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Original Article

Effect of RNAi silencing *Notch1* on chemosensitivity in human glioma cells

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Abstract: *Objective:* To investigate the effect of *Notch1* on the proliferation and apoptosis of human glioma cells as well as the resistance to chemotherapeutics, so as to explore its possible mechanism. *Methods:* The expression vector of *Notch1*-siRNA was established to silence the expression of *Notch1*. The human glioma cell line U373 was divided into 5 groups for different RNA intervention and drug intervention methods. The proliferation capability and apoptosis rate of the cells in each group were detected by MTT and Flow Cytometer respectively. The expressions of MMP2 and VEGF which were related to the proliferation and invasion of glioma cells were detected by RT-PCR and Western-blot respectively. Transplanted tumor models of human glioma cells in nude mice were established to observe the effects of RNAi silencing *Notch1* combined with TMZ on the growth of implanted tumor. *Results:* Compared with control U373 cells, the proliferation capability of cell transfected with *Notch1*-siRNA was significantly reduced and the apoptosis rate was significantly increased ($P<0.05$). Compared with normal U373 cells with the administration of TMZ, the apoptosis rate of the U373 cells which transfected with *Notch1*-siRNA followed by the administration of TMZ was significantly increased ($P<0.05$). The expression of MMP2 and VEGF in U373 cell transfected with *Notch1*-siRNA was significantly lower than that in control U373 cells. The expression of MMP2 and VEGF in U373 cells which transfected with *Notch1*-siRNA combined with TMZ was significantly lower than that in control U373 cells ($P<0.05$). The *Notch1*-siRNA combined with TMZ significantly inhibited the growth of mouse implanted tumors, and the difference was statistically significant compared to the mouse used TMZ alone ($P<0.05$). *Conclusions:* RNAi silencing *Notch1* can effectively inhibit the proliferation of the glioma cell, promote the apoptosis and increase the sensitivity to chemotherapeutic drug TMZ, and inhibit the proliferation and invasion of tumor cells by reducing the expression of MMP2 and VEGF.

Keywords: *Notch1*, RNAi, human glioma U373 cells, temozolomide, chemosensitivity

Introduction

Glioma, characterized by high degree of malignancy, strong invasiveness and a high recurrence rate, is the most common malignant tumor in human brain. Currently, surgery combined with radiotherapy and chemotherapy is a significant therapeutic method in the treatment of glioma [1], but the infinite proliferation, invasive growth and the insensitivity to drug treatment of residual tumor cells ultimately lead to the failure of chemotherapy or tumor relapse. Therefore, finding effective ways to enhance the targeted killing or inhibitory effects of chemotherapeutic drugs on tumor cells is the key to improve therapeutic effect of glioma. *Notch1* signaling pathway played an crucial role in cell proliferation, differentiation and apoptosis, and is closely related to the occurrence and development of tumor as well as the sensitivity of tumor cells to chemotherapeutic drugs [2-4]. Many studies have shown that *Notch1* in glioma is highly expressed and *Notch1* knockdown can inhibit proliferation and growth of glioma cell [5-7]. To further understand the relationship between *Notch1* signaling and the occurrence and development of glioma and to investigate the effect of *Notch1* on the chemosensitivity of glioma, the U373 cells model of RNAi silencing *Notch1* was established and the proliferation and apoptosis of U373 cells was detected to explore its effect on the sensitivity to chemotherapeutic Temozolomide (TMZ), which was commonly used in clinics. The expression of

Table 1. Cell transfection and administration among groups

Group	Transfection	Administration
A	Untransfected (normal U373 cells)	100 μL medium
B	Transfected *Notch1*-NC plasmid	100 μLmedium
C	Transfected *Notch1*-siRNA plasmid	100 μL medium
D	Untransfected (normal U373 cells)	70 μL medium+30 μL 200 μmol/L TMZ
E	Transfected *Notch1*-siRNA plasmid	70 μL medium+30 μL 200 μmol/L TMZ

Matrix Metalloproteinase-2 (MMP2) and vascular endothelial growth factor (VEGF) were detected to explore the potential effects on the proliferation and invasion of tumor cells.

