

RETRACTED ARTICLE: NR5A2 Promotes Cell Growth and Resistance to Temozolomide Through Regulating Notch Signal Pathway in Glioma

This article was published in the following Dove Press journal:
OncoTargets and Therapy

Quanxi Yang¹
Lei Deng²
Jialiang Li¹
Pengfei Miao¹
Wenxiang Liu¹
Qi Huang¹

¹Department of Neurosurgery, The First People's Hospital of Shangqiu in Henan Province, Shangqiu Clinical College, Xuzhou Medical University, Shangqiu 476100, People's Republic of China;

²Department of Neonatology, The First People's Hospital of Shangqiu in Henan Province, Shangqiu Clinical College, Xuzhou Medical University, Shangqiu 476100, People's Republic of China

Background: Glioma is a fatal primary malignant tumor. We aimed to explore the effect of nuclear receptor subfamily 5 group A member 2 (NR5A2) on glioma.

Methods: NR5A2 expression in glioma tissues and cell lines was detected using qRT-PCR and immunohistochemistry (IHC)/Western blot. SPSS 22.0 was performed to explore the relationship between NR5A2 expression and glioma clinicopathologic features. The down-expressed plasmid of NR5A2 was transfected into glioma cells, and the cell viability, proliferation, apoptosis, migration, and invasion were respectively determined by MTT, EdU, flow cytometry, wound healing and transwell assays. Cell cycle was analyzed using flow cytometry. Temozolomide (TMZ)-resistant glioma cells were established to define the effect of NR5A2 on drug resistance. The expressions of Notch pathway-related proteins were assessed by Western blot. Glioma nude mice model was constructed to explore the role of NR5A2 played in vivo.

Results: NR5A2 was highly expressed in glioma tissues and cell lines. NR5A2 overexpression was related to the poor prognosis of glioma patients. NR5A2 knockdown inhibited cell viability, proliferation, migration, and invasion, induced cell cycle arrest and promoted cell apoptosis in U138 and U251 cells. In U138/TMZ and U251/TMZ cell lines, NR5A2 upregulation enhanced TMZ resistance while NR5A2 downregulation reduced it. The knockdown of NR5A2 influenced the expressions of Notch pathway-related proteins. NR5A2 knockdown suppressed tumor growth and facilitated apoptosis in glioma mice model.

Conclusion: NR5A2 affected glioma cell malignant behaviors and TMZ resistance via Notch signaling pathway and it might be a novel target in glioma therapy.

Keywords: glioma, Notch signaling pathway, temozolomide (TMZ), animal model, NR5A2

Introduction

Glioma is a common malignant brain tumor in central nervous system.^{1,2} The 2018 Global Cancer Statistics estimates there will be 296,851 new cases of brain tumors worldwide and 241,037 deaths from the disease.³ In China, the incidence of glioma was three-to-eight per 100,000 people.⁴ Currently, the most commonly used classification system for glioma is developed by the World Health Organization (WHO), which includes pilocytic astrocytoma (Grade I), diffuse astrocytoma (Grade II), anaplastic astrocytoma (Grade III) and glioblastoma (Grade IV).^{5,6} Patients with low-grade glioma often have a course of several months or even years, while patients with high-grade glioma usually have a course of only a few weeks or months.^{7,8}

Correspondence: Quanxi Yang
Department of Neurosurgery, The First People's Hospital of Shangqiu in Henan Province, Shangqiu Clinical College, Xuzhou Medical University, No. 292 South Kaixuan Road, Shangqiu 476100, People's Republic of China
Tel +86-17737590198
Email yangquanxish@163.com

At present, the diagnosis of glioma mainly depends on cranial computed tomography (CT) and magnetic resonance imaging (MRI).⁶ Neither surgical excision nor combined chemoradiotherapy can cure glioma thoroughly because of its high invasiveness and proliferation.⁹ The research on the pathogenesis, diagnosis and treatment of glioma has always been a worldwide problem. In recent years, it has been found that changes in some specific genes and proteins have a great impact on the prognosis and biological characteristics of glioma with the in-depth study of the molecular mechanism of glioma.¹⁰ Therefore, some new precise treatment methods, such as targeted therapy, gene therapy and immunotherapy, have been proposed, which are expected to provide more efficient and targeted treatment for glioma.

Nuclear receptors (NRs) are the largest members of the transcription factor family.¹¹ They regulate the expression of target genes in a ligand-dependent manner and participate in the regulation of metabolism, development, and reproductive functions. Many hormones, cholesterol, and other lipid metabolites can be used as ligands for NR.¹² NR is collectively referred to as orphan nuclear receptor. Nuclear receptor subfamily 5 group A member 2 (*NR5A2*), also known as liver receptor homolog 1 (*LRH-1*), is an important orphan nuclear receptor. The encoding gene of *NR5A2* is located on chromosome 1 q32.11, and contains eight exons and is longer than 150 kb.¹³ *NR5A2* is involved in follicular development, female reproduction, and steroid hormone production in mammals, and mainly expressed in liver, intestine, and ovaries of mammals.¹⁴ In recent years, research reports that *NR5A2* plays a crucial role in regulating cell proliferation and is highly expressed in a variety of tumors, such as breast cancer,¹⁵ non-small-cell lung cancer,¹⁶ pancreatic cancer,¹⁷ and gastric cancer.¹⁸ However, to our knowledge, the expression and biological role of *NR5A2* in glioma remains unclear.

In this study, we aimed to explore the effect of *NR5A2* on glioma, with focus on its effects on cell viability, proliferation, apoptosis, migration, invasion, and temozolomide (TMZ) sensitivity. We also defined the role of *NR5A2* in vivo by glioma nude mice model establishment. In addition, we confirmed that *NR5A2* could modulate the activity of Notch signaling pathway. We proved that *NR5A2* accelerated malignant behaviors and resistance of TMZ in glioma via modulating Notch signaling pathway. These findings provided a novel insight of glioma diagnosis and treatment.

Materials and Methods

The Cancer Genome Atlas (TCGA) Analysis

The expressions of *NR5A2* in 156 cases of glioma tumor samples and in 15 cases of normal samples were downloaded from the TCGA database (<https://portal.gdc.cancer.gov/>). The *NR5A2* expressions in all of the above samples were compared.

Glioma Patients and Tissues

Glioma tissues (n=50) and peritumoral brain edema (PTBE) tissues (n=12) were collected from glioma patients (26 female and 24 male patients; age 19 to 71 years old) after surgery between September 2013 and May 2017 at The First People's Hospital of Shangqiu in Henan Province. The PTBE tissues served as the normal group. Liquid nitrogen was used to freeze all specimens immediately after surgery and then the specimens were stored at -80°C. The research protocol was authorized by the Ethics Committee of The First People's Hospital of Shangqiu in Henan Province (QFPH01278) and carried out in accordance with the guidelines of The First People's Hospital of Shangqiu in Henan Province and adhered to the ethical guidelines of the 1975 Declaration of Helsinki. All patients enrolled in the study gave written informed consent. The median *NR5A2* expression level in 50 glioma tissues was calculated using SPSS 22.0 (IBM Corporation, Armonk, NY, USA) and then taken as truncation value. Those higher than the median *NR5A2* expression level were considered as *NR5A2* high expression group, while those lower than the median *NR5A2* expression level were considered as *NR5A2* low expression group. The overall survival was defined as the time from surgery to death, and then the survival curve was plotted.

