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ORIGINAL ARTICLE

Effects of TNFα on cell viability, proliferation and apoptosis of glioma cells U251

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Summary

Purpose: *To analyse the relationship between the expression of TNFa and the characteristics of glioma cell line U251.*

Methods: *U251 cell line was transfected with overexpressed TNFa or depleted TNFa constructs. The cell viability, proliferation and apoptosis were determined with MTT assay and flow cytometry analysis. The expressions of TNFa, caspase or microRNA (miR) were detected by Western blot analysis or real-time PCR (RT-PCR). The activity of caspase3 was examined by the absorbance of p-nitroaniline (pNA) production.*

Results: *Overexpression of TNFa could decrease cell viability, suppress cell proliferation and promote cell apoptosis, whereas knockdown of TNFa displayed the opposite effects. In addition, the effects of TNFa on the expression of caspase3, miR-9-3p, miR-9-5p, miR-17-3p, miR-17-5p, miR-31-3p and miR-31-5p were also probed. Overexpression of TNFa could raise the expression of caspase3 and miR-9 and knockdown of TNFa had an opposite effect. Our results also showed the overexpression of miR-9 could increase the expression of caspase3, while the inhibition of miR-9 had the opposite effect.*

Conclusion: *These results suggested that TNFa might be involved in the attenuation of growth, proliferation and promotion of apoptosis in U-251 cells by regulating the expression of miR-9 and caspase3 and that miR-17 and miR-31 perhaps were not correlated with the apoptosis of U251 cells or not acted as important factors in mediating the apoptosis of U251 cells induced by TNFa. Furthermore, the caspase3 could be regulated by miR-9 in an indirect way.*

Key words: *apoptosis, caspase3, miR-9, TNFa, U251*

Introduction

Glioblastomas are the most common and lethal primary human malignancies of the central nervous system (CNS). They have high proliferative and invasive capabilities and have a poor prognosis despite aggressive surgery, radiotherapy, and chemotherapy. Compared to systemic neoplasms, gliomas have a high macrophage content, which secretes a wide variety of molecules, including cytokines, which affect survival, growth and proliferation of the tumors [1,2]. An inherent resistance to apoptosis and proliferation may account for the low success rate of glioma treatments [3].Therefore, innovative treatment modalities that target the apoptosis and proliferation of gliomas are urgently needed.

TNFα (tumor necrosis factor α) is a pleiotropic cytokine which is extensively studied for its role in the pathogenesis of a variety of diseases. It is produced by a variety of cells such as macrophages monocytes, lymphocytes, natural killer (NK) cells and cancer cells [4]. TNFα elicits a wide spectrum of cellular responses, which mediates inflammation, regulates immune response and also induces apoptosis in certain types of cancer cells [5].In the CNS, the proinflammatory cytokine TNFα is considered as the principal mediator of neuroinflammation that elicits a cascade of cellular events culminating in neuronal death. At the same time, TNFα affords neuroprotection in certain neurological conditions. Thus, TNFα appears to exhibit a dual role in the brain [6]. Nitta et al.

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showed that the gene expression of cytokines in tumor and surrounding lesions included TNFα but not in normal brains. It is suspected that astroglial cell-derived cytokines may participate in local immune reactions accompanying glioma in the brain [4].

miRs are short, non-coding RNA regulators of gene expression that have been identified in a broad range of eukaryotes. In addition to regulating growth, development, differentiation, and metabolism, some miRs have also been reported to be involved in tumor growth, proliferation or apoptosis [7]. TNFα was reported to be correlated with the expression and regulation of some miRs such as miR-9, miR-17 and miR-31. These miRs have been regarded as important mediators in proinflammatory signals or control of Inflammation [8,9]. Furthermore, some reports showed that miR-9 could inhibit cell proliferation, migration and invasion, suggesting its tumor-suppressive potential in neuroblastoma and gastric cancer [10,11]. Transfection of miR-17-5p led to increased cell proliferation compared to control values [12]. However, miR-17-5p also acted as both an oncogene and a tumor suppressor in different cellular contexts [7]. Overexpression of miR-31 significantly inhibited tumor cell migration and invasion, whose targets included oncogenic kinases such as SRC, MET, NIK (MAP3K14) and the melanoma specific oncogene RAB27a [13,14]. In addition, miR-31 was shown to promote chemotherapeutic agents-induced apoptosis in prostate cancer cells, suggesting its tumor suppressive potential [15]. Consequently, whether the endogenous over- expression of TNFα could promote cell apoptosis and inhibit cell proliferation and what role would be played by miR-9, miR-17, and miR-31 induced by TNFα in glioma cell line U251 still remain to be elucidated.