Materials and methods

Experimental materials

Human glioma cell line U373 was purchased from Shanghai Cell Bank of Chinese Academy of Sciences. BALB/c nude mice (SPF grade) were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences.

Experimental methods

Construction of expression vector of Notch1 shRNA: According to the design principles of siRNA and the characteristics of plasmid vector of PLKO.1-puro polyclonal site, the *Notch1*-shRNA sequence and the negative control (NC) of shRNA sequence were designed on the basis of the *Notch1* (NM-017617.4) in GenBank, synthesized and constructed on PLKO.1-puro vector. The recombinant plasmids were identified and screened out: *Notch1*-siRNA plasmid and *Notch1*-NC plasmid.

Cell culture and transfection: Human glioma U373 cells were cultured in DMEM medium containing 10% fetal bovine serum. *Notch1*-siRNA and *Notch1*-NC expression vector were steadily transfected, screening the steadily transfected cell lines. The grouping and each transfection were shown in **Table 1**: Normal U373 cells in group A and D, U373 cells transfected with *Notch1*-NC plasmid in group B, U373 cells transfected with *Notch1*-siRNA plasmid in group C and E.

The detection of cell proliferation ability by MTT assay: The transfected cells in five groups were inoculated into 96-well plates, repeated with 5-well in each group. Cells in each group were treated with **Table 1**. The cells were treated with 100 μL medium in group A, B and C, and 70 μL medium plus 30 μL TMZ at a concentration of 200 μmol/L were added to group D and E, then cultured in DMEM medium subsequently. 20 μL 5 mg/mL MTT solution was added in each well and incubated at 37°C for 5 h. Added 150 μL DMSO in each well and shocked 10 min and down to dissolve crytals. Then the optical density (OD) value of cells in each well at 570 nm was detected using spectrophotometer after 24 h, 48 h and 72 h, respectively. The cell growth inhibition rate was calculated.

Detection of apoptosis rate by annexin V/PI double staining method: The transfected cells were inoculated into 6-well plates, with the same administration in **Table 1**. After culture for 72 h, the cells were collected and washed with PBS to formulate cell suspension. Adding Annexin V/PI for 15 min in the dark, the apoptosis rate was measured by Cyto FLEX flow cytometry.

Detection of mRNA expressions of Notch1, MMP2 and VEGF with RT-PCR: The cells cultured for 48 h after transfection and 48 h after administration were collected, in which RNA were extracted and reverse transcribed into cDNA. The relative expressions of *Notch1*, MMP2 and VEGF mRNA were detected by RT-PCR. The primers of *Notch1*, MMP2, VEGF and β-actin were showed in **Table 2.** And the amplification procedure: predenaturation at 95°C for 1 min; 35 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 25 s and extension for 30 s at 72°C. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression.

Detection of protein expression of MMP2 and VEGF by western-blot: Cells in each group cultured for 72 h after administration were collected and total protein were extracted. Polyacrylamide gel electrophoresis was used to separated the protein of all sizes, then transferred the protein to the membrane and incubated antibody. The primary antibodies: Anti-*Notch1* monoclonal antibody, Anti-VEGF polyclonal antibody and Anti-MMP2 polyclonal antibody (Abcam) were incubated with 1:1000 at

Table 2. The primers used for RT-PCR

Gene	Primer sequence	Product length
Notch1	Sense: 5'-CGACGCACAAGGTGTCTTCCA-3'	145 bp
	Antisense: 5'-GGCGTGTGAGTTGATGAGGT-3'	
MMP2	Sense: 5'-AGCTGGCCTAGTGATGATGTT-3'	176 bp
	Antisense: 5'-TTCAGCACAAACAGGTTGCAG-3'	
VEGF	Sense: 5'-TGGAGCGTGTACGTTGGTG-3'	153 bp
	Antisense: 5'-GCAACGCGAGTCTGTGTTTTT-3'	
β-actin	Sense: 5'-GGACTTCGAGCAGGAGATGG-3'	139 bp
	Antisense: 5'-CAGGAGCAAGGCTGGAAGA-3'	