Cell Culture

Human normal astrocytes cell line (NHA) and glioma cell lines (U138, U251, A172 and U87), which were all purchased from Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, China), were seeded in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) that consisted of 1% penicillin (Invitrogen, Shanghai, China), 1% streptomycin (Invitrogen) and 10% FBS (Invitrogen) at 37°C in a humidified atmosphere with 5% CO₂. Cell passage was performed until the cell fusion rate reached about 90%.

The establishment of temozolomide (TMZ)-resistant cell lines referred to previous studies.^{19,20} Briefly, U138 and U251 cells were treated with 10 μ M TMZ, and the concentration of TMZ doubled every two weeks until the concentration reached 640 μ M. Then the TMZ-resistant cells were treated with TMZ of different concentrations. The IC₅₀ value was detected by MTT to confirm the effectiveness of TMZ-resistant cells. Experiments were done in triplicate.

Cell Transfection

The *NR5A2* silence plasmid (short hairpin *NR5A2*, *NR5A2*-sh1# and *NR5A2*-sh2#), silence control plasmid (Scramble), *NR5A2* overexpression plasmid (*NR5A2*) and overexpression control plasmid (Vector) were all synthesized and provided by Sangon Biotech Co., Ltd (Shanghai, China). Human glioma cell lines U138 and U251 were transfected with *NR5A2*-sh/Scramble or *NR5A2*/Vector. Glioma cell lines (3×10^5 cells/well) were seeded in six-well plates using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. After 48 h, the transfected glioma cell lines were harvested and used for the following experiments.

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

The total RNA of glioma tissues and cell lines were collected using TRIzol kit (Invitrogen) and then cDNA was synthesized using reverse transcriptase that provided by Takara (Dalian, China) and oligonucleotide (dT) according to the manufacturer's protocol. SYBR Premix Ex TaqTM II kit, which was provided by Takara, was used to operate qRT-PCR. The conditions of qRT-PCR are as follows: 94°C for 10 seconds, 94°C for five seconds, 52°C for 30 seconds and 72°C for 15 seconds, and the procedure was cycled 40 times. The primer sequences used in this study were as follows: *NR5A2* forward primers 5'-CAGGCAGTATCCCTCATCTT-3' and reverse primers 5'-AGCCACATTTTCAGCAACAG-3', β -actin forward primers 5'-GCTCGTCGTCGACAACGGCTC-3' and reverse primers 5'-CAAACATGATCTGGGTCATCTTCTC-3'. The relative expression of *NR5A2* was calculated by $2^{-\Delta\Delta Ct}$ method with β -actin as the internal reference. Experiments were done in triplicate.

Immunohistochemistry (IHC) Staining

In brief, the glioma tissues were immersed in the target retrieval solution with water bath for 30 min. The endogenous peroxidase was blocked by H₂O₂ (3%) for 15 min, and the nonspecific bindings were blocked by goat serum for 50 min. Then, primary antibodies (*NR5A2*, Ki67 and Notch1) and secondary antibody polymer HRP were respectively applied to stain the slides. After that, the slices were stained DAB and methyl green. The slices were observed and photographed under a inverted microscope (IX71, Olympus Corporation, Tokyo, Japan). Experiments were done in triplicate.

Western Blot

After 48 h transfection, the glioma cells were collected and resuspended. The total protein of was extracted from glioma tissues and cell lines by 500 μ L Radioimmunoprecipitation (RIPA) lysate. The concentration of total protein concentration was determined according to the BCA kit (Takara). Then total protein (60 μ g) of each sample was subjected to SDS-PAGE and subsequently transferred to PVDF membrane for one hour of closure using skimmed milk (5%). After the skimmed milk powder was removed, the PVDF membrane was treated with primary antibodies for 12 h at 4°C. Subsequently, the membrane was incubated using horseradish peroxidase-labeled secondary antibody (1:2000) purchased from Zhongshan Jinqiao Biotechnology Co., Ltd (Beijing, China) for two hours at room temperature. The membrane was treated with enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA) and the relative expression of proteins was analyzed by Quantity-One software (Bio-Rad, CA, USA). β -actin or *GAPDH* served as the internal reference. The details and dilutions of primary antibodies, which were all obtained from Abcam (Cambridge, MA, USA) or Cell Signaling Technology (Danvers, MA, USA), were as follows: anti-*NR5A2* (ab189876, 1:1000), anti-Notch1 (ab52627, 1:1000), anti-hES (ab71559, 1:1000), anti-Cyclin D1 (ab16663, 1:200), anti-p21 (ab109520, 1:1000), anti-cleaved caspase-3 (ab2302, 1:1000), anti-cleaved PARP (ab32064, 1:1000), anti-MMP2 (ab97779, 1:1000), anti-E-cadherin (ab231303, 1:1000), anti-cleaved Notch1 (anti-NICD1, cst#4147, 1:1000), anti- β -actin (ab8227, 1:1000) and anti-*GAPDH* (1:500; ab8245). Experiments were done in triplicate.

MTT Assay

The cell viability of human glioma cell lines (U138 and U251) was detected using the MTT Cell Proliferation and Cytotoxicity Assay Kit that purchased from Dojindo Laboratories (Tokyo, Japan) according to the manufacturer's instructions. Cell lines (1×10^4 cells/well) were all cultured in 24-well plates and then transfected with Vector/*NR5A2* or Scramble/*NR5A2*-sh2#. The transfected U138 and U251 cells were cultured for 12, 24, 48, and 72 h. After that, the MTT solution was removed and replaced with 150 μ L 4% dimethyl sulfoxide (DMSO; Sigma). Moreover, the half maximal inhibitory concentration (IC_{50}) was detected to determine the cytotoxicity of TMZ. A microplate reader (Bio-Tek, Instruments, Neufahrn, Germany) was used to measure the absorbance at 490 nm. Experiments were done in triplicate.

EdU Assay

The cell proliferation activity was measured through EdU assay. Human glioma cell lines (U138 and U251) were seeded in 96-well plates with cover glass. The growth of cell lines was determined using an EdU incorporation assay according to the manufacturer's protocol. Briefly, U138 and U251 cell lines were implanted in DMEM with EdU labeling for four hours. Then, the cell lines were fixed, infiltrated and stained with EdU antibody. Lastly, the experimental cell lines were observed under a microscope (IX71, Olympus Corporation, Tokyo, Japan). Experiments were done in triplicate.

