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Overexpression of lncRNA IRAIN restrains the progression and Temozolomide resistance of glioma via repressing IGF-1R-PI3K-NF-κB signaling pathway

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Summary. Background. Increasing studies have found that long noncoding RNAs (lncRNAs) contribute to regulating tumor progression. This study explores the expression characteristics, effects, and related mechanisms of lncRNA IGF1R antisense imprinted non-protein coding RNA (IRAIN) in glioma.

Methods. Quantitative real-time PCR (qRT-PCR) was implemented to testify the IRAIN profile in glioma tissues and paracancerous tissues, and the link between the IRAIN level and the clinicopathological indicators of glioma was analyzed. IRAIN overexpression and knockdown cell models were constructed in glioma cells. Cell proliferation was verified by the colony formation experiment, while flow cytometry was implemented to monitor apoptosis. Transwell assay was performed to examine cell invasion and migration. Western blot (WB) was adopted to compare the profiles of the apoptosis-related proteins (Bax, Bcl2, and Caspase3) and IGF-1R-PI3K-NF-κB pathway.

Results. IRAIN was down-regulated in glioma tissues (compared with adjacent normal tissues), and the low IRAIN expression was significantly linked with the larger tumor volume and higher pathological stages. Functionally, overexpressing IRAIN abated glioma cell proliferation, invasion, and migration, promoted apoptosis, and attenuated IGF-1R-PI3K-NF-κB expression and temozolomide (TMZ) resistance, which was also confirmed in the xenograft tumor experiment. The WB result showed that overexpressing IRAIN inactivated the IGF-1R-PI3K-NF-κB pathway. Additionally, the IGF-1R knockdown model was established in U251 cells. Si-IGF-1R induced cell proliferation inhibition, promoted cell death, and reduced cell migration and TMZ resistance, whereas Si-IGF-1R+IRAIN group showed no additional effects on glioma cells compared with the Si-IGF-1R group.

Conclusion. IRAIN repressed glioma development and TMZ resistance by inactivating the IGF-1R-PI3K-NF-κB axis.

Key words: Glioma, IRAIN, Signaling pathway, Temozolomide, Resistance

Introduction

Background

Glioma is the commonest primary brain tumor worldwide with different biological and clinical characteristics (Leng et al., 2018). It is mainly derived from astrocytes or oligodendrocytes and shows an invasive growth pattern, lacking a clearly identifiable tumor boundary (Cordier et al., 2016). Glioma treatment includes surgical resection, followed by adjuvant external irradiation or systemic chemotherapy, depending on the degree of resection and WHO grade (Morshed et al., 2019). Despite the great progress in surgical and clinical treatment, the molecular complexity of glioma and its different pathological stages lead to inaccurate prediction of the disease progression and treatment failure (Malta et al., 2018). Therefore, glioma treatment is still challenging, and it is particularly important to probe the molecular mechanism of glioma to provide some reference for its treatment and prognosis.

Long noncoding RNAs (lncRNAs) are over 200

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Abbreviations. lncRNAs, long noncoding RNAs; qRT-PCR, Quantitative real-time PCR; WB, western blot; TMZ, temozolomide; IGFs, Insulin-like Growth Factors; IGFRs, IGF receptors; NF-κB, nuclear factor B; WHO, World Health Organization; PVDF, polyvinylidene fluoride; RT, room temperature; HRP, horseradish peroxidase

nucleotides in length and are not translated into proteins (Lu et al., 2019). It is reported that the abnormal expression of various lncRNAs contributes to glioma development (Zhang et al., 2021). For example, lncRNA growth arrest specific 5 (GAS5) effectively inhibits glioma cell proliferation, migration and invasion and facilitates apoptosis by targeting glutathione S-transferase mu 3 (GSTM3) (Li et al., 2019). In addition, lncRNA cancer susceptibility candidate 7 (CASC7) dampens glioma progression by regulating the Wnt/β-catenin pathway (Gong et al., 2019). LncRNA IRAIN, an antisense imprinted non-protein coding RNA of IGF-1R, is a member of the lncRNA family. It is located at 15q26.3 and is 5359 bp in length, but its role in glioma remains elusive.

Insulin-like Growth Factors (IGFs) are a type of multifunctional cell proliferation regulators, which contribute to promoting cell differentiation, proliferation, and growth (Zapf and Froesch, 1986). IGF receptors (IGFRs) mainly include IGF-1R and IGF-2R (Benarroch, 2012). It was found that IGF-1R, a transmembrane tyrosine protein receptor, is homologous to the amino acid sequence of the insulin receptor. It is widely distributed in most body tissues, such as the brain, kidney, and heart, and it contributes to regulating cell mitosis, proliferation and anti-apoptosis after binding with IGFRs (Rajapaksha et al., 2012). PI3K is an intracellular phosphatidylinositol kinase, and nuclear factor κB (NF-κB) is a protein complex controlling cytokine production and cell survival. Several pieces of research have revealed that the IGF-1R-PI3K-NF-κB signaling pathway affects various tumors. For instance, IGF-1R knockdown inhibits pancreatic cancer growth and enhances its chemosensitivity by inhibiting the PI3K/AKT and NF-κB pathways (Ma et al., 2010) Additionally, Wu CM et al. found that IGF-I increases the expression of α5β1 integrin through the IGF-1R/PI3K/Akt/NF-κB axis, thus strengthening chondrosarcoma cell migration (Wu et al., 2011). However, whether the IGF-1R-PI3K-NF-κB signaling pathway has the same effect in glioma needs further study.

It is reported that temozolomide (TMZ), a commonly used chemotherapy drug, is widely utilized in the clinical treatment of glioma. Studies have found that knocking down lncRNA MIR155HG increases the TMZ sensitivity of glioma by down-regulating polypyrimidine tract binding protein 1 (PTBP1) to inactivate Wnt/β-catenin (He et al., 2021). Besides, lncRNA CCAT2 enhances glioma sensitivity to TMZ through the miR-424/Chk axis (Ding et al., 2020). These reports illustrate that multiple lncRNAs have important values in glioma sensitivity to TMZ, but whether IRAIN has the same effect is still unclear. Overall, we intend to probe the function and mechanism of IRAIN in glioma. It was found that IRAIN is downregulated in the glioma tissues and glioma cell lines. Further mechanism studies have confirmed that IRAIN inhibits glioma progression as a tumor suppressor and enhances TMZ sensitivity by inactivating IGF-1R-PI3K-NF-κB pathway, which provides referenceable molecular markers for the clinical treatment and prognosis evaluation of glioma.