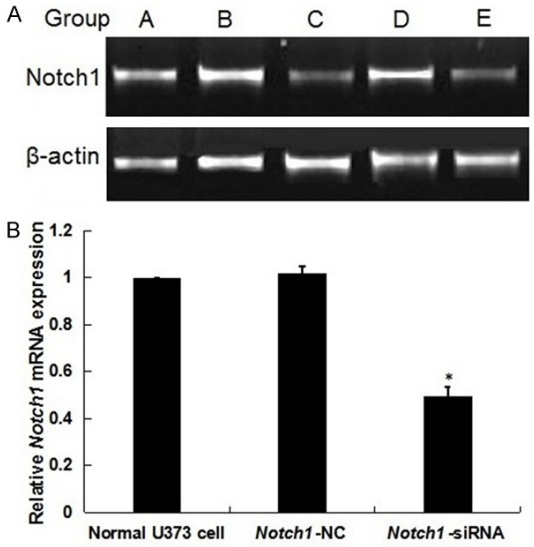

Figure 1. The expression of *Notch1* mRNA. A: Electrophoretic results of *Notch1* mRNA. B: Relative expression of *Notch1* mRNA. Group A and D: Normal Human glioma U373 cells, Group B: U373 cells transfected with *Notch1*-NC, Group C and E: U373 cells transfected with *Notch1*-siRNA.

4°C overnight. Secondary antibody: HRP Goat anti-Rabbit IgG antibody (1:2000). Using Beyo-ECL Plus to detected the protein bands. The relative expression of MMP2 and VEGF protein were calculated by ImageJ2x.

Establishment of implanted tumor model of human glioma cells in nude mice: 32 BALB/c nude mice were randomly divided into four groups: group A, group B, group C and group D on average. The suspension of cells in the logarithmic growth phase (1×10^7/L) with an amount of 0.2 mL was inoculated in subcutaneous tissue in the groin of each nude mouse to establish implanted tumor. Normal U373 cells were inoculated in group A and B, and U373 cells transfected with *Notch1*-siRNA were inoculated in group C and D. After 20 d, mice in group B and D were intravenously injected with TMZ (12.5 mg/ml). The dose was 40 mg/kg/d and 3 d/time, totally 6 times (18 d). At the same time, group A and C were injected with the equal saline. The Animal experiment had been approved by Animal Ethics Committee of Shandong University.

Approximately 100 mm^3 tumor was observed on the tenth day after inoculation. Long diameter (a) and short diameter (b) of tumor were measured every other day, calculated tumor volume=0.5a * b^2. After drug administration for 18 days and staying for 7 days, the mice were sacrificed and the intact tumor was peeled and weighed. The tumor inhibition rare was calculated.

Data analysis

Group A as control group, the cell growth inhibition rate=(OD value of the control group-OD value of the experimental group)/OD value of the control group×100% and the tumor inhibition rate=(tumor weight in the experimental group-tumor weight in control group)/tumor weight in control group×100%. The protein relative expression was calculated by ImageJ2X. All data was analyzed by SPSS 19.0. Measurement data was shown as mean ± SD. The comparison between two groups was performed by *t* test, data comparison among multiple groups was performed with single factor analysis of variance. $P<0.05$ represented the difference was statistically significant.

Results

The expression level of Notch1 mRNA

The expression level of *Notch1* mRNA was detected by RT-PCR. The result was shown in **Figure 1**. It can be observed that the relative expression level of *Notch1* mRNA in group C and E (0.495±0.04) was significantly lower than that in other groups, demonstrating that the *Notch1*-siRNA could down-regulate the expression of *Notch1* mRNA, which caused *Notch1* gene silencing in human glioma U373 cells.