Flow Cytometry Assay

Flow cytometry analysis was performed to explore the cell apoptosis of glioma cells. After 48 h of transfection, the U138 and U251 cells were washed with absolute ethanol overnight at -4°C , and then subjected to RNase. Next, the propidium iodide (PI, Sigma-Aldrich, St Louis, MO, USA) was employed to incubate the cell lines. Apoptosis of U138 and U251 cells were examined using an Annexin-VFITC/PI Apoptosis Detection Kit obtained from Sigma-Aldrich according to the manual. Experiments were done in triplicate.

For cell cycle analysis, glioma cells were harvested, washed, and fixed in cold ethanol (70%) overnight. PI (50 $\mu\text{g/mL}$)/RNase buffer (BD Biosciences, San Jose, CA, USA) were used to resuspend the cells (1×10^6 cells). Then, cells were cultured at 37°C for 30 min

in the dark. A FACSCalibur flow cytometer (BD Biosciences) was subsequently performed to assess the cell cycle. Experiments were done in triplicate.

Wound Healing Assay

Wound healing assay was accomplished to analyze the glioma cells migration activity. Experimental cell lines (U138 and U251) were seeded in 12-well plates at a density of 1.0×10^6 cells/well. After cell lines had bred to assemblage, a pipette tip (200 μ L) was used to shave off the glioma cell monolayer to build a scratch. Next, the cells were washed three times with PBS, and then incubated in FBS-free medium. The U138 and U251 cell lines were shot at 0 and 24 h by an IX71 inverted microscope (Olympus, Tokyo, Japan). Image J software provided by National Institutes of Health (Bethesda, MD, USA), was applied to calculate the scratch area. The migration rate = (area of original wound - area of wound at different times) / area of original wound $\times 100\%$. Experiments were done in triplicate.

Transwell Assay

The transwell chambers were placed in 24-well plates. The DMEM complete medium (500 μ L) was added to the lower chamber and serum-free DMEM medium containing 1×10^5 cells (200 μ L) was added to the upper chamber. These plates were then placed at 37°C , CO_2 (5%) and humidity (95%) for 48 h. Then chambers were taken out, and a cotton swab was used to remove the cells from the upper chambers. Then, the cells in the lower chambers were fixed and stained with formaldehyde and crystal violet (0.01%), respectively. Invasive cell lines were counted under a microscope (Olympus, Tokyo, Japan) in five nonoverlapping random fields. Experiments were done in triplicate.

Glioma Animal Model

BALB/c nude mice (six mice/group, four-to -six weeks old) were obtained from Shanghai Laboratory Animal Center (Shanghai, China). A suspension of human glioma cell lines U251 with a density of 5.0×10^6 cells/100 μ L was transfected with shNC or sh*NR5A2*. The mice were divided into shNC group and sh*NR5A2* group (n=6). A BALB/c mice model of glioma was established by subcutaneous injection of shNC or sh*NR5A2* transfected U251 cells in the the right flanks of mice. The tumor volume was measured after every seven days. At day 35, air embolization was performed

to kill the mice and the glioma tissues were collected. Then, the tumor weight was calculated. Experiments were done in triplicate. Next, IHC was applied to monitor the expression levels of *NR5A2*, Ki67 and Notch1. This animal study was authorized by the Ethics Committee of The First People's Hospital of Shangqiu in Henan Province and carried out in accordance with the Guidelines for Animal Use in the National Institutes of Health.

Terminal Deoxynucleotidyl Transferase (TdT)-mediated Deoxyuridine Triphosphate (dUTP) Nick End Labeling (TUNEL) Staining

An in situ cell death detection kit, purchased from Roche (Basel, Switzerland), was used to monitor the apoptosis of mice glioma tissues. Briefly, the glioma tissue sections were blocked via 3% H₂O₂ for five minutes and then fixed by TdT labeling reaction mix for one hour. The experiment was performed at 37°C. DAB was applied to stain the tumor sections for 15 min. A light microscope (Olympus, Tokyo, Japan) was employed to observe the sections. Experiments were done in triplicate.

Statistical Analysis

SPSS 22.0 (IBM) was used to analyze the quantitative data. The data were expressed by mean $\pm$ SD of at least three independent experiments. Student's *t*-test or one-way ANOVA were used to compare the differences among groups. Pearson's chi-squared tests were applied to explore the relationship between *NR5A2* expression and glioma clinical pathology containing patients' age, sex, tumor size, PTBE and WHO stages. Kaplan–Meier survival analysis was performed to measure survival rate. The log rank test was used to calculate the *P*-value, and *P* < 0.05 presented statistically significant. Normality of the data was checked using Kolmogorov–Smirnov test, *P* > 0.05 was considered to be in line with normal distribution. Student's *t*-test was performed to examine data that were normal distributed while non-parametric rank sum test was performed to examine data that were not normal distributed. Experiments were done in triplicate.

Results

NR5A2 Overexpression in Glioma Tissues and Cell Lines was Associated with Poor Prognosis

Firstly, the data from TCGA database showed that *NR5A2* was highly expressed in glioma tissues compared to normal tissues (Figure 1A). Then, a total of 50 cases of glioma patients tumor tissues and 12 cases of normal tissues were collected and then subjected to qRT-PCR to determine the *NR5A2* expression level. The expressions of *NR5A2* in different WHO stages were also monitored using qRT-PCR. Next, PTBE tissues, WHO grade I and II glioma tissues and WHO grade III and IV glioma tissues were randomly selected (*n*=5) and the expression of *NR5A2* was detected via IHC. Data from qRT-PCR and IHC both showed that the expression levels of *NR5A2* were significantly upregulated in glioma tissues compared to that in normal tissues (Figure 1B and C, *P* < 0.01). And the *NR5A2* expressions in WHO grade III and IV tissues were markedly higher than that in WHO grade I and II tissues (Figure 1C and D, *P* < 0.01). Moreover, the 50 cases of glioma tissues were divided into *NR5A2* high expression group and *NR5A2* low expression group according to the median *NR5A2* expression level, and overall survival curves of the two groups were produced. Memorably, the *NR5A2* high expression group exhibited shorter survival time when compared to the *NR5A2* low expression group (Figure 1E, *P* < 0.01). Furthermore, the relationship between *NR5A2* expression and glioma clinical pathology (patients' age, sex, tumor size, PTBE and WHO stages) were analyzed via SPSS, and the results showed that the expression of *NR5A2* was associated with glioma patients' tumor size, PTBE and WHO stages, but not with their age or sex (Table 1). In addition, the expressions of *NR5A2* in human glioma cell lines, including U138, U251, A172, and U87 and human normal astrocytes cell line NHA, were assessed using qRT-PCR and Western blot. As shown as Figure 1F and G, *NR5A2* was obviously overexpressed in glioma cell lines when compared to that in NHA cell lines (*P* < 0.01). U138 as well as U251 cell lines were chosen for the following experiments because they showed the highest *NR5A2* expression among these cell lines. These results illustrated that *NR5A2* was highly expressed in glioma tissues and cell lines and its high

