was found to contain a complementary sequence for miR-126-3p in 3′UTR (Fig. 3A). Here, we focused on SOX2 for its oncogenicity in GBM [16]. To confirm the direct binding between miR-126-3p and SOX2 3′UTR, we established the wild type or mutant luciferase reporters containing the potential binding site of SOX2 and conducted luciferase assay experiments. The results discovered that ectopic expression of miR-126-3p resulted in a marked suppression on luciferase activity of SOX2-WT reporter rather than SOX2-MUT reporter in U87/TR and U251/TR cells (Fig. 3B and C). Subsequently, Western blot assays were applied to verify the actual effects of miR-126-3p on SOX2 expression. Results indicated that overexpression of miR-126-3p obviously inhibited SOX2 protein level, while knockdown of miR-126-3p substantially promoted SOX2 protein expression in U87/TR and U251/TR cells (Fig. 3D and E). Moreover, SOX2 mRNA expression was observed to be higher in the tumors from patients showing no response to TMZ than that in patients showing response to TMZ (Fig. 3F). Consistently, SOX2 protein level was up-regulated in U87/TR and U251/TR cells compared with parental GBM cells (Fig. 3G and H). These evidences suggested that miR-126-3p could bind to SOX2 3′UTR and inhibited its expression.

3.4. SOX2 up-regulation attenuates miR-126-3p-mediated TMZ sensitivity in TMZ-resistant GBM cells

To further illuminate whether miR-126-3p affects TMZ sensitivity by modulating SOX2, U87/TR and U251/TR cells were transfected with miR-NC or miR-126-3p, together with or without SOX2-overexpressing plasmid, followed by treatment with indicated dose of TMZ. As expected, SOX2 protein level was increased after transfection with pcDNA-SOX2, and miR-126-3p-mediated suppression of SOX2 protein expression was greatly rescued by reintroduction of SOX2 (Fig. 4A and