Determination of cell proliferation ability

The optical density values (OD) of cells in each group were measured by ELIASA at 24 h, 48 h

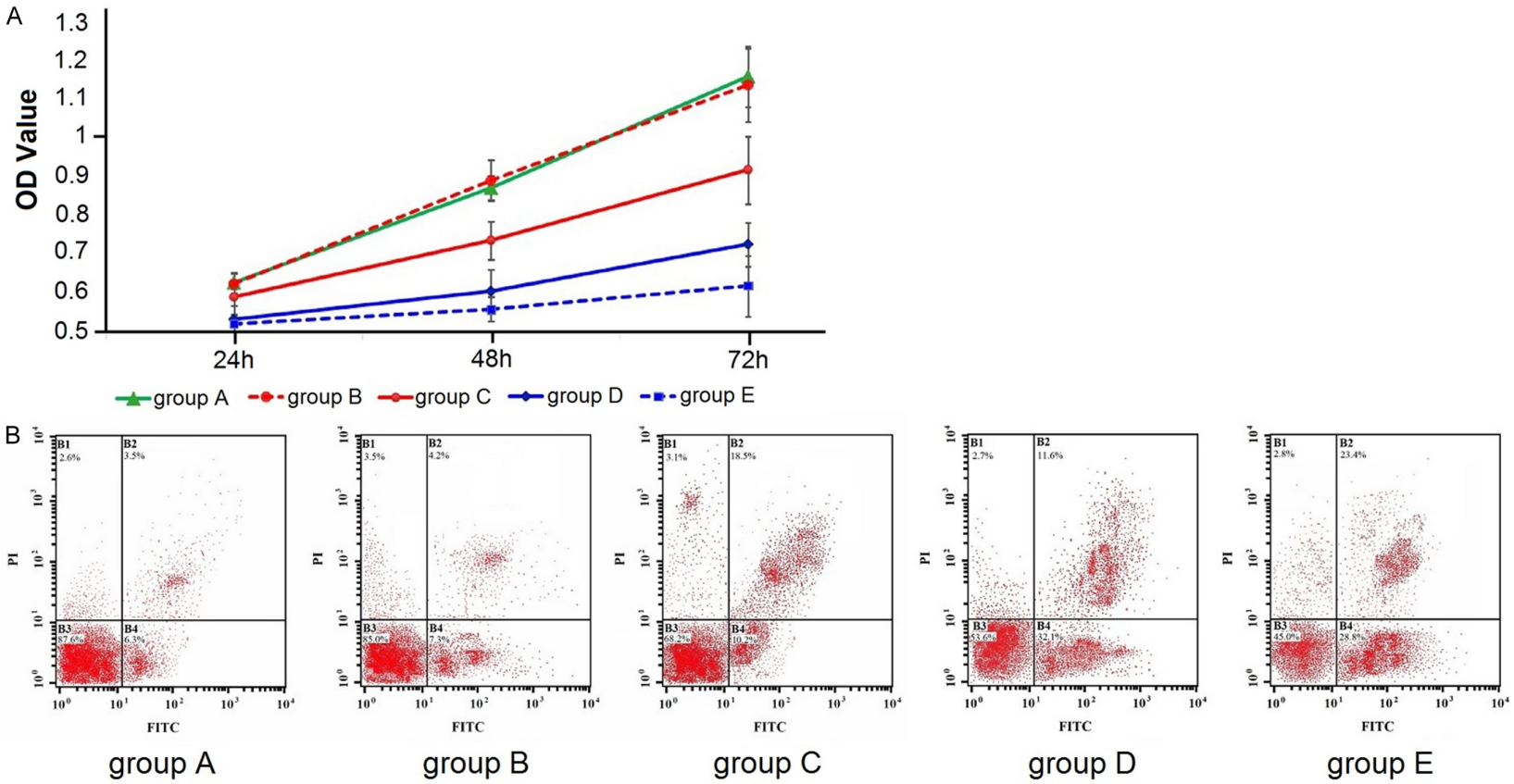

Figure 2. The growth curve and apoptosis rate of glioma cells in each group. A: The growth curve of glioma cells in each group, B: The apoptosis rate of glioma cells in each group detected by Annexin V/PI double staining. Group A: normal human glioma cells U373, Group B: cells transfected with *Notch1*-NC, Group C: cells transfected with *Notch1*-siRNA, Group D: normal human glioma cells U373+TMZ, Group E: cells transfected with *Notch1*-siRNA+TMZ.