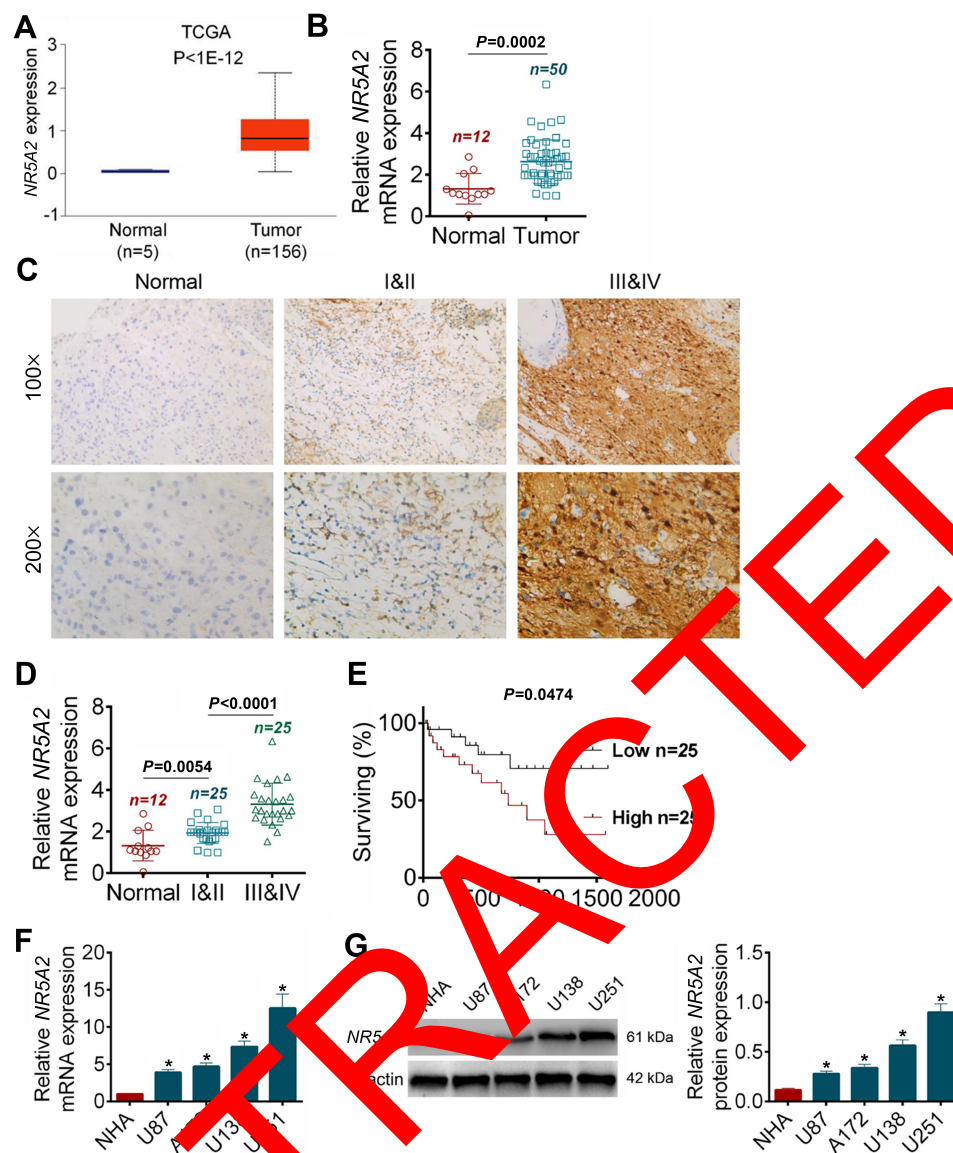


Figure 1 NR5A2 was highly expressed in glioma tissues and cell lines. (A) The data of NR5A2 expressions in 156 cases of glioma tumor tissues and 15 cases of adjacent nontumor tissues were harvested from TCGA database. (B) The NR5A2 mRNA expressions in 50 cases of glioma tissues and 12 cases of PTBE tissues were detected using qRT-PCR. (C) The NR5A2 expressions in different grades of glioma tissues and PTBE tissues were determined using IHC. (D) The NR5A2 expressions in different grades of glioma tissues and PTBE tissues were measured using qRT-PCR. (E) The 50 cases of glioma tissues were divided into NR5A2 high expression group and low expression group, the correlation between the survival rate of glioma patients and the expression of NR5A2 was assessed using Kaplan–Meier survival analysis. (F) The NR5A2 expression levels in human glioma cell lines (U138, U251, A172 and U87) and human normal astrocytes cell line (NHA) were analyzed using qRT-PCR. (G) The NR5A2 expression levels in human glioma cell lines (U138, U251, A172 and U87) and human normal astrocytes cell line (NHA) were evaluated using qRT-PCR. Data were shown as mean $\pm$ SD. * $P < 0.05$ vs normal tissues or normal cell lines.

expression was correlated with the shorter overall survival time and poor prognosis of glioma patients.

NR5A2 Knockdown Inhibited Cell Proliferation, Promoted Cell Apoptosis and Induced Cell Arrest in Glioma

After NR5A2-sh1#, NR5A2-sh2# or Scramble transfection, the NR5A2 expressions in U138 and U251 cell lines were detected using qRT-PCR and Western blot. Data from qRT-

PCR and Western blot both revealed that NR5A2-sh1# or NR5A2-sh2# transfection could effectively downregulate the NR5A2 expression level in U138 and U251 cells ($P < 0.01$) and NR5A2-sh2# showed better knockdown efficiency (Figure 2A and B). Therefore, NR5A2-sh2# plasmid was selected for the following experiments. MTT assay was performed to explore the cell viability, Figure 2C exhibited that the silence of NR5A2 significantly decreased the cell viability compared to Scramble group

Table I Correlation of Expression of *NR5A2* with Glioma Clinicopathologic Features

Characteristics	Number of Patients	<i>NR5A2</i> Low Expression (<median)	<i>NR5A2</i> High Expression (≥median)	P-value
Number	50	25	25	
Ages (years)				0.569
<45	22	12	10	
≥45	28	13	15	
Sex				0.571
Female	26	14	12	
Male	24	11	13	
Tumor size				0.021
<5 cm	20	14	6	
≥5 cm	30	11	19	
Peritumor brain edema				0.023
<1 cm	26	17	9	
≥1 cm	24	8	16	
WHO stage				<0.01
I and II	25	22	3	
III and IV	25	3	22	

both in U138 and U251 cell lines ($P<0.01$). The EdU assay was then applied to monitor cell proliferation. The EdU positive cells in *NR5A2*-sh2# groups were obviously less than that in Scramble groups (Figure 2B, $P<0.01$). Furthermore, flow cytometry assay proceeded to assess cell apoptosis and the data from flow cytometry assay was shown in Figure 2E. Markedly, the cell apoptosis of U138 and U251 cell lines in *NR5A2*-sh2# groups was enhanced when compared with that of Scramble groups (Figure 2E, $P<0.01$). Flow cytometry was also performed to analyze cell cycle. As shown as Figure 2F, *NR5A2* knockdown induced cell arrest in G1 phase in both U138 and U251 cells ($P<0.01$). These data confirmed that *NR5A2* knockdown could suppress cell viability and proliferation, induce cell arrest and accelerate cell apoptosis in glioma cell lines.