In this study, the effects of TNFα on cell viability, cell cycle distribution and apoptosis of U251 cells were detected by MTT method, flow cytometry analysis and annexin V-FICTC assay. In addition, the expression of miR-9, miR-17 and miR-31 induced by TNFα was also measured by RT-PCR. At the same time, the caspase3 activity was analyzed by the pNA production method. In addition, the relationship between the caspase3 and miR-9 was also detected. These data might benefit the glioma prognosis and therapies in the future.

Methods

Reagents

All cell culture components were purchased from Thermo Scientific Inc. (Shanghai, China). U251 cell line was obtained from the American type culture collection (ATCC; Rockville, MD, USA). Human TNFα siRNA and TNFα overexpression pCMV-TNFα constructs were purchased from OriGene Technologies (Rockville, MD, USA). miR primers and miR-9 overexpression and inhibition vectors were purchased from Shanghai Genepharma Company. The transfection reagent Lipofectamine™ 2000 was purchased from Life technologies (Shanghai, China). Protein extraction buffer, Annexin V-FITC, Propidium iodide (PI) and Crystal violet were obtained from Beyotime Iinstitute of Biotechnology (Haimen, China). Whatman nitrocellulose membranes were purchased from GE Healthcare (Shanghai, China). The ECL chemiluminescence kit was purchased from Boster Biology (Wuhan, China). The antibodies include rabbit anti-human TNFα polyclonal antibody (LifeSpan BioSciences, Seattle, WA), rabbit anti-GAPDH polyclonal antibody (Boster Biology, Wuhan, China), and horseradish peroxidase conjugated goat anti-rabbit IgG polyclonal antibody (KPL, Chester, USA).

Cell culture and transfection

Human glioma cell line U251 was cultured in Dulbecco's modified Eagle's medium, including 10 % fetal bovine serum (FBS), 50 U/ml penicillin, and 50 mg/ml streptomycin. The culture condition was at 37 °C in a humidified atmosphere containing 5% CO_2. On the day of transfection, cells reaching 70-90% confluency were changed to serum-free medium just before the experiments. Transient transfections were performed using Lipofectamine™ 2000 transfection reagent following the manufacturer's recommendation (Life Technologies).

Western blot

A Protein Extraction Kit was used to extract total protein from U251 cell line transfected with TNFα overexpression vector or TNFα knockdown vector or only treated with Lipofectamine™ 2000 and the total protein was quantified by a BCA assay kit. The protein was separated by SDS-PAGE and transferred to a Whatman nitrocellulose membrane. The membrane was blocked for 2 hrs in PBST solution containing 5% skimmed milk and probed with primary antibody at 4 °C overnight. Then it was washed 5 min for 3 times in PBST and probed with the corresponding secondary antibody at room temperature for 2hrs. After being washed with PBST, autoradiography was conducted with ECL chemiluminescence reagents. The relative expression of the target protein was valuated with the gray value ratio of target protein content to GAPDH (target protein/GAPDH) content.

Determination of U251 cell viability

The effect of TNFα on the viability of U251 glioma cells was determined by MTT assay. The U251 cells were seeded into 96-well plates at the density of

1×10^3 cells/well, and, after reaching 80% confluence, they were transfected with TNFα overexpression vector or TNFα knockdown vector or only treated with Lipofectamine™ 2000. Another 24-h incubation was allowed and 10μl MTT (5 mg/ml) were added to the cells and incubated for an additional 4hrs. Media were then removed and 150 μl DMSO were added and thoroughly mixed to dissolve the crystals. OD values were measured by microplate reader at a wavelength of 570 nanometers. The relative cell proliferation was calculated and the experiment was repeated three times.