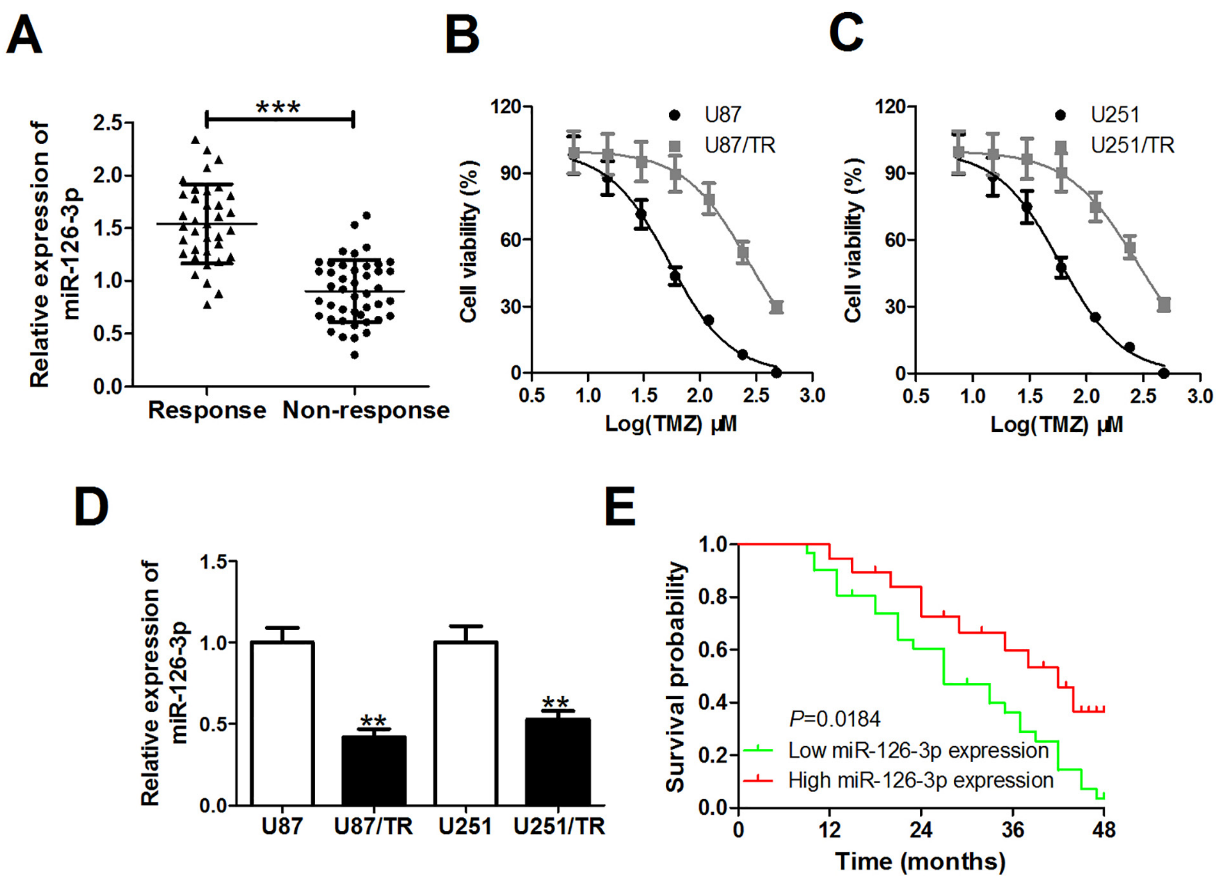

Fig. 1. MiR-126-3p expression is decreased in TMZ-resistant GBM tissues and cells. (A) qRT-PCR analysis was performed to measure the expression level of miR-126-3p in patients showing response to TMZ (n = 36) and patients showing no response to TMZ (n = 44). (B–C) Cell survival was determined by CCK-8 assay in TMZ-sensitive and TMZ-resistant GBM cells after treatment with different concentration of TMZ. (D) The expression level of miR-126-3p was detected in TMZ-resistant GBM cells (U87/TR and U251/TR) and mathed parental GBM cells (U87 and U251) by qRT-PCR analysis. (E) GBM patients were classified into high miR-126-3p expression (n = 40) and low miR-126-3p expression (n = 40) groups. Kaplan-Meier overall survival curves according to the relative miR-126-3p expression level. $^{**}P < 0.01$, $^{***}P < 0.001$.

B). Functionally, SOX2 overexpression exhibited the opposite effects in regulating cell viability and IC50 values (Fig. 4C and D), colony forming ability (Fig. 4E and F), and apoptosis (Fig. 4G and H), when compared with miR-126-3p. Furthermore, CCK-8 assay uncovered that miR-126-3p-induced decrease of cell viability and IC50 values were remarkably abated following SOX2 up-regulation (Fig. 4C and D). Colony formation assay elaborated that the inhibition of colony forming ability triggered by miR-126-3p was evidently abolished by SOX2 overexpression with TMZ treatment (Fig. 4E and F). Flow cytometry assay showed that miR-126-3p-elicited apoptosis was apparently reversed by restoration of SOX2 under TMZ treatment (Fig. 4G and H). Similarly, increased cell viability and IC50 values (Fig. 4C and D), enhanced colony forming potential (Fig. 4E and F), and decreased apoptosis (Fig. 4G and H) mediated by SOX2 overexpression were greatly abrogated by co-transfection with miR-126-3p. These data supported that miR-126-3p enhanced TMZ sensitivity in GBM cells by down-regulating SOX2.

3.5. miR-126-3p inhibited Wnt/β-catenin signaling in TMZ-resistant GBM cells by targeting SOX2