NR5A2 Promoted Cell Migration and Invasion in Glioma

To explore the effect of *NR5A2* knockdown on cell migration in glioma, wound healing assay was performed. As shown in Figure 3A, the relative wound width (24 h/0 h) of *NR5A2*-sh2# groups was significantly wider than that of Scramble groups in both U138 and U251 cell lines

($P<0.01$). The transwell assay was used to examine the cell invasion activity. Obviously, the invading cell number of *NR5A2*-sh2# groups was fewer than that of Scramble groups (Figure 3B, $P<0.01$). Besides, the expression of *NR5A2* was significantly upregulated in U138 and U251 cell lines by *NR5A2* transfection (Figure 3C, $P<0.01$). The cell migration and invasion were examined via wound healing assay and transwell assay, respectively. Data from the wound healing assay showed that the relative wound width of *NR5A2* groups was remarkably less than that of Vector groups (Figure 3D, $P<0.01$). Results from the transwell assay displayed that the invading cell number of *NR5A2* groups was obviously more than that of Vector groups (Figure 3E, $P<0.01$). The above results revealed that the downregulation of *NR5A2* could suppress cell migration and invasion in glioma.

NR5A2 Knockdown Increased the Sensitivity of Glioma Cells to TMZ

To determine the effect of *NR5A2* on TMZ-resistance of glioma cells, U138 and U251 cell lines were established as TMZ-resistant glioma cell lines. IC₅₀ value that was detected by MTT assay, and data from MTT assay proved that the TMZ-resistant glioma cell lines were successfully constructed (Figure 4A). Then, *NR5A2*/Vector or *NR5A2*-

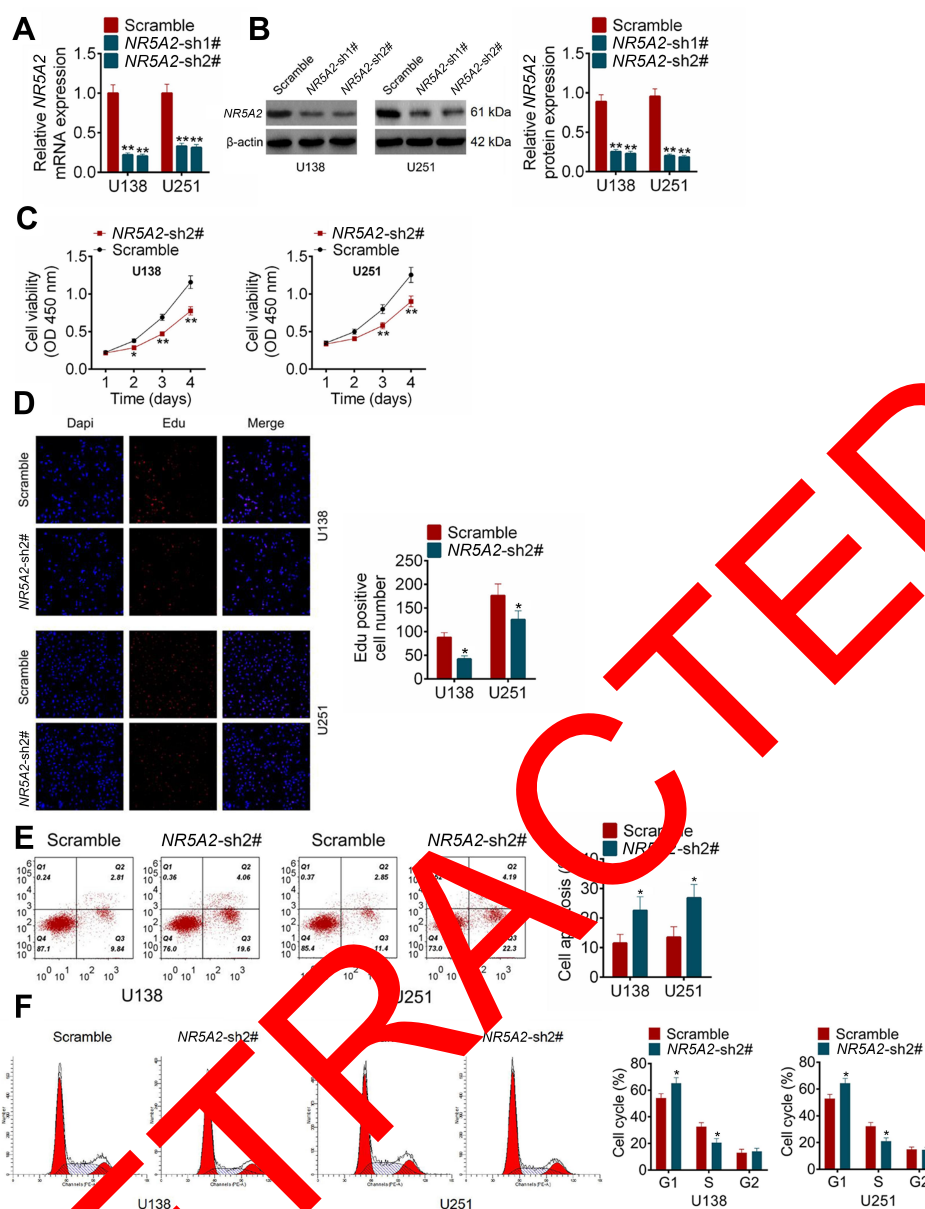


Figure 2 NR5A2 knockdown inhibits glioma cell viability and proliferation and promoted cell apoptosis. (A and B) NR5A2 expression was downregulated in U138 and U251 cells with NR5A2-sh1#/NR5A2-sh2# transfection. (C) Cell viability was detected using MTT assay. (D) Cell proliferation was measured using EdU assay (magnification: 200×). (E) Cell apoptosis was assessed using flow cytometry assay. (F) Cell cycle was analyzed using flow cytometry assay. Data were shown as mean $\pm$ SD. * P <0.05, ** P <0.01 vs Scramble group.

sh2#/Scramble plasmids were effectively transfected into U138/TMZ or U251/TMZ cell line. MTT assay was again performed to IC_{50} value to define the effect of NR5A2 on TMZ resistant in glioma. As shown in Figure 4B and C, the IC_{50} values of NR5A2 groups were significantly higher than that of the Vector group while IC_{50} values of NR5A2-sh2# groups were obviously less than that of the Scramble groups in both U138/TMZ and U251/TMZ cell lines (P <0.01). These results suggested that the downregulation

of NR5A2 promoted the sensitivity of glioma cell lines to TMZ and the upregulation of NR5A2 repressed it.