Table 3. The cell growth inhibition rate in each group

Time	Group					Statistics
	A	B	C	D	E	
24 h	0	0.5±0.1	5.9±1.1*,▲	15.3±4.2*,▲	17.2±3.9*	F=163.082, P<0.001
48 h	0	-2.2±0.8	15.7±3.7*,▲	30.9±7.4*,▲	36.3±8.6*	F=201.777, P<0.001
72 h	0	1.9±0.7	20.9±4.8*,▲	37.6±9.2*,▲	47.0±11.6*	F=156.209, P<0.001

*represents significant differences compared with group A; ▲represents significant differences compared with group E, (P<0.05). Group A: normal human glioma cells U373, Group B: cells transfected with *Notch1*-NC, Group C: cells transfected with *Notch1*-siRNA, Group D: normal human glioma cells U373+TMZ, Group E: cells transfected with *Notch1*-siRNA+TMZ.

and 72 h, respectively. The cell growth curves were drew as shown in **Figure 2A**. Meanwhile, the cell growth inhibition rate was calculated as shown in **Table 3**, group A as the control group. The results showed that the cell proliferation capacity of group C, D and E was significantly lower than that of group A and B, and the proliferation capacity of group E was significantly lower than that of group C and D. These results indicated that the proliferation of the cells transfected with *Notch1*-siRNA was inhibited and was more sensitive to TMZ.

Determination of cell apoptosis rate

The apoptosis rate in each group was detected using Annexin V/PI double staining, and the result of flow cytometry was shown in **Figure 2B**. B2 and B4 represented apoptosis rate on middle-late stage and early stage, respectively. The total apoptosis rate which was shown in **Table 4**. The results showed that there was no difference in apoptosis rates between group A and B (P > 0.05). The apoptosis rates of the cells in group C, D and E was significantly higher than that in group A and B, and the apoptosis rate of the cells in group E was significantly higher than that in group C and D (P<0.05). The results suggested that cells transfected with *Notch1*-siRNA were susceptible to apoptosis and were more sensitive to TMZ.

Detection of expression level of MMP2 and VEGF

Expression level of MMP2 and VEGF mRNA: Relative expression of MMP2 and VEGF mRNA in different groups were detected by RT-PCR, as shown in **Figure 3A** and **3B**, respectively. It can be found that the expression level of MMP2 and VEGF mRNA in group C, D and E were decreased compared with group A, and the expression in group E was lower than that in group C and D, indicating that *Notch1*-siRNA could reduce the expression of MMP2 and VEGF, and *Notch1*-siRNA combined with TMZ could further reduce the expression of MMP2 and VEGF mRNA.

Expression level of MMP2 and VEGF Protein: The expressions of MMP2, VEGF and internal control protein were detected by Western-blot. The SDS-PAGE gels was shown in **Figure 3C** and the relative expression of MMP2 and VEGF protein was calculated using ImageJ2x, as shown in **Figure 3D**. Results showed that the expressions of MMP2 and VEGF protein in group C, D and E decreased significantly compared with group A, and the expressions in group E was significantly lower than those in group C and D, which were consistent with the result of mRNA expression.

The tumor volume changes in nude mice

Implanted tumor Model of human glioma cells in nude mice was successfully established. The changes of tumor volume were shown in **Figure 4C**. The tumor weight and tumor inhibiting rate were shown in **Table 5**. The results showed that the tumor volume and tumor weight of group B, C and D were significantly smaller than those of group A (P<0.05). The tumor volume and tumor weight of group D were significantly lighter than those of other groups, with tumor inhibiting rate more than 50%. The results showed that *Notch1*-siRNA reduced the tumorigenic ability of U373 cells *in vivo* and was more sensitive to TMZ, which significantly inhibited the growth of tumor *in vivo*.