Materials and methods

Clinical specimen collection

Cancerous tissues and adjacent normal tissues of 45 patients who underwent glioma resection in Zhangzhou Municipal Hospital of Fujian Province and Zhangzhou Affiliated Hospital of Fujian Medical University from July 2015 to August 2018 were collected. None of the patients received chemotherapy or radiotherapy before the surgery. The control samples were from the same patients (at least 3 cm away from the surgical resection margin), and no cancer cells were discovered in the postoperative examination. The diagnosis of glioma was pathologically confirmed according to World Health Organization (WHO) standards. All specimens were immediately preserved in -196°C liquid nitrogen until they were used for RNA extraction. The Research Ethics Committee of Zhangzhou Affiliated Hospital of Fujian Medical University authorized this study, and all participating patients signed the informed consent.

Cell culture

Human normal glial cell HEB and glioma cell lines (U251, A172, SF-539, and Hs683) were bought from ATCC (Rockville, USA). Then, they were cultured in DMEM supplemented with 5% FBS (Thermo Fisher Scientific, MA, USA) in an incubator with 5% CO_2 at 37°C. The medium was altered once every 2 days, and the cells were sub-cultured once every 5 days. Experiments were carried out when the cells covered about 90% of the bottle bottom. It was found that IRAIN had the lowest expression in U251 and the highest expression in A172. Therefore, U251 and A172 cells were selected as research objects in subsequent studies.

Cell transfection

After trypsinization and passage, U251 and A172 cells at the logarithmic growth stages were seeded in 6-well plates at 5×10^6/well. Cells were transfected after they grew stable. IRAIN overexpression plasmids, siRNA targeting IGF-1R (Si-IGFR) and corresponding controls, including negative control (NC), small inference RNA negative control (si-NC), were provided by GenePharma (Shanghai, China). U251 and A172 were inoculated into 24-well plates at 3×10^5 cells/well. They were cultured at 37°C with 5% CO_2 for 24 h before cell transfection using Lipofectamine® 3000 (Invitrogen; ThermoFisherScientific, Inc.). The transfection validity was calculated by quantitative real-time PCR (qRT-PCR). The cells were incubated at 37°C with 5% CO_2 for 24 h for subsequent analysis (Shi et al., 2020).

Quantitative real-time polymerase chain reaction (QRT-PCR)

Total cellular RNA was isolated with the TRIzol reagent and reversely transcribed into cDNA with the PrimeScript™ RT Reagent kit (Invitrogen, Shanghai, China). Bio-Rad CFX96 quantitative PCR system and SYBR were used for qPCR, according to the manufacturer's regulations. The conditions for PCR were predenaturation for 5 min at 95°C, denaturation for 15 s at 95°C, and annealing for 30 s at 60°C. GAPDH was the endogenous control of IRAIN, and the $2^{(-\Delta\Delta Ct)}$ method was adopted for statistics. Each test was done three times. The primer sequences are exhibited in Table 1.

Western blot (WB)

After cell treatment, the culture medium was discarded, and the protein lysates (Roche) were added, and the total proteins were separated. Next, 50 μg total protein was loaded on 12% polyacrylamide gel at 100 V for electrophoresis for 2 h and then transferred to polyvinylidene fluoride (PVDF) membranes. After the membranes were blocked with 5% skimmed milk for 1 h at room temperature (RT), they were rinsed with TBST three times (10 min each time) and incubated with the primary Anti-Bcl2 antibody (ab182858), Anti-Bax antibody (ab32503), Anti-Caspase3 antibody (ab13847), Anti-PI3K antibody (ab191606), Anti-p-PI3K antibody (ab182651), Anti-p-NF-κB antibody (ab28849), Anti-NF-κB antibody (ab32360), Anti-IGF-1R antibody (ab182408) (concentration: 1:1000), Anti-GAPDH antibody (ab8245) at 4°C overnight. After being cleared with TBST, the membranes were incubated with horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibodies (1:300) for 1 h at RT. All the above antibodies were bought from Abcam (Cambridge, UK). After, the membranes were rinsed with TBST three times (10 min/time). Finally, WB special reagents were employed for color imaging, and Image J (Media Cybem ethics, America) was used to analyze the gray intensity of each protein.

Colony formation experiment

U251 and A172 cells at the logarithmic growth stage were inoculated in 6-well plates with 1×10^6 cells/well. After culturing for 24 h, they were inoculated in 60 mm culture dishes with 200 cells/well. The medium was discarded after culturing for 8 days, and then the cells were washed with PBS three times, immobilized with methanol for 20 min, dyed with 1% methylene blue for 40 min, cleared twice with deionized water, and dried. The number of colonies over 50 cells was counted under 40× microscope (Zheng et al., 2020). The test was conducted three times to take the mean value.

Table 1. Primer sequences of each gene.

Gene name	primer sequence (5'→3')
lncRNA IRAIN	forward: CAAACTCCTCTCTCCTGCCA reverse: AAGTCTCCGCGGTTGTTTTC
GADPH	forward: AGAAGGCTGGGGCTCATTTG reverse: AGGGGCCATCCACAGTCTTC

Transwell assay

U251 and A172 cells were inoculated in a 24-well plate equipped with small chambers, and each group was provided with two repetitive wells. U251 and A172 were suspended in a serum-free medium in the upper chamber (approximately 10^5 cells/well) (8 μm pore size; BD Biosciences), and the lower chamber was supplemented with 500 μL RMPI-1640 containing 10% fetal bovine serum. Twenty-four hours later, the cells on the upper chamber (i.e., the unmigrated cells) were gently swabbed, and the cells that migrated to the lower chamber were fixed with methanol and stained with crystal violet. Five fields (including the center and periphery of the membranes) were randomly selected under an inverted microscope to count the number of migrated cells. In the invasion experiment, the bottom of the transwell chamber was pre-coated with matrigel, and the other steps were the same as that of the migration experiment. All experiments were done in triplicate (Fei et al., 2020).

Flow cytometry

U251 and A172 cells in the logarithmic phase were collected to make the single-cell suspension, which was then seeded in a 25-cm^2 culture bottle overnight. After cell adherence, the old medium was removed. The experimental group was supplemented with the culture medium containing 0.3% FBS, while the control group was supplemented with the medium containing an equal volume of PBS. The two mediums were incubated at 37°C with 5% CO_2 for 24 h, and the cell supernatant was harvested and cleaned with cold PBS. After, the cells were trypsinized with EDTA-free trypsin and harvested. The remaining steps were performed following the AnnexinV-PI Apoptosis Kit (Southern Biotechnology, Birmingham, Al, USA), and cell apoptosis was determined by flow cytometry within 1 h (Fei et al., 2020).