NR5A2 Regulated the Activity of Notch Signaling Pathway in Glioma Cell Lines

To understand the mechanism of NR5A2 in glioma, the expression levels of Notch signaling pathway-related proteins, including Notch1, hES, CyclinD1, p21, cleaved caspase-3, cleaved PARP, MMP2 and E-cadherin, were

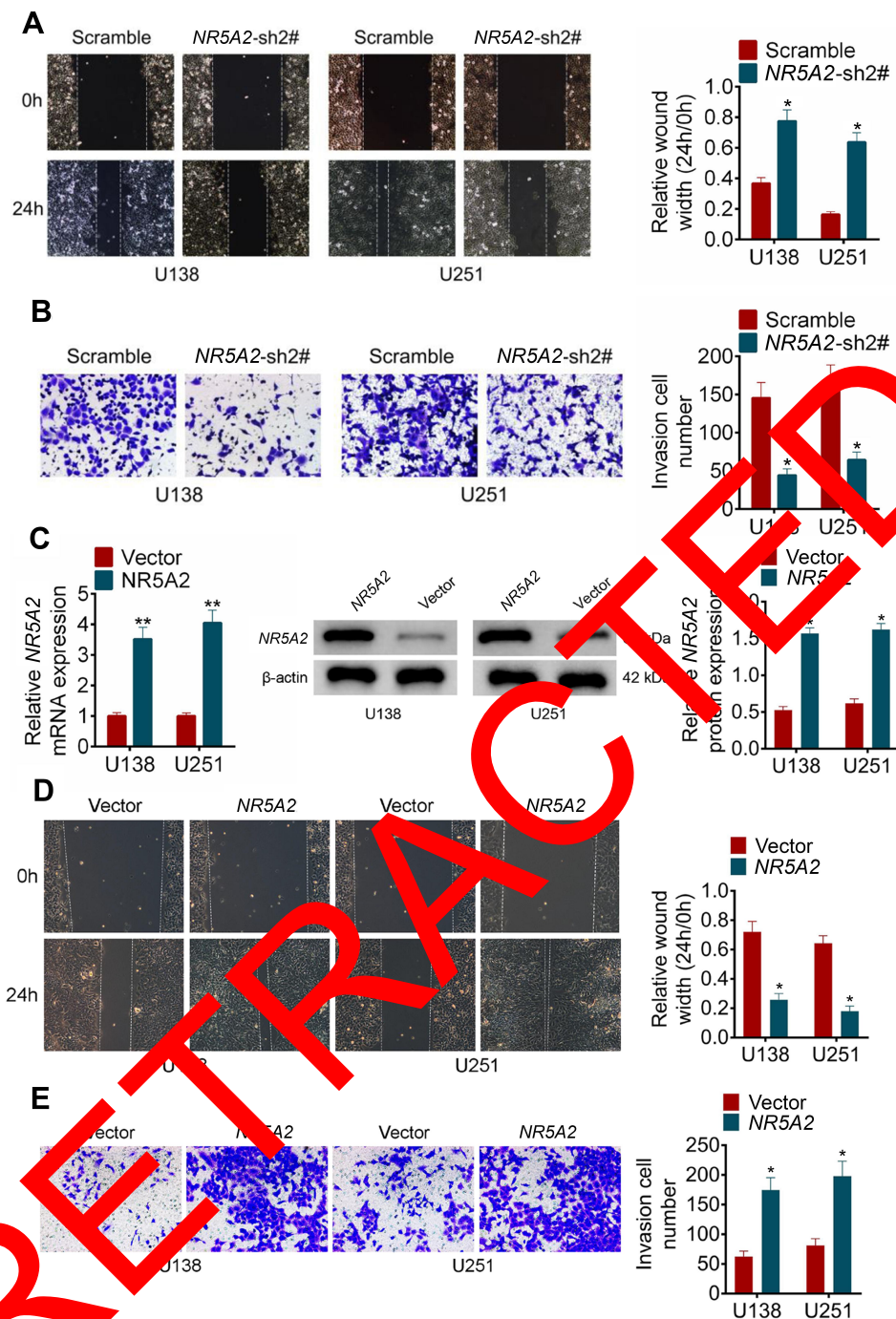


Figure 3 NR5A2 promoted cell migration and invasion in glioma cell lines (U138 and U251). (A) Cell migration was detected using wound healing assay (magnification: 200×). (B) Cell invasion was measured using transwell assay (magnification: 200×). (C) The mRNA and protein expression level of NR5A2 were respectively assessed using qRT-PCR and Western blot. (D) Cell migration was detected using wound healing assay (magnification: 200×). (E) Cell invasion was measured using transwell assay (magnification: 200×). Data were shown as mean ±SD. * $P < 0.05$ ** $P < 0.01$ vs Scramble group or Vector group.

detected using Western blot. When NR5A2 was knocked down in U138 and U251 cell lines, the protein expressions of Notch1, hES, CyclinD1 and MMP2 were significantly decreased as well as the protein expressions of p21,

cleaved caspase-3, cleaved PARP and E-cadherin were remarkably increased (Figure 5, $P < 0.01$). The above data demonstrated that the knockdown of NR5A2 inhibited the activation of Notch signaling pathway.