Discussion

Notch1 signaling pathway is closely related to cell proliferation, differentiation and apoptosis. At present, abnormal expression of *Notch1* receptor has been found to be associated with the development and progression of various human tumors, such as breast cancer, colon cancer, lung cancer, etc [8-10]; and studies also confirmed the higher expression of *Notch1* receptor in human glioma cells [7, 11]. Glioma

Table 4. Statistical results of total apoptosis rate of cells in each group

Group	A	B	C	D	E	Statistics
Apoptosis rate	9.8±1.6	11.5±2.7	28.7±4.8*,▲	43.7±8.9*,▲	52.2±12.6*	F=210.642, P<0.001

*represents significant differences compared with group A; ▲represents significant differences compared with group E, (P<0.05). Group A: normal human glioma cells U373, Group B: cells transfected with *Notch1*-NC, Group C: cells transfected with *Notch1*-siRNA, Group D: normal human glioma cells U373+TMZ, Group E: cells transfected with *Notch1*-siRNA+TMZ.

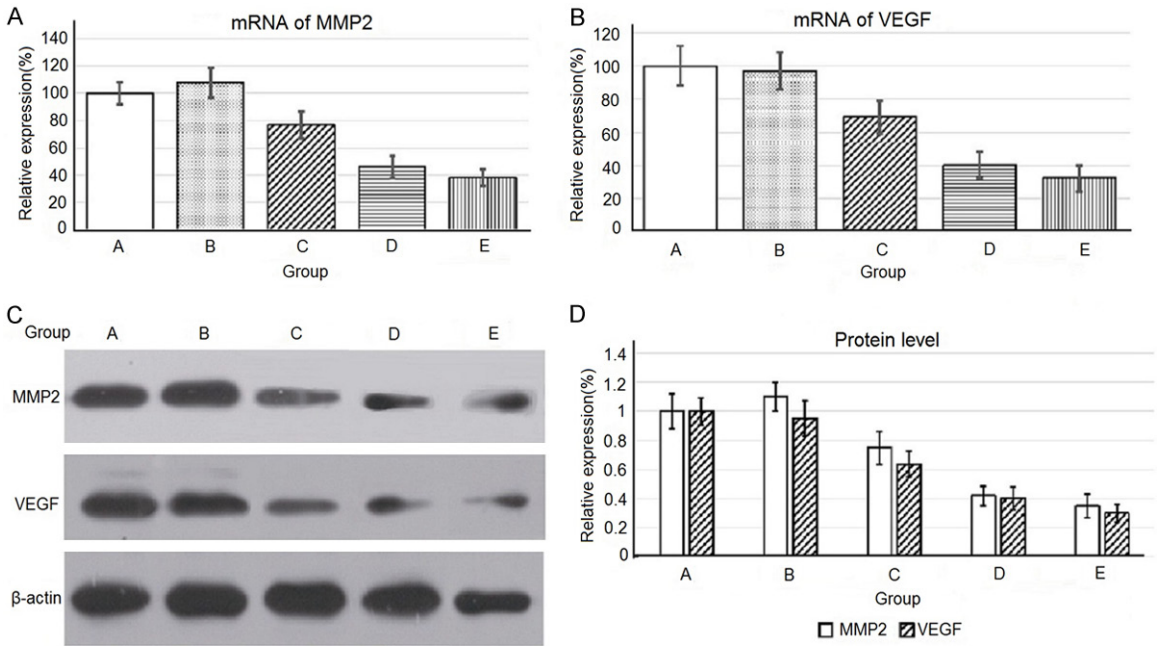

Figure 3. Relative expression of MMP2, VEGF. A: Relative expression of MMP2 mRNA in each group, B: Relative expression of VEGF mRNA in each group, C: SDS-PAGE gels of MMP2 and VEGF Protein in each group, D: Relative expression of MMP2 and VEGF protein. Group A: normal human glioma cells U373, Group B: cells transfected with *Notch1*-NC, Group C: cells transfected with *Notch1*-siRNA, Group D: normal human glioma cells U373+TMZ, Group E: cells transfected with *Notch1*-siRNA+TMZ. *, significant difference compared with group A; ▲, significant difference compared with group E, (P<0.05).