Tumor formation experiment in nude mice

Six-week-old BALB/c-nu nude mice were employed to construct the tumor formation model. A172 cells transfected with si-NC or si-IRAIN at the logarithmic growth stage were treated with 0.25% trypsin and collected. Then, the cells were rinsed and resuspended in serum-free medium to make single-cell suspension (3×10^7/mL). Subsequently, 0.1 mL suspension was subcutaneously injected in the left forearm armpit of each nude mouse (20 mice in total, 5 mice in each

group). After the injection of tumor cells, TMZ (200 μmol/kg) was injected three times within the first week (at day 1, day3, and day 6) (Wedge and Newlands, 1996). The mice were anesthetized on day 36, and the tumors were removed and weighed. The longest diameter (a) of the tumor and the shortest diameter (b) perpendicular to (a) were measured with the vernier caliper. The tumor volume was calculated according to the formula V (mm^3)=0.5× a × b^2. All experiments were approved by the Ethics Review Committee of Zhangzhou Municipal Hospital.

Immunohistochemistry

The tumors were collected and fixed in 4% paraformaldehyde solution (Beyotime, Wuhan, China) for 24 h at room temperature, embedded in paraffin, deparaffinized in a series of xylene and graded alcohols, heated in citrate buffer (10 mM, pH 6.0) in sub-boiling temperature for 10 min for antigen retrieval. After that, the sections were incubated with primary antibody (Ki-67, ab15580, 1:250, Abcam, China) for 12 h at 4°C, washed by washing buffer for 15min (twice) and incubated with HRP-labeled secondary antibody at room temperature for 1 hour. Finally, the sections were observed using a microscope (Olympus, Japan).

Statistical Analysis

Experimental data were processed by the SPSS18.0 statistical software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software, USA). Student's t test was adopted to compare the mean difference between the two groups, and analysis of variance was used to compare the difference between multiple groups. P<0.05 presented statistical significance.

Results

Expression of lncRNA IRAIN in glioma tissues and cells

We implemented qRT-PCR to test the IRAIN profile in glioma tissues. Our results showed that IRAIN was down-regulated in glioma tissues compared with t the normal tissues adjacent to cancer (P<0.05, Fig. 1A). Also, the IRAIN levels in different glioma cell lines were detected. It turned out that IRAIN was down-

Table 2. Correlation between IRAIN levels and clinical features in glioma patients.

Pathological Parameters	n	Profile of IRAIN		p-Value
		High (n=24)	Low (n=21)	
Age (years)				0.807
<45	27	14	13	
≥45	18	10	8	
Gender				0.936
Male	26	14	12	
Female	19	10	9	
Tumor stage (WHO)				0.012*
I-II	24	17	7	
III-IV	21	7	14	
Tumor volume				0.046*
≥5 cm	34	21	13	
<5 cm	11	3	8	
Invasion Depth				0.028*
T1-T2	27	18	9	
T3-T4	18	6	12	
Lymphatic metastasis				0.033*
positive	16	5	11	
Negative	29	19	10	

*P<0.05 indicates statistical difference.

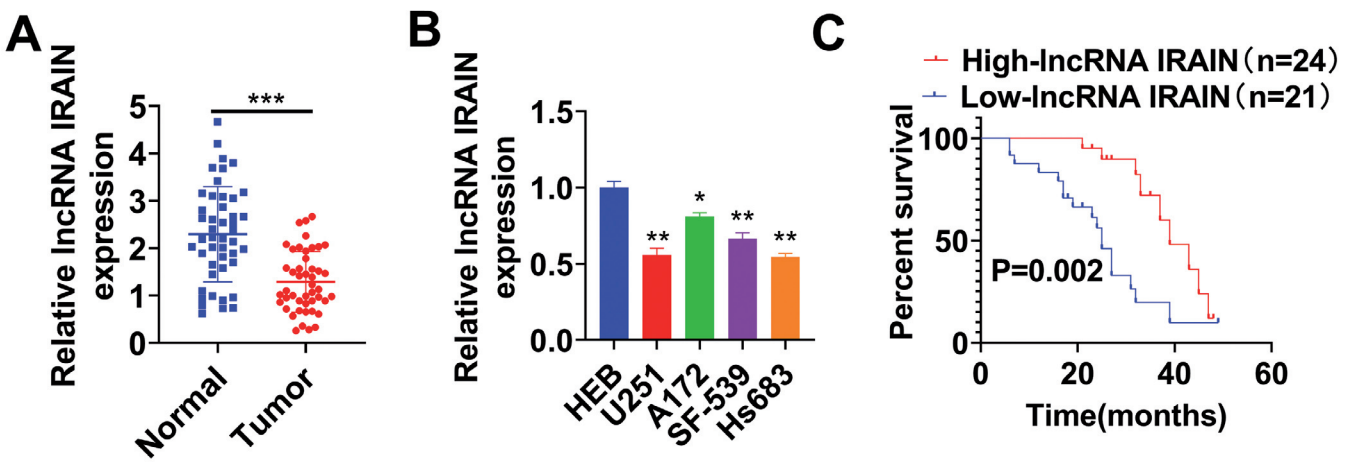

Fig. 1. The IRAIN profile in glioma tissues and cells. **A.** QRT-PCR was adopted to detect the IRAIN level in glioma tissues. ***P<0.001. **B.** QRT-PCR was conducted to compare the IRAIN levels in different glioma cell lines. **C.** The survival and prognosis of glioma patients and IRAIN level. *P<0.05, **P<0.01, ***P<0.001 (vs. HEB group). N=3