Wnt/β-catenin signaling is associated with glioma progression, and facilitates clinical malignancy grade and poor prognosis of glioma patients [17]. A previous document stated that SOX2 promotes tumor metastasis in breast cancer by stimulating epithelial-to-mesenchymal transition via activation of Wnt/β-catenin signaling [18]. Thus, we further investigated whether miR-126-3p could regulate Wnt/β-catenin signaling in GBM by SOX2. As we might expect, miR-126-3p overexpression resulted in a significant suppression of nuclear β-catenin protein level, and a decrease of c-myc and cyclin D1 protein expression, while these effects were dramatically counteracted by increased SOX2 expression (Fig. 5A and B). On the contrary, SOX2 up-regulation led to an accumulation of nuclear β-catenin protein, and an increase of c-myc and cyclin D1 protein expression, while these effects were greatly abated after co-transfection with miR-126-3p (Fig. 5A and B). In addition, miR-126-3p overexpression inhibited the activity of Wnt/β-catenin signaling, which was obviously reversed following increased SOX2 expression, as evidenced by TOP/FOP luciferase activity (Fig. 5C and D). Moreover, SOX2 up-regulation promoted the activity of Wnt/β-catenin signaling, which was evidently abrogated by miR-126-3p up-regulation (Fig. 5C and D). These results proved that miR-126-3p inactivated Wnt/β-catenin signaling by inhibition of SOX2. All these data prompt us to draw a conclusion that miR-126-3p sensitized GBM cells to TMZ via inactivation of Wnt/β-catenin signaling by targeting SOX2.

4. Discussion

TMZ is considered as a standard chemotherapy for GBM together with radiotherapy or as a single agent for maintenance therapy [19]. However, the developed resistance to TMZ is still a major obstacle for the effective treatment of glioma patients [20]. Hence, it is of great significance to figure out the possible molecular mechanisms of the innate or the acquired resistance for improving the drug efficiency and clinical outcomes.