is the most common neurological malignant tumor. Surgery invariably fails to remove the tumor completely due to its special location, strong invasiveness and less sensitivity to radiotherapy and chemotherapy, resulting in recurrence of glioma, high mortality rate and poor prognosis [12]. TMZ is well tolerated and is emerging as a feasible first-line choice in the treatment of glioma and clinical studies confirmed that TMZ can improve the prognosis of patients with glioma. But due to drug resistance, the chemosensitivity in tumor cells is reduced, causing the recurrence of tumor [13, 14]. Therefore, improving the chemosensitivity of anti-cancer drugs (such as TMZ) in glioma tumor cell is the key to the treatment of malignant glioma. RNA interference (RNAi) technology can efficiently and specifically down-regulated the expression of target genes, which was an important method for gene function research and oncogene therapy [15]. With high silence efficiency, the recombinant vector was successfully constructed and transfected with human glioma tumor U373 cell in this study. The result found that *Notch1*-siRNA could effectively down-regulate the expression of *Notch1* and silence *Notch1* through detecting the expression of *Notch1* mRNA.

So far, multiple studies have shown that inhibition of *Notch1* expression in breast cancer [16, 17], colon cancer [18] and many other cancers would increase the sensitivity of tumor cells to chemotherapeutic drugs. Therefore, whether or not RNAi silencing *Notch1* had an impact on the proliferation, apoptosis of U373 cells as well as on its sensitivity to TMZ needs to be further researched. In this study, the growth curve of

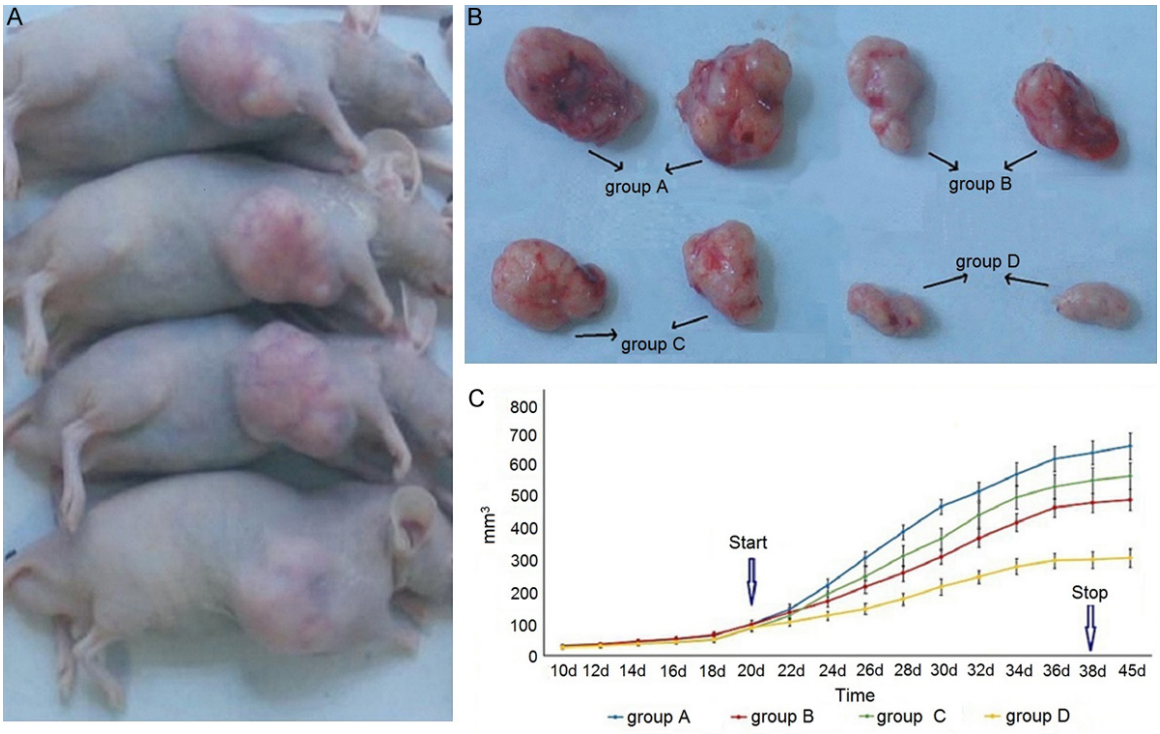

Figure 4. Implanted tumor model in nude mice and determination of tumor volume. A: Implanted tumor model in nude mice, B: Collected intact tumor, C: Determination of tumor volume; Group A: inoculation of normal U373 cells and injection of saline, Group B: inoculation of normal U373 cells and injection of TMZ. Group C: inoculation of U373 cells transfected with Notch1-siRNA and injection of saline. Group D: inoculation of U373 cells transfected with Notch1-siRNA and injection of TMZ.