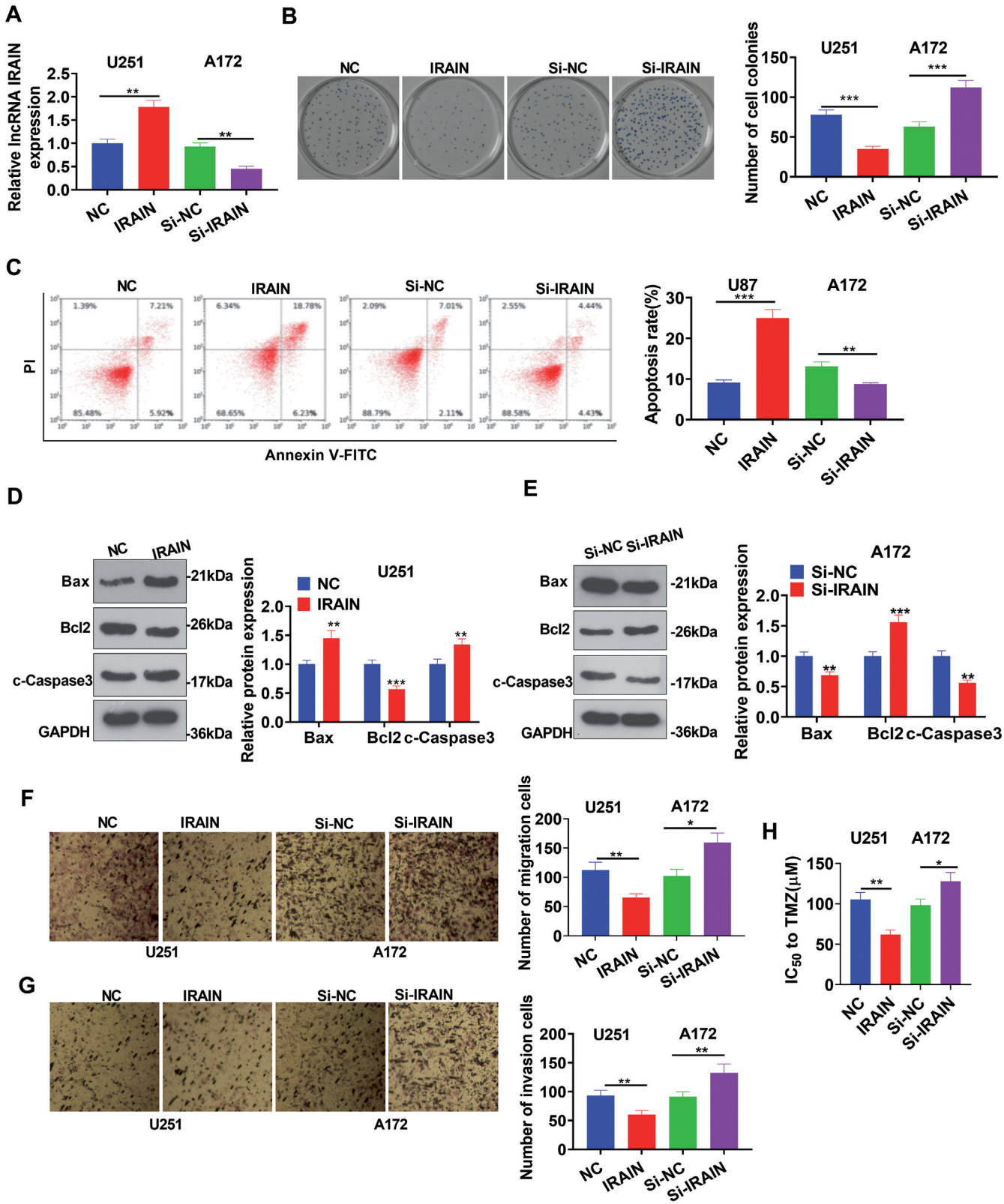

Fig. 2. Influence of regulating IRAIN on glioma progression. IRAIN overexpression and knockdown models were constructed in U251 and A172, respectively. **A.** The IRAIN profile was determined by qRT-PCR. **B.** Colony formation assay was carried out to test cell proliferation. **C.** Flow cytometry was implemented to verify apoptosis. **D, E.** The profiles of Bax, Bcl2, and c-Caspase3 were monitored by WB. **F, G.** Transwell assay was conducted to investigate the influence of IRAIN on glioma cell migration and invasion. **H.** Regulation of IRAIN in changes of TMZ IC50 in glioma cells. *P<0.05, **P<0.01, ***P<0.001. N=3.

regulated in glioma cell lines (U251, A172, SF-539 and Hs683) compared with the HEB group (P<0.05, Fig. 1B). Moreover, patients with low expression of IRAIN had higher WHO grades, larger tumor volume, deeper invasion, and more lymphatic metastasis (Table 2). Meanwhile, glioma patients with higher IRAIN level had longer survival time than those with low IRAIN levels (Fig. 1C). These results suggested that IRAIN might modulate the progression of glioma.

Effects of regulating IRAIN on glioma development in vitro

We constructed IRAIN overexpression and knockdown models in U251 and A172 cells, respectively, to investigate the effect of IRAIN in glioma (P<0.05, Fig. 2A). By comparing with the NC or si-NC group, the colony formation experiment revealed that glioma cell proliferation was dampened after overexpressing IRAIN, and it was enhanced after knocking down IRAIN (P<0.05, Fig. 2B). Besides, Flow cytometry verified that up-regulating IRAIN in cells significantly increased apoptosis, while knocking down IRAIN had the opposite effect (P<0.05, Fig. 2C). Moreover, WB results confirmed that overexpressing IRAIN elevated the levels of pro-apoptotic proteins Bax and cleaved Caspase3 (c-Caspase3) and hindered the profiles of anti-apoptotic protein Bcl2, while knocking down IRAIN weakened the effect (P<0.05, Fig. 2D-E). Furthermore, the transwell assay was carried out, and it proved that compared with the control group overexpressing IRAIN reduced glioma cell migration and invasion, while knocking down IRAIN had the opposite effect (P<0.05, Fig. 2F-G). Meanwhile, the TMZ IC50 in the IRAIN overexpression group was abated, while IRAIN knockdown led to the opposite results (P<0.05, Fig. 2H). These conclusions proved that IRAIN repressed glioma cell proliferation, migration, and invasion and enhanced apoptosis and TMZ sensitivity.

IRAIN inactivated IGF-1R-PI3K-NF-κB pathway

We performed WB to further explore the impact of IRAIN on theIGF-1R-PI3K-NF-κB axis. Our results showed that compared with the NC group, IGF-1R level, phosphorylated PI3K and NF-κB were down-regulated after overexpressing IRAIN, while they were up-regulated after knocking down IRAIN (vs. si-NC group, P<0.05, Fig. 3A-B). This indicated that up-regulating IRAIN inactivated the IGF-1R-PI3K-NF-κB pathway.

IRAIN dampened the progression of glioma by inhibiting IGF-1R-PI3K-NF-κB axis

We intervened U251 cells with the IRAIN overexpression plasmid and/or Si-IGF-1R to explore the specific mechanism of IRAIN in glioma. The colony formation experiment and flow cytometry proved that, compared with the control group, either overexpressing IRAIN or knocking down IGF-1R inhibited cell proliferation and promoted cell apoptosis (P<0.05, Fig. 4A-B). However, compared with the Si-IGF-1R group, the combined intervention of the two had no significant difference in cell proliferation and apoptosis (P>0.05, Fig. 4A-B). Also, WB confirmed that overexpressing IRAIN or knocking down IGFR abated Bcl2 and the IGF-1R-PI3K-NF-κB pathway and elevated the levels of Bax and c-Caspase3 (P<0.05, Fig. 4C). In contrast, compared with the Si-IGF-1R group, there was no significant difference in the expression of the above-mentioned proteins in the IRAIN+Si-IGF-1R group (P>0.05, Fig. 4C). Moreover, transwell assay demonstrated that overexpressing IRAIN or knocking down IGF-1R attenuated cell migration and invasion (P<0.05, Fig. 4D-E), while there was no distinct