In recent years, miRNAs have been extensively studied for their

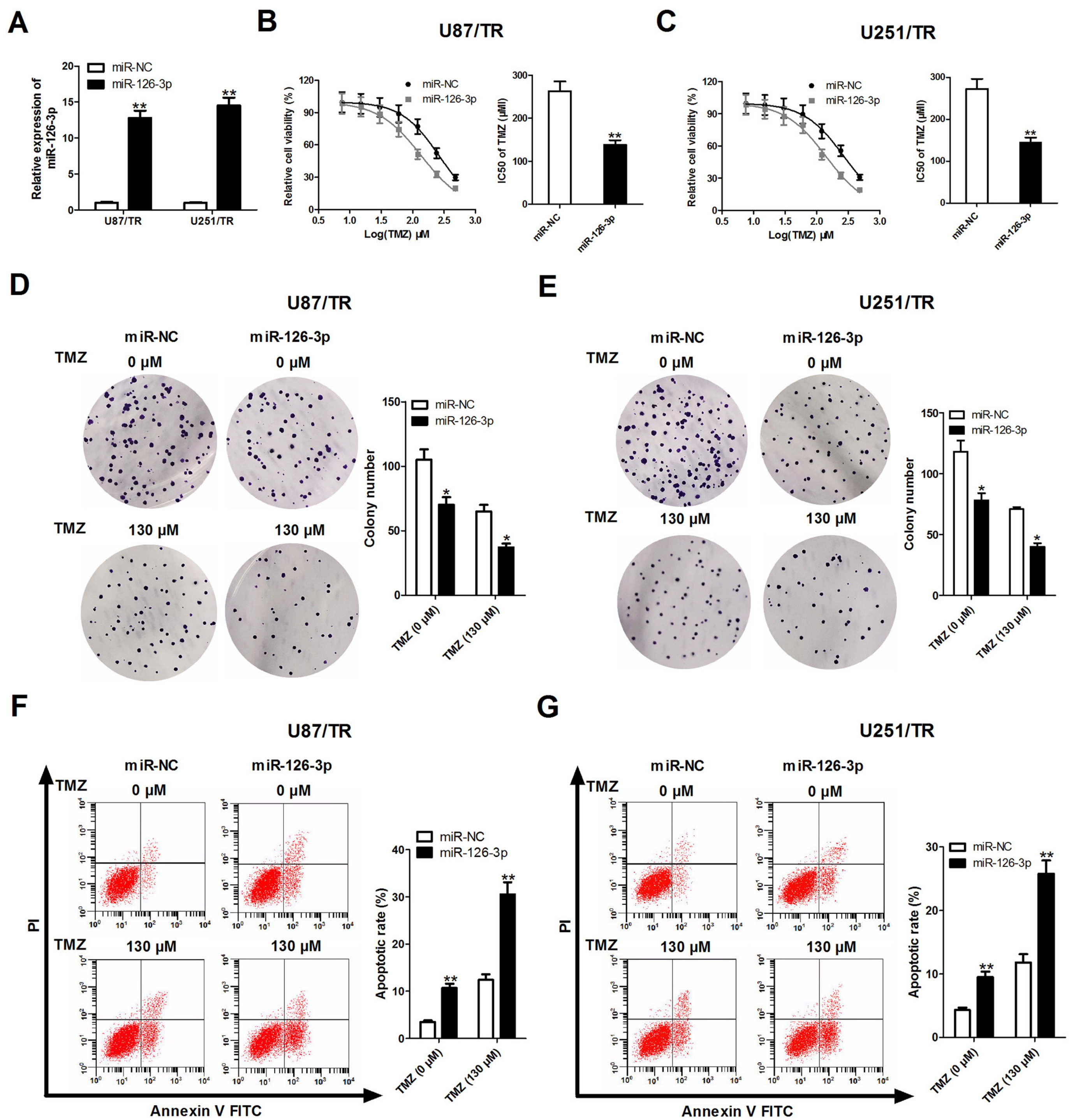

Fig. 2. Ectopic expression of miR-126-3p increases the chemosensitivity to TMZ in TMZ-resistant GBM cells. (A) qRT-PCR analysis was used to assess the transfection efficiency of miR-126-3p in U87/TR and U251/TR cells. (B and C) CCK-8 assay was applied to evaluate the effect of miR-126-3p on cell viability and IC50 values in U87/TR and U251/TR cells after treatment with different doses of TMZ. (D and E) Colony formation assay was carried out to determine the effect of miR-126-3p on the colony forming ability in U87/TR and U251/TR cells with or without TMZ treatment. (F and G) Flow cytometry analysis was conducted to confirm the effect of miR-126-3p on apoptosis in U87/TR and U251/TR cells with or without TMZ treatment. $^{*}P < 0.05$, $^{**}P < 0.01$.

implication in tumorigenesis [21]. A growing number of miRNAs have been demonstrated to participate in the regulation of chemoresistance in different malignant tumors through various mechanisms and pathways [22]. MiR-126-3p was previously found to exert anti-tumor activity in several human cancers [23,24]. Moreover, recent documents elucidated the anticancer effects of miR-126-3p in GBM genesis and development [13,14]. Also, Han et al. revealed that miR-126 expression was significantly lower in glioblastoma samples than that in paired non-tumoral controls, and patients with higher intratumoral miR-126 expression improved postsurgical prognosis [25]. Luan et al. reported that miR-126 exerted tumor-suppressive effects in glioma cells by targeting insulin receptor substrate 1 (IRS-1) via the PI3K/AKT signaling pathways [26]. Cui et al. clarified that miRNA-126 down-regulation phenomenon in patients with glioma was associated with higher level of methylation [27]. Xu et al. discovered that miR-126 suppressed the migration and invasion of glioma cells possibly via regulating GATA4