Table 5. Volume, weight and inhibition rate of implanted tumor

Group	Tumor volume (mm^3)	Tumor weight (g)	Tumor inhibition rate
A	654.3±96.5	1.22±0.24	0
B	386.4±62.3*,▲	0.78±0.18*,▲	36.1±6.8*,▲
C	560.2±87.4*,▲	1.02±0.20*,▲	16.7±2.4*,▲
D	304.8±46.7*	0.60±0.16*	50.8±7.6*
Statistics	F=123.034, P<0.001	F=58.769, P<0.001	F=154.283, P<0.001

*represents significant differences compared with group A; ▲represents significant differences compared with group E, (P<0.05). Group A: inoculation of normal U373 cells and injection of saline, Group B: inoculated of normal U373 cells and injection of TMZ. Group C: inoculated of U373 cells transfected with *Notch1*-siRNA and injection of saline. Group D: inoculated of U373 cells transfected with *Notch1*-siRNA and injection of TMZ.

cells showed that the proliferation of cells transfected with *Notch1*-siRNA was reduced compared to normal U373 cells, and the cells transfected with *Notch1*-siRNA+TMZ were significantly suppressed, indicating that *Notch1*-siRNA combined with TMZ could further inhibit the proliferation of glioma cells. Detection results of apoptosis demonstrated that the apoptosis rate of cells transfected with *Notch1*-siRNA significantly increased compared to the normal U373 cells. The apoptosis rate of cells transfected with *Notch1*-siRNA+TMZ was significantly higher than that of cells transfected with *Notch1*-siRNA (P<0.05). The results suggested that RNAi silencing *Notch1* could inhibit the proliferation of the glioma cells, induce apoptosis and enhance its sensitivity to TMZ. The detection results of volume and weight of the implanted tumor also suggested that RNAi silencing *Notch1* decreased tumorigenic ability of U373 cells *in vivo*, and the inhibiting tumor growth ability of *Notch1*-siRNA combined with TMZ was more evident than used TMZ alone. The results of this study were consistent with the results of Zhang et al [19]: Combined RNAi with chemotherapeutic drugs could enhance the sensitivity of tumor cell to chemotherapy and promote its apoptosis [20, 21].

In addition, the results in the study found that the expressions of MMP2 and VEGF in U373 cells transfected with *Notch1*-siRNA were lower than those of normal U373 cells, and the expression of MMP2 and VEGF in cells transfected with *Notch1*-siRNA+TMZ decreased exponentially/sharply. Therefore, the pathway was presumed: *Notch1*-siRNA→inhibition MMP2 and VEGF expression→inhibition proliferation and invasion and induction apoptosis of glioma cell→increasing the sensitivity of glioma cell to TMZ.

In conclusion, *Notch1* played an important role in the proliferation, invasion and apoptosis of glioma. *In vitro* or *in vivo*, RNAi silencing *Notch1* could effectively inhibit the proliferation of glioma cells, induce its apoptosis, inhibit its tumorigenic ability and upgrade its sensitivity to TMZ, probably through reducing the expression of MMP2 and VEGF. Therefore, RNAi silencing *Notch1* in glioma cells increased the sensitivity to chemotherapy, improving the chemotherapeutic effect.

Disclosure of conflict of interest

None.

Abbreviations

RNAi, RNA interference; TMZ, Temozolomide; MMP2, Matrix MetalloProteinase-2; VEGF, Vascular Endothelial Growth Factor; NC, Negative Control; OD, Optical Density.

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