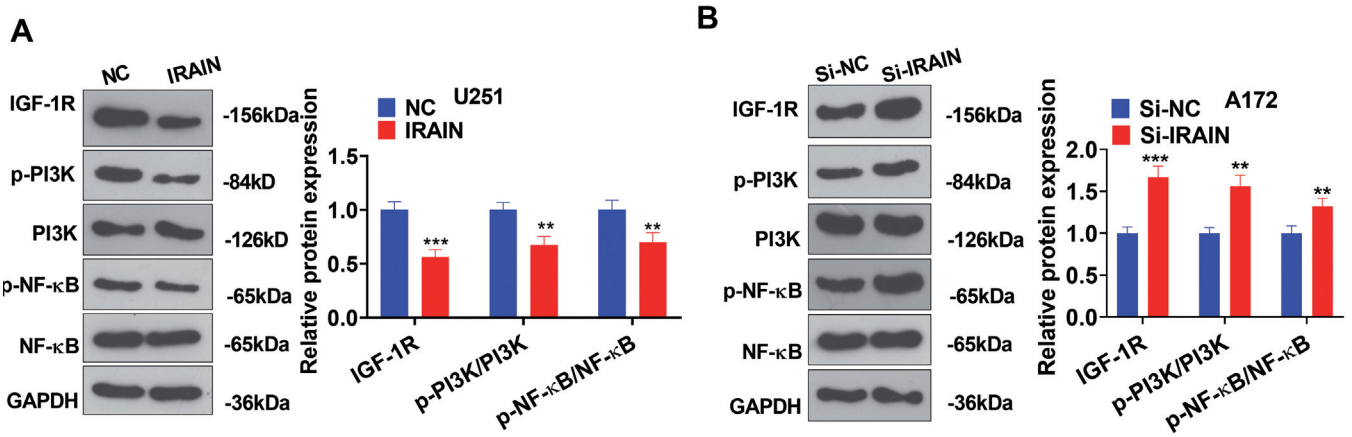

Fig. 3. IRAIN inactivated IGF-1R-PI3K-NF-κB axis. IRAIN overexpression and knockdown models were constructed in U251 and A172 cells, respectively. **A, B.** WB was employed to test the IGF-1R-PI3K-NF-κB level. **P<0.01, ***P<0.001. N=3.

difference in cell migration and invasion between the IRAIN+Si-IGF-1R group and the Si-IGF-1R group (P>0.05, Fig. 4D-E). Meanwhile, compared with the control group, the IC50 of the TMZ in the IRAIN group and the Si-IGF-1R group was reduced. On the other hand, compared with the Si-IGF-1R group, the IC50 of the TMZ in the IRAIN+Si-IGF-1R group was not significantly different (P>0.05, Fig. 4G). Furthermore, WB proved that IGF-1R level, phosphorylated PI3K and NF-κB were abated by overexpressing IRAIN or knocking down IGF-1R (P<0.05, Fig. 4G), while their expressions in the IRAIN+Si-IGF-1R group were not significantly altered in the Si-IGF-1R group (P>0.05, Fig. 4G). These findings proved that IRAIN repressed glioma cell proliferation, migration and invasion and strengthened TMZ sensitivity by attenuating IGF-1R-

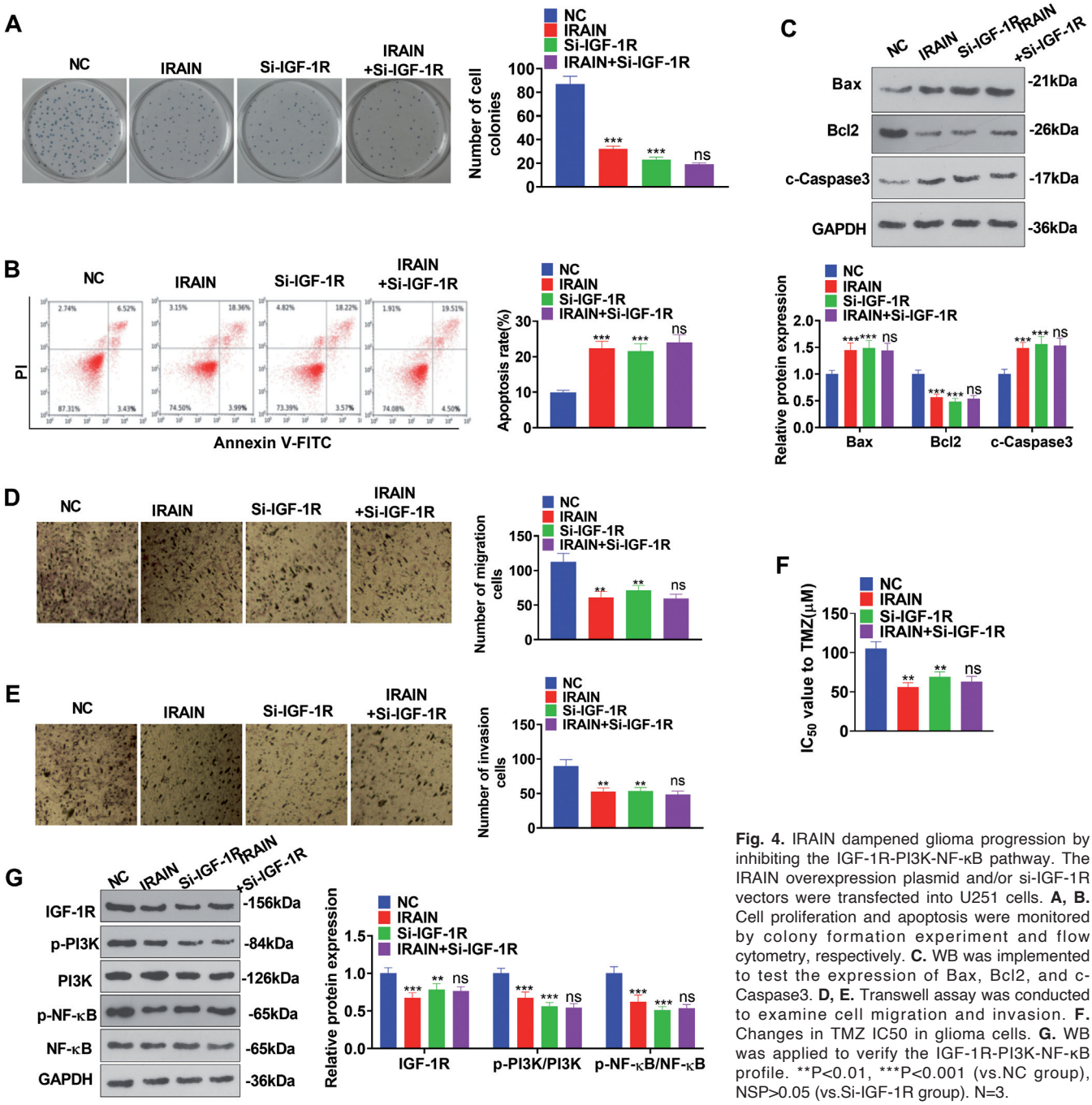

Fig. 4. IRAIN dampened glioma progression by inhibiting the IGF-1R-PI3K-NF-κB pathway. The IRAIN overexpression plasmid and/or si-IGF-1R vectors were transfected into U251 cells. **A, B.** Cell proliferation and apoptosis were monitored by colony formation experiment and flow cytometry, respectively. **C.** WB was implemented to test the expression of Bax, Bcl2, and c-Caspase3. **D, E.** Transwell assay was conducted to examine cell migration and invasion. **F.** Changes in TMZ IC50 in glioma cells. **G.** WB was applied to verify the IGF-1R-PI3K-NF-κB profile. **P<0.01, ***P<0.001 (vs.NC group), NSP>0.05 (vs.Si-IGF-1R group). N=3.