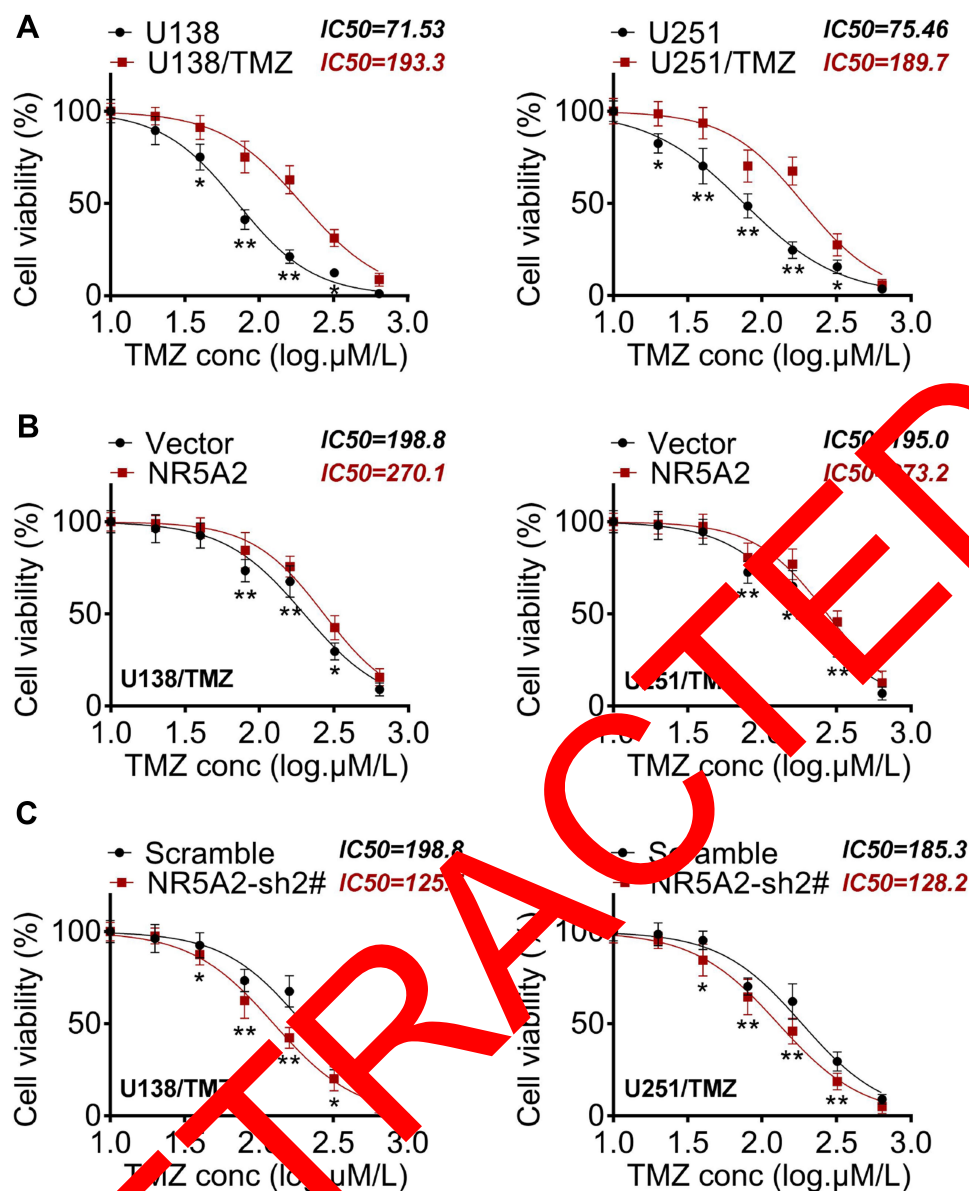


Figure 4 NR5A2 knockdown increased the sensitivity of glioma cells (U138 and U251) to TMZ. (A) Detection of IC_{50} by MTT to determine the effectiveness of TMZ-resistant cells. (B and C) IC_{50} values were detected using MTT assay. Data were shown as mean $\pm$ SD. * $P<0.05$, ** $P<0.01$ vs U251 group, Vector group or Scramble group.

NR5A2 Knockdown Inhibited Tumor Proliferation and Notch Activity and Promoted Apoptosis of Glioma in vivo

To define the effect of NR5A2 on glioma in vivo, a glioma mouse model was constructed. The tumor size, volume and weight of mice in shNR5A2 group were all notably decreased compared to that in the shNC group (Figure 6AC, $P<0.01$). The relative protein expression levels of Ki-67, NR5A2 and Notch1 were measured by IHC. Data from IHC showed that shNR5A2 transfection

inhibited Ki-67, NR5A2 and Notch1 expressions in vivo (Figure 6D). In addition, TUNEL assay was used to evaluate tumor apoptosis, and the results exhibited that NR5A2 knockdown facilitated tumor apoptosis in glioma mice model (Figure 6D). Furthermore, Western blot was performed to examine the expression of NICD1 and hES that were related to Notch signaling pathway. Results from Western blot showed that the expressions of NICD1 and hES in shNR5A2 group was significantly lower than that in the shNC group (Figure 6E, $P<0.01$). The above data indi-

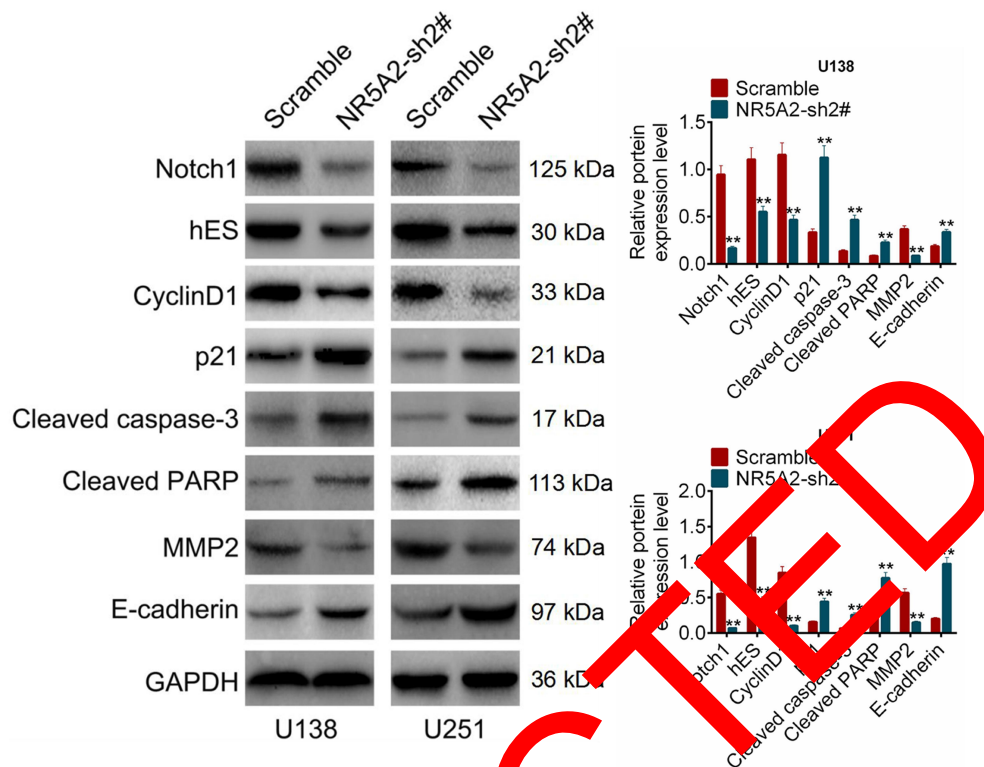


Figure 5 NR5A2 knockdown inhibited Notch signaling pathway activity. The expression levels of Notch signaling pathway-related proteins including NOTCH1, hES, Cyclin D1, p21, cleaved caspase-3, cleaved PARP, MMP2 and E-cadherin were detected using Western blot. Data were shown as mean $\pm$ SD. ** $P < 0.01$ vs Scramble group.