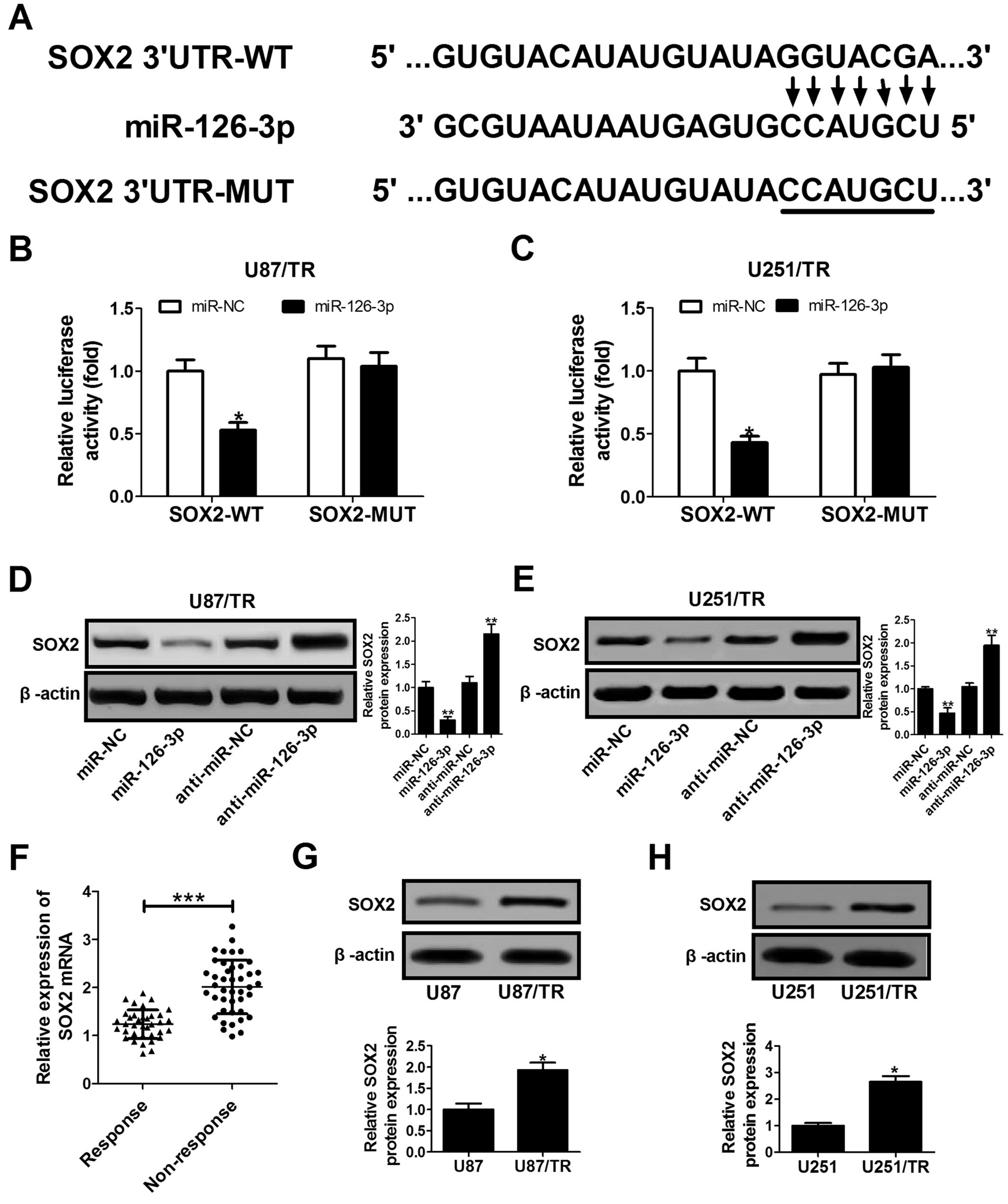

Fig. 3. SOX2 is identified as a direct target of miR-126-3p in TMZ-resistant GBM cells. (A) The predicted miR-126-3p target sequence in the 3′UTR of SOX2, as well as the mutant containing altered nucleotides in the 3′UTR of SOX2. (B and C) Luciferase activity assay in U87/TR and U251/TR cells transfected with SOX2-WT or SOX2-MUT reporter and miR-NC or miR-126-3p. (D and E) Western blot analysis was performed to examine the effect of miR-126-3p overexpression or knockdown on SOX2 protein level in U87/TR and U251/TR cells. (F) qRT-PCR analysis was used to test the expression level of SOX2 mRNA in tumor tissues from patients sensitive to TMZ (n = 36) and patients insensitive to TMZ (n = 44). (G and H) SOX2 protein expression analysis in TMZ-resistant GBM cells and corresponding parental GBM cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