PI3K-NF-κB.

Overexpressing IRAIN dampened TMZ resistance

To explore the specific mechanism by which IRAIN repressed TMZ resistance in glioma, we gave 200 μM TMZ and IRAIN overexpression plasmids to treat U251 cells alone or in combination. Colony formation experiment and flow cytometry manifested that compared with the control group, TMZ alone abated cell proliferation and enhanced cell apoptosis, and the above effect was facilitated after overexpressing IRAIN on this basis (P<0.05, Fig. 5A-B). By comparing with the TMZ group, WB showed that TMZ alone knocked down Bcl2 and elevated Bax and c-Caspase3, and overexpressing IRAIN further enhanced the effect of TMZ (P<0.05, Fig. 5C). Besides, transwell assay confirmed that cell migration and invasion were repressed by TMZ alone, and they were further attenuated after overexpressing IRAIN (P<0.05, Fig. 5D-E). Furthermore, WB proved that TMZ alone hindered the IGF-1R-PI3K-NF-κB level, and overexpressing IRAIN further decreased the IGF-1R-PI3K-NF-κB profile (P<0.05, Fig. 5F). These findings demonstrated IRAIN facilitated the TMZ sensitivity in glioma.

IRAIN inhibited glioma progression and enhanced TMZ sensitivity in vivo

We conducted in vivo experiments to further verify the inhibition of IRAIN on glioma and TMZ sensitivity. As a result, compared with the control group, TMZ (200 μmol/kg) or IRAIN overexpression abated tumor volume and weight (Fig. 6A-C). Meanwhile, the combined intervention of the two further lessened tumor volume and weight compared with the TMZ group (P<0.05, Fig. 6A-C). Next, Ki67 staining was used to label the growth of tumor cells in vivo. As the data shows, both TMZ treatment and IRAIN overexpression reduced Ki-67-positive cells, while the combination of TMZ administration and IRAIN overexpression markedly reduced Ki-67-positive cells (p<0.05 compared with TMZ group, Fig. 6 D). Additionally, the IGF-1R-PI3K-NF-κB expression was detected by WB. It

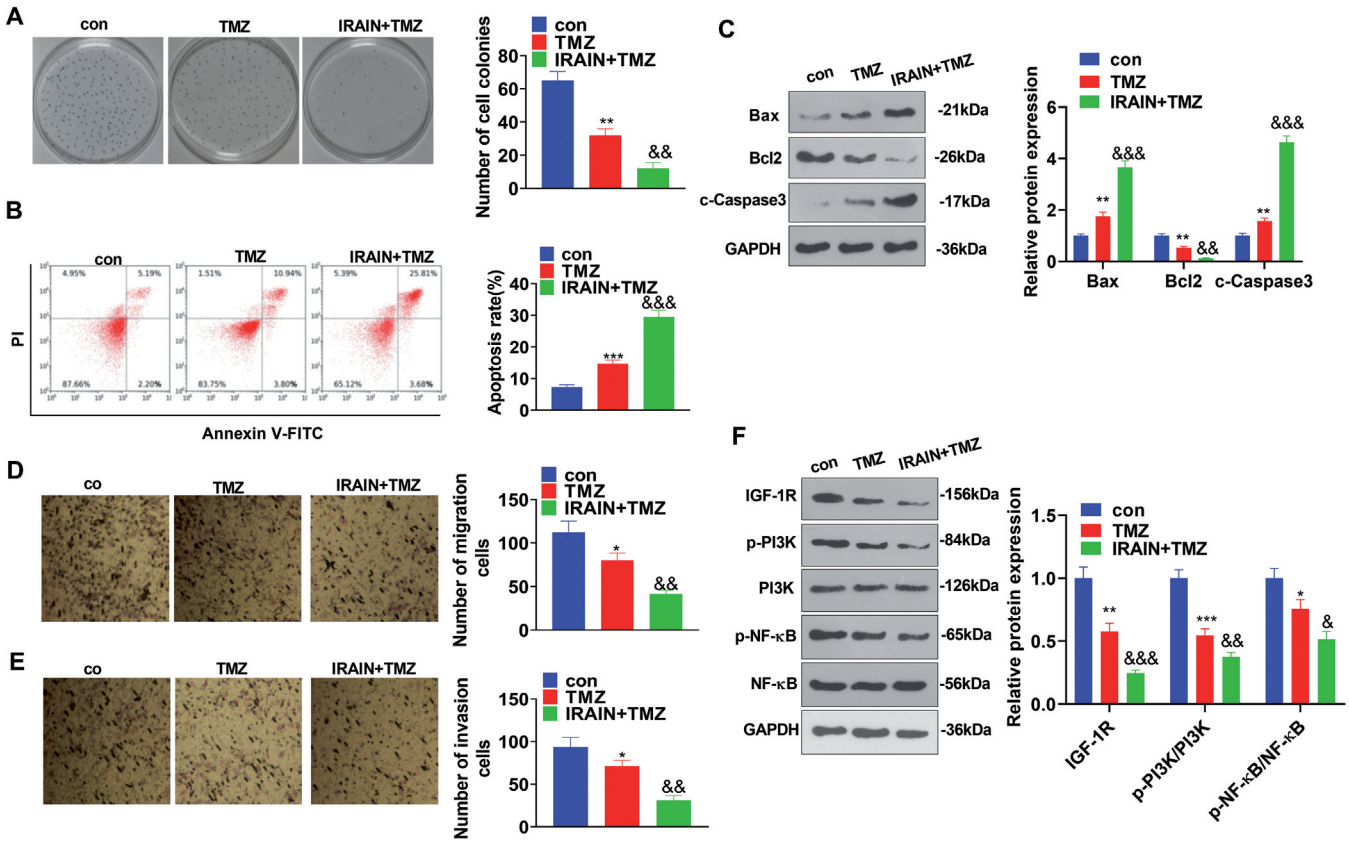

Fig. 5. IRAIN attenuated TMZ resistance by inhibiting the IGF-1R-PI3K-NF-κB pathway. U251 cells were treated with 200 μM TMZ and IRAIN overexpression plasmids alone or in combination. **A, B.** Cell proliferation and apoptosis were monitored by the colony formation assay and flow cytometry, respectively. **C.** WB was conducted to verify the expression of Bax, Bcl2, c-Caspase3. **D, E.** Transwell assay was employed to testify cell migration and invasion. **F.** WB was employed to test the IGF-1R-PI3K-NF-κB level. *P<0.05, **P<0.01, ***P<0.001 (vs. con group). &P<0.05, &&P<0.01, &&&P<0.001 (vs. TMZ group), N=3.