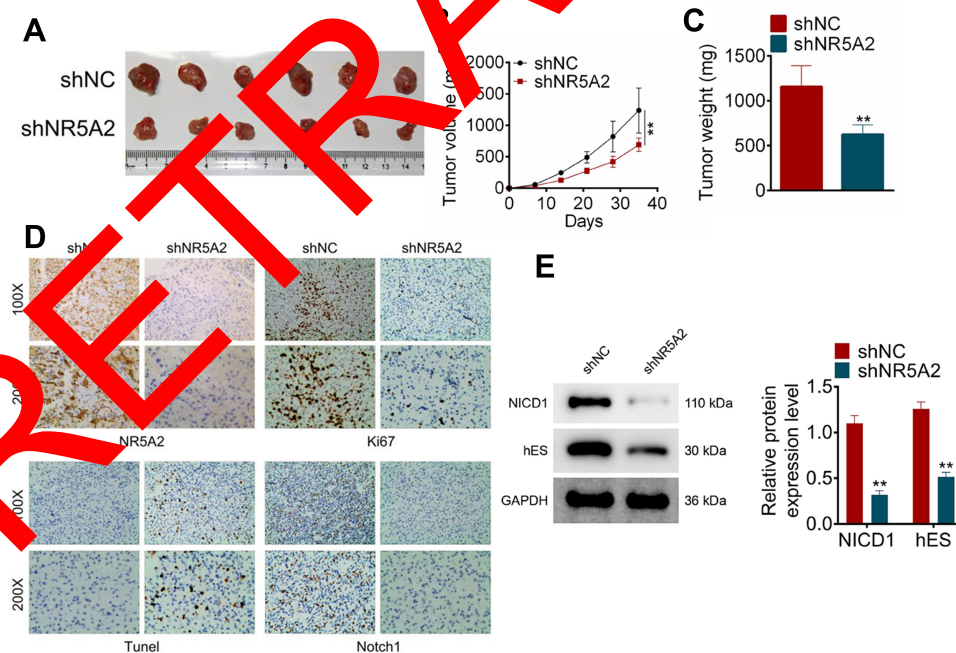


Figure 6 NR5A2 knockdown inhibited glioma tumor growth and attenuated Notch activation in vivo. (A) The pictures of the resected tumors of mice injected with U251 cells infected with shNR5A2 vectors (n = 6) and shNC (n = 6), captured after 35 days. (B) The tumor volume was measured every seven days. (C) The weight of the tumor was detected immediately after resected. (D) Ki67, Notch1, NR5A2 and TUNEL expressions were determined using IHC. (E) The expression levels of Notch signaling pathway-related proteins (NICD1 and hES) were examined using Western blot. Data were shown as mean $\pm$ SD. ** $P < 0.01$ vs shNC group.

cated that *NR5A2* knockdown could repress glioma tumor proliferation and Notch activity and promoted apoptosis in vivo.

Discussion

Glioma is the most common primary intracranial malignant tumor.^{1,2} The occurrence and development of glioma is a multistep process that requires cell adhesion, migration, proliferation, and angiogenesis.⁹ The research of the molecular mechanisms and important targets that regulate the progression of glioma is helpful for the development of new effective therapeutic strategies. Several biomarkers have been reported to be involved in the regulation of glioma. However, the current understanding of the molecular mechanism of glioma is still limited, and there are many potential genes that need to be explored.

NR5A2 is a kind of orphan nuclear receptor (ONR), which regulates gene transcription in the form of monomer or polymer through the action of solitary nuclear receptor acting elements.¹³ This process is regulated by ONR-related cofactors, thus regulating various physiological activities of the body.²¹ Previous studies have reported the effect of *NR5A2* on a variety of cancers. Liu et al,²² confirmed that *NR5A2* was related to non-small-cell lung cancer metastasis, progression, and poor prognosis. Meinsohn et al,²³ found that *NR5A2* promoted cell proliferation and played as a regulator in ovarian cancer. Luo et al,²⁴ reported that *NR5A2* affected cancer stem cell (CSC) stemness and epithelial-mesenchymal transition (EMT) in pancreatic cancer, which may be related to the poor prognosis. As far as we know, the role of *NR5A2* in the occurrence and development of glioma has not been reported.

In the present study, *NR5A2* was firstly found to be overexpressed in glioma tissues and cells, and the abnormal high expression of *NR5A2* contributed to a worse clinical outcome and lower overall survival of glioma patients. In order to explore the role of *NR5A2* exerted in glioma, the expressions of *NR5A2* were downregulated in human glioma cell lines U138 and U251. Then, we demonstrated that the knockdown of *NR5A2* inhibited cell viability, proliferation, migration and invasion. And *NR5A2* knockdown was proved to promote cell apoptosis and induce cell arrest in G1 phase by flow cytometry assay. We also found that the overexpression of *NR5A2* enhanced cell migration and

invasion in glioma cell lines. Furthermore, TMZ-resistant U138 and U251 cell lines were established and then transfected with *NR5A2*/Vector or *NR5A2*-sh2#/Scramble. TMZ is an important drug in the chemotherapy of glioma.²⁵ The IC₅₀ values were assessed by MTT assay to present the level of TMZ resistance, and the data from MTT assay elucidated that *NR5A2* overexpression increased the TMZ-resistant level while *NR5A2* downexpression decreased it.

To further explore the functional mechanism of *NR5A2*, we predicted that *NR5A2* affected glioma through Notch signaling pathway. Notch, a receptor, could mediate intercellular signaling via a conserved pathway conserved in metazoa.²⁶ Notch signaling pathway has been reported to be related to TMZ resistance²⁷ and cell activity²⁸ in glioma. In several studies, NOTCH1,²⁹ hES,³⁰ Cyclin D1 and MMP2³² were proved to promote Notch signaling pathway activation, while p21,³³ cleaved caspase-3,³⁴ cleaved PARP³⁵ and E-cadherin³⁶ were reported to interdict it. To verify our prediction, the expression of these above proteins that related to Notch signaling pathway in U138 and U251 cells that after transfection were detected using Western blot. Results from Western blot exhibited that proteins positively related to Notch signaling pathway (NOTCH1, hES, Cyclin D1 and MMP2) expressions were suppressed in *NR5A2*-sh2# groups while proteins negatively related to Notch signaling pathway (p21, cleaved caspase-3, cleaved PARP and E-cadherin) expressions were enhanced. These data confirmed that the knockdown of *NR5A2* suppressed the activation of Notch signaling pathway.

In addition, glioma nude mice model was conducted to verify the function and mechanism of *NR5A2* in vivo. A Nude mouse model is a common animal model for glioma research in vivo.³⁷ The Ki-67 assay is a common method to detect proliferation in tissues.³⁸ We found that *NR5A2* knockdown in glioma mice model inhibited tumor growth and proliferation, reduced the expressions of Notch signaling pathway-related proteins (NICD1 and hES) and promoted apoptosis.

In conclusion, the current study revealed that *NR5A2* promoted glioma cell proliferation, migration, invasion, and TMZ-resistant in vivo and in vitro through regulating Notch signaling pathway. These results provided a theoretical basis for the application of *NR5A2* in the diagnosis and treatment of glioma and *NR5A2* might be a novel molecular therapeutic target in glioma.

Data Sharing Statement

All data generated and/or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate

The project protocol was authorized by the Ethics Committee of The First People's Hospital of Shangqiu in Henan Province (SQFPH01278) and carried out in accordance with the Guidelines of The First People's Hospital of Shangqiu in Henan Province and adhered to the ethical guidelines of the 1975 Declaration of Helsinki.

Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Funding

No funding was received.

Disclosure

The authors report no competing interests in this work.

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