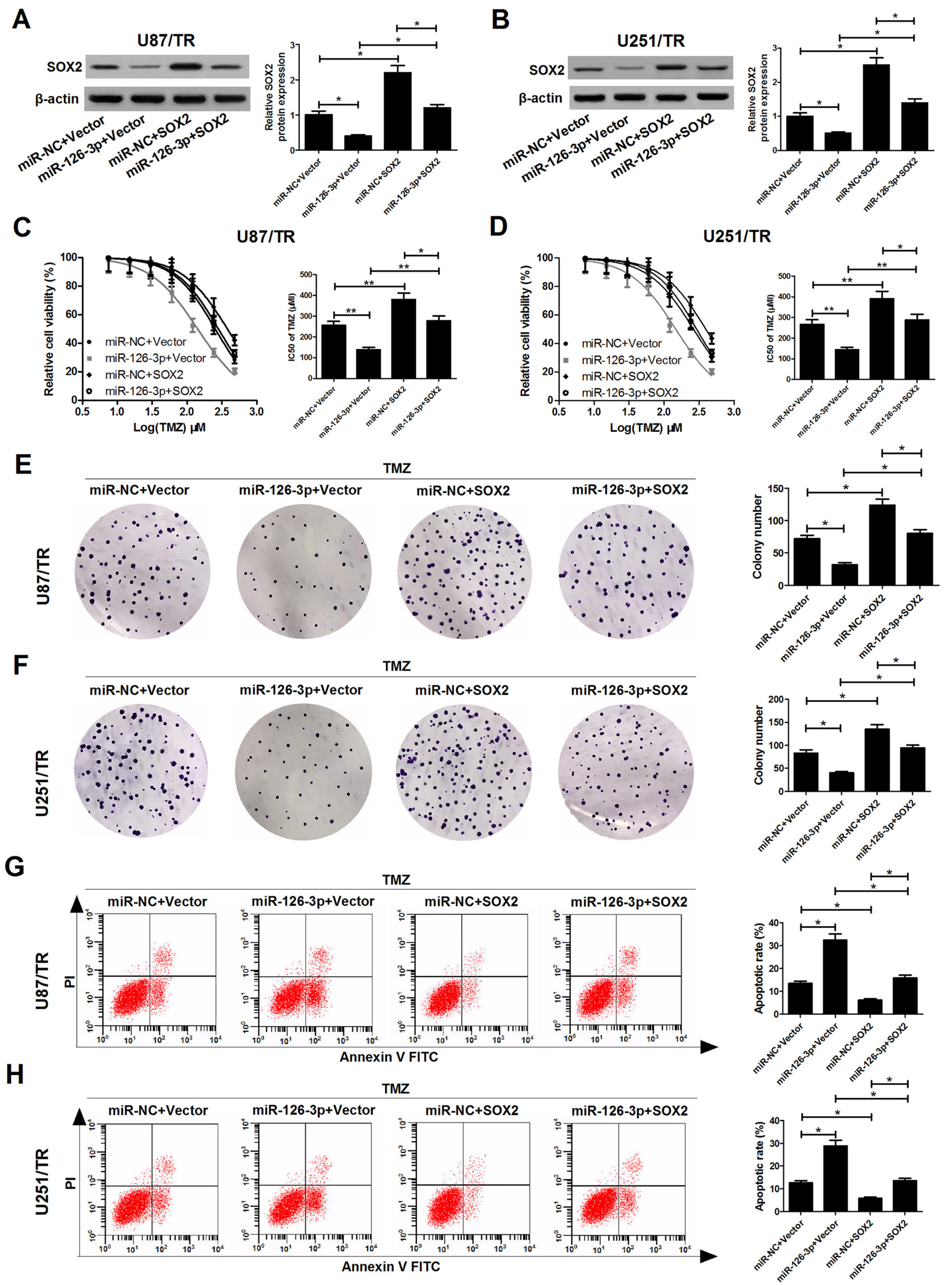

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Fig. 4. SOX2 overexpression reversed miR-126-3p-mediated TMZ sensitivity in TMZ-resistant GBM cells. (A and B) U87/TR and U251/TR cells were transfected with miR-NC or miR-126-3p, together with or without SOX2-overexpressing plasmid, followed by Western blot analysis of SOX2 protein level. (C and D) Transfected U87/TR and U251/TR cells were treated with a serial dose of TMZ for 48 h, then the cell viability and IC_{50} value of TMZ were monitored by CCK-8 assay. (E and F) Transfected U87/TR and U251/TR cells were treated with indicated dose of TMZ for 48 h, then the colony forming ability was identified at day 12. (G and H) After treatment with indicated dose of TMZ for 48 h, the apoptotic rate was measured by flow cytometry. *$P < 0.05$, **$P < 0.01$.