turned out that compared with the control group, the use of 200 μmol/kg TMZ alone or the IRAIN overexpression dampened the IGF-1R-PI3K-NF-κB profile. Besides, the combined intervention of the two further down-regulated the pathway compared with the TMZ group ($P<0.05$, Fig. 6E). These findings confirmed that IRAIN inhibited glioma growth and TMZ resistance.

Discussion

Glioma is one of the commonest tumors in the central nervous system (Saxena and Jha, 2017). Due to its aggressiveness, the morbidity and mortality of glioma are still high despite the maximum surgical resection and chemotherapy (Ostrom et al., 2015; Cahill and Turcan, 2018). Therefore, finding ways to inhibit glioma progression at multiple levels and exploring its molecular mechanisms will help identify potential therapeutic targets. Here, we discovered that IRAIN alleviates glioma and enhances TMZ sensitivity by attenuating IGF-1R-PI3K-NF-κB (Fig. 7), which provides new ideas for the clinical treatment and prognosis of glioma.

Emerging studies have shown that the abnormal expression of IRAIN has significant value in various cancers. For example, IRAIN is a tumor suppressor in breast cancer and is down-regulated in cells. It targets IGF1R in breast cancer through antisense lncRNA-mediated promoter cis competition (Pian et al., 2018). Also, Wang et al. found that IRAIN is significantly

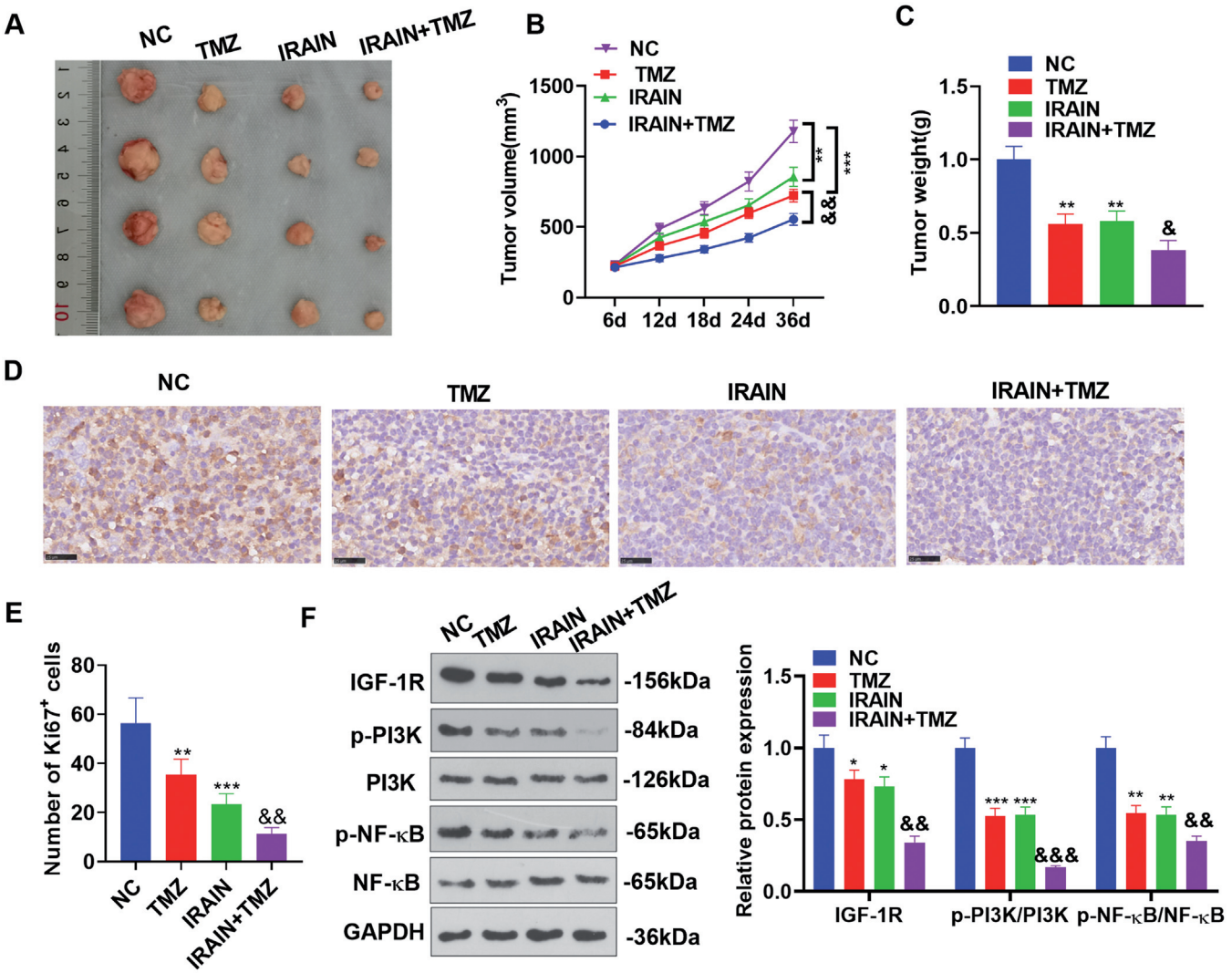

Fig. 6. IRAIN abated glioma progression and enhanced TMZ sensitivity *in vivo*. U251 cells transfected with IRAIN overexpression plasmids were subjected to tumor-formation experiment in nude mice, which were treated with 200 μmol/kg of TMZ. **A.** Tumor formation in nude mice. **B, C.** Tumor volume and weight. **D.** IHC was used to test the proliferation (labeled by Ki67) of U251 cells *in vivo*. **E.** WB was carried out to examine the IGF-1R-PI3K-NF-κB profile. *P<0.05, **P<0.01, ***P<0.001 (vs. Vector group); &P<0.05, &&P<0.01, &&&P<0.001 (vs. TMZ group), N=5.

down-regulated in laryngeal cancer tissues compared with that in paracancerous tissues, which is an early diagnostic marker for laryngeal cancer (Wang et al., 2020). This suggests that IRAIN serves as a tumor suppressor. However, there is no report on the role of IRAIN in glioma. Chen H et al. showed that lncRNA CPS1-IT1 is knocked down in glioma, the low expression of CPS1-IT1 is an independent prognostic factor for the overall survival of glioma patients, and overexpressing CPS1-IT1 dampens glioma cell invasion, migration, and proliferation (Chen et al., 2019). Meanwhile, lncRNA-135528 up-regulates CXCL10 through the JAK/STAT pathway, thereby promoting glioma cell apoptosis and inhibiting cell proliferation and cell cycle (Wang et al., 2018). Moreover, multiple lncRNAs, such as lncRNA PTENP1 (Hu et al., 2018a), lncRNA PLAC2 (Hu et al., 2018b), and lncRNA TSLC1-AS1 (Qin et al., 2014), are down-regulated in glioma, and their up-regulation abates glioma cell proliferation, migration and invasion and facilitates apoptosis. Based on the above studies, we posit that IRAIN may also inhibit glioma. Fortunately, this study proved that IRAIN is down-regulated in glioma tissues compared with the paracancerous tissues. By constructing IRAIN overexpression and knockdown models in glioma cell lines, we discovered that overexpressing IRAIN inhibits cell proliferation, migration, and invasion, and elevates apoptosis, and knocking down IRAIN exerts an opposite role. The above findings were also confirmed in vivo, indicating that IRAIN attenuated the malignant phenotype of glioma.

IGFRs (including IGF1R and IGF2R) play an increasingly prominent role in glioma. Some studies have illustrated that miR-422a represses glioma cell proliferation and invasion by targeting IGF1 and IGF-1R (Jiang et al., 2017). Also, several studies have shown that IGF-1R is closely related to glioma status, and the inhibition of IGF-1R by GSK1838705A dampens glioma cell proliferation and promotes apoptosis in vivo (Zhou et al., 2015). Similarly, Zhou et al. showed that the IGF-1R inhibitor PQ401 induces glioma cell apoptosis and inhibits cell growth, proliferation, and migration (Zhou et al., 2016). This indicates that IGF-1R may be the oncogenic gene in glioma, and its inhibition slows down the malignant progression of glioma. PI3K-NF-κB is a classic signaling pathway, and its role in glioma has also been studied extensively. Interleukin 17 (IL-17), a pro-inflammatory cytokine, facilitates glioma cell proliferation and migration through PI3K/Akt1/NF-κB-p65 activation (Wang et al., 2019). Also, Ji et al. reported in 2018 that AnnexinA5 strengthens glioma cell proliferation, migration and invasion by activating PI3K/Akt/NF-κB (Ji et al., 2018). These studies have shown that inactivating PI3K-NF-κB attenuates malignant behaviors of glioma. Similar to the above results, we found that overexpressing IRAIN inactivates IGF-1R-PI3K-NF-κB. By knocking down IGF-1R, we discovered that compared with Si-IGF-1R intervention alone, there was no significant difference in glioma cell proliferation, migration, invasion and apoptosis after overexpressing IRAIN on this basis. It proved that IRAIN exerted its tumor-suppressive effect by inactivating IGF-1R-PI3K-NF-κB pathway.

As a first-line chemotherapy drug, TMZ has been employed to treat glioma for more than a decade due to its advantages of oral administration, easy penetration of the blood-brain barrier, acidic environmental stability, and overlapping non-toxicity with other drugs. However, its drug resistance is a serious obstacle to chemotherapy failure for glioma (Yin and Cui, 2020; Hu et al., 2020). Therefore, it is of great value to enhance the TMZ sensitivity of glioma and prevent drug resistance. This study revealed that TMZ alone or its joint use with IRAIN restrains glioma. Meanwhile, by knocking down IGF-1R, it was found that compared with the combined intervention of lncRNA IRAIN+TMZ, there was no significant difference in glioma cell proliferation, migration, invasion and apoptosis after knocking down IGF-1R on this basis. It proved that IRAIN inhibited TMZ resistance by repressing IGF-1R-PI3K-NF-κB, which was consistent with a previous study (Zhang et al., 2018).

Overall, our study suggests that IRAIN is down-regulated in glioma, and overexpressing IRAIN dampens glioma cell proliferation, migration and invasion and

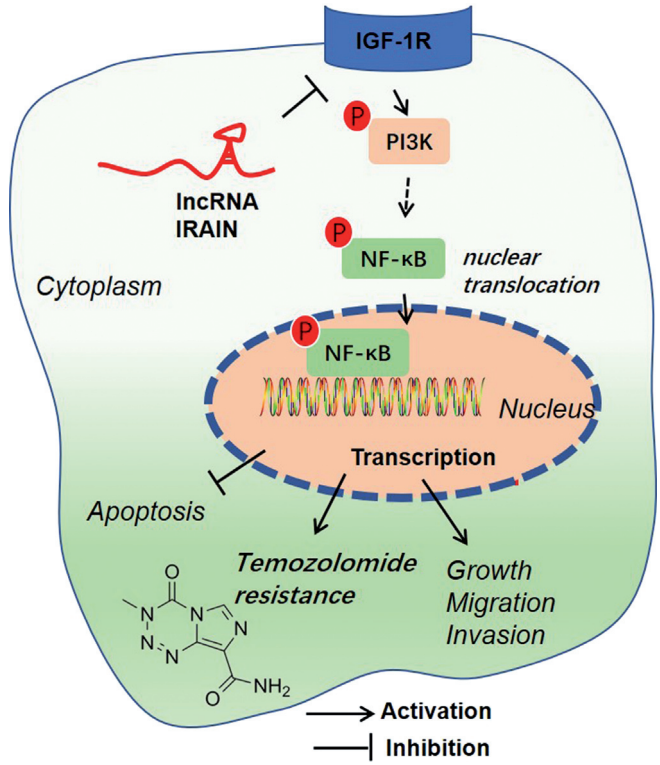

Fig. 7. Schematic diagram of lncRNA IRAIN-IGF-1R-PI3K-NF-κB axis in glioma progression.

strengthens apoptosis and TMZ resistance (Fig. 7). It is believed that IRAIN is a potential molecular diagnostic marker in glioma and functions as a tumor suppressor in glioma by repressing cell growth and enhancing chemotherapy sensitivity. However, in vivo assays need to be conducted to confirm whether IRAIN exerts its tumor-suppressive effect through IGF-1R-PI3K-NF-κB pathway.

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Availability of data and materials. The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests. The authors declare that they have no competing interests.

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Author's contribution. Conceived and designed the experiments: YLL;
Performed the experiments: ASG, RSL, SSZ;
Statistical analysis: GXF, ZRL, ZJZ;
Wrote the paper: ASG.
All authors read and approved the final manuscript.

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