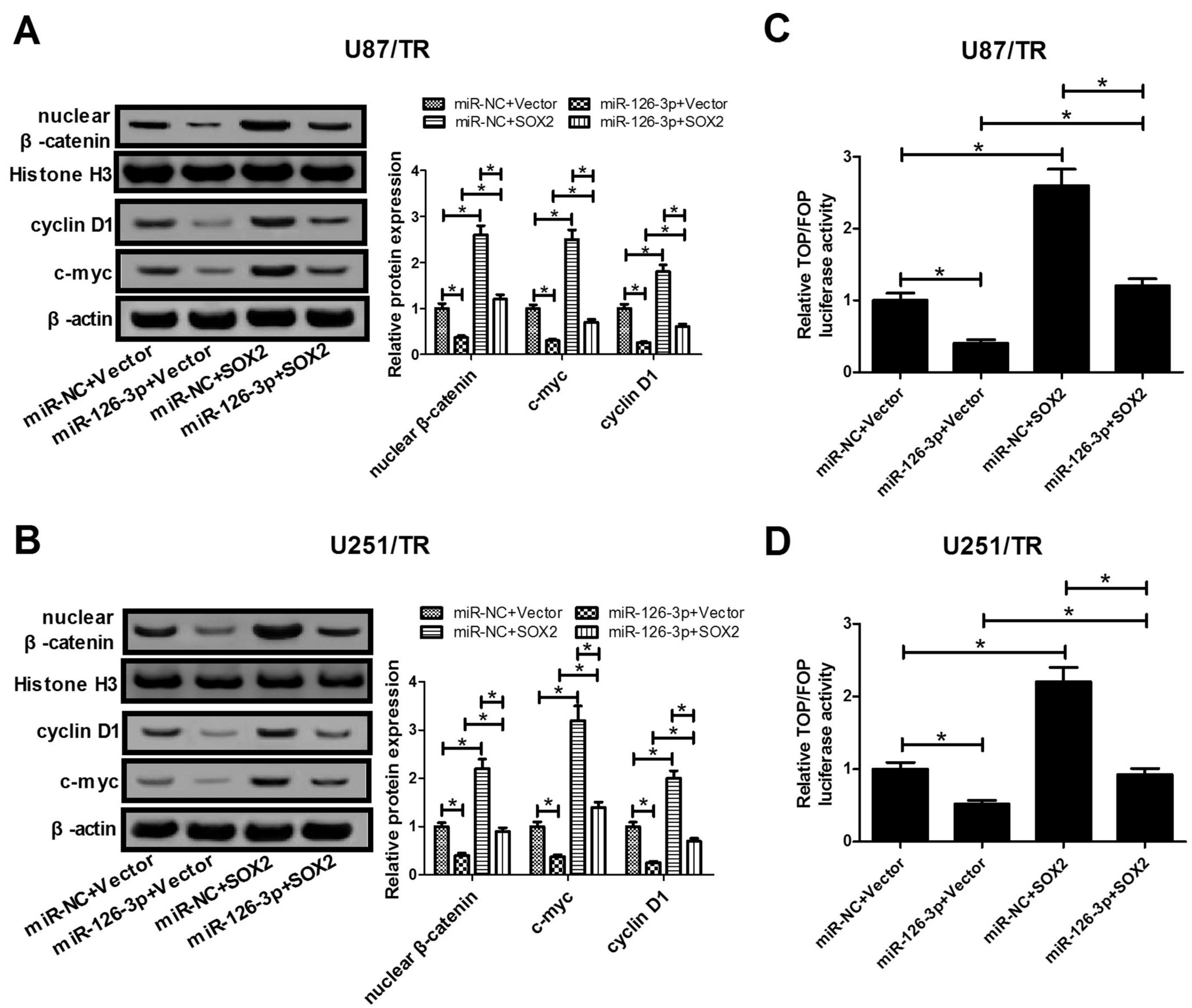

Fig. 5. miR-126-3p-mediated inactivation of Wnt/β-catenin signaling was abated by SOX2 up-regulation. (A and B) Western blot assay was used to assess the effects of miR-126-3p or SOX2 on the protein levels of nuclear β-catenin, c-myc, and cyclin D1 in both U87/TR and U251/TR cells. (C and D) TOP/FOP luciferase activity assay was performed to explore the effects of miR-126-3p or SOX2 on Wnt/β-catenin signaling pathway. *$P < 0.05$.

[28]. Here, we aim to further investigate the biological functions of miR-126-3p in modulating TMZ resistance phenotype of GBM. In the present study, we found that miR-126-3p expression was lowered in TMZ-resistant GBM tissues and cells. Gain-of-function assays manifested that overexpression of miR-126-3p decreased cell viability, inhibited colony forming potential, and facilitated apoptosis in U87/TR and U251/TR cells with TMZ treatment. These data suggested that miR-126-3p could increase TMZ sensitivity of GBM cells. In accordance with our results, Caporali et al. found that depletion of miR-126 expression is associated with acquired resistance to dabrafenib in melanoma cells, and restoration of miR-126 in dabrafenib-resistant melanomas repressed tumor growth and metastasis [29]. Wang et al. disclosed that ectopic expression of miR-126 induced the sensitivity of SGC7901/VCR and SGC7901/ADR cells to vincristine (VCR) and adriamycin (ADR) in gastric cancer through targeting EZH2 [30]. Zhang et al. described that miR-126 impaired drug resistance to TNF-related apoptosis-inducing ligand (TRAIL) by suppressing the expression of c-FLIP in cervical cancer [31]. All these data indicated that miR-126-3p may be applied as a sensitizing agent for anti-tumor drugs.

Subsequently, we used on-line bioinformatic softwares and luciferase reporter experiments to identify the candidate targets of miR-126-3p. As a result, SOX2 was proved as a direct target of miR-126-3p, and could be inhibited by miR-126-3p. Moreover, SOX2 expression was enhanced in TMZ-resistant glioma tissues and cells, consistent with a recent report elucidating that SOX2 was up-regulated in U87/TR cells [32]. SOX2, a transcription factor belonging to the SOX gene family and containing a high-mobility group (HMG) domain, was found to be overexpressed in a variety of cancer types and promote tumor aggressiveness and worse prognosis [33]. Also, SOX2 was validated as a cancerigenic agent in glioma through affecting cell proliferation, migration and invasion in glioma [34,35]. Moreover, SOX2 was linked to the resistance development of different chemotherapy drugs, such as tamoxifen [36], adriamycin [37] and paclitaxel [38], via regulating various signaling pathways. In this study, we found that enforced expression of SOX2 promoted cell viability, increased colony forming capability, and reduced apoptosis in U87/TR and U251/TR cells with TMZ treatment, suggesting that SOX2 rendered TMZ resistance in GBM cells. Similarly, Garros-Regulez et al. showed that high levels of SOX2 and SOX9 were correlated with TMZ resistance in glioma stem cells [39]. Furthermore, miR-126-3p-mediated TMZ sensitivity was obviously abated by overexpression of SOX2 in U87/TR and U251/TR cells. These findings illustrated that miR-126-3p induced TMZ sensitivity in GBM cells via inhibiting SOX2 expression.

Wnt/β-catenin signaling plays an important part in cell proliferation, migration, invasion, and angiogenesis, thereby contributing to glioma progression [40]. Wnt/β-catenin signaling was previously clarified to be related to SOX2-mediated carcinogenesis [41,42]. Herein, we found that miR-126-3p overexpression inhibited Wnt/β-catenin signaling, while SOX2 up-regulation activated Wnt/β-catenin signaling. Moreover, miR-126-3p-induced suppression of Wnt/β-catenin signaling was reversed following increased expression of SOX2. In summary, miR-126-3p sensitized GBM cells to TMZ possibly through inactivation of Wnt/β-catenin signaling by targeting SOX2.

In summary, our present data demonstrated that restoration of miR-126-3p resulted in a suppression of SOX2 expression and Wnt/β-catenin signaling, thereby sensitizing GBM cells to TMZ. Combination of miR-126-3p and TMZ may be a potential strategy advantage for GBM patients.

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Conflicts of interest

The authors declare that no competing interest exists.

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