Determination of U251 cell cycle

The effect of TNFα on cell cycle of U251 glioma cell was investigated with flow cytometry. Briefly, the cells were detached by trypsinization, washed twice in PBS, and fixed in 70% cold ethanol overnight at -20 °C. The next day, after being washed by citrate phosphate buffer, followed by PBS, U251 cells were incubated with RNAse A solution for 1 h at 37 °C. Then, the U251 cells were incubated in PI solution at room temperature for 30 min. The cell cycle was detected by flow cytometry and the experiment was performed in triplicate.

Determination of U251 cell apoptosis

U251 cell apoptosis was detected by flow cytometry according to the manufacturer's instructions. Briefly, U251 cells were harvested, washed twice with PBS, and resuspended in 195 μl Annexin V binding buffer. A volume of 5 μl Annexin V-FITC was added and gently mixed, and U251 cells were stained in the dark at room temperature for 10 min. Then, U251 cells were centrifuged at 1,000×g for 5 min, and gently resuspended in 190μl of Annexin V binding buffer. Finally, 10 μl PI staining solution were added and gently mixed, and U251 cells were kept on ice in the dark and immediately subjected to flow cytometry analysis. Cell Quest software was used to analyze the results and the experiment was performed three times.

Caspase3 activity assay

Caspase3 activity was assayed depending on the pNA production. Briefly, all the cells cultured in dish were collected and lysed in recommended lysis buffer. After centrifugation at 16,000- 20,000g for 10min the supernatant was gathered. The yellow product pNA would be formed after substrates Ac-DEVD-pNA being added into the supernatant. Caspase3 activity was measured on the solution absorbance at wavelength 405 nanometre. The results were calculated and the experiments were repeated three times.

RT-PCR analysis

A total of 2×10^5 U251 cells was independently cultured in each well of 6-well plates to 80% confluence. After transfection with siTNFα, pCMV-TNFα or only Lipofectamine™ 2000, the total RNA was extracted with Trizol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed (Takara; Dalian, China) and the cDNA was subjected to PCR amplification for the miR-9-3p, miR-9-5p, miR-17-3p, miR-17-5p, miR-31-3p and miR-31-5p in ABI PRISM 7500 fast Real-Time system and the miRNA-U6 was used as internal reference. The PCR cycling conditions were 40 cycles of 95 °C for 12 s, and 62 °C for 40 s.

The effect of miR-9 on the expression of caspase3

In order to clear the relationship between the miR-9 and capsase3, the U251 cells were divided into three groups including miR-9 overexpression group, miR-9 inhibition group and control group. The three groups were respectively transfected with miR-9 overexpression vectors, miR-9 inhibition vectors or only treated with transfection reagents. The total RNA was extracted with Trizol reagent and the expression of miR-9 and caspase3 were detected by RT-PCR.

Statistics

The data were analyzed by SPSS 20.0 software package (SPSS Inc, Chicago, IL). One-way ANOVA and Student's t test, were used to analyze the related data. All p values were two-sided and the results were considered to be statistically significant if $p<0.05$.

Results

TNFa protein expression in U251 glioma cell line

In order to exploit the function of TNFα, the U251 cells were divided into three groups: control group (only treated with Lipofectamine™ 2000), overexpression group (infected with overexpression vector) and knockdown group (infected with siRNA). Three groups of glioma cells (control group, overexpression group and knockdown group) were lysed by RIPA lysis buffer and the protein expression was examined by Western blot. Our results showed that the protein level of TNFα in the overexpression group was significantly higher than that in the control group ($p< 0.05$) and that in the knockdown group it was significantly lower than that in the control group ($p< 0.05$) (Figure 1). These data demonstrated that the TNFα gene in different cell groups was successfully overexpressed or depleted.

Effect of TNFa on U251 cell viability

As stated above, U251 cells were divided equally into three groups: control group, overexpression group and knockdown group. U251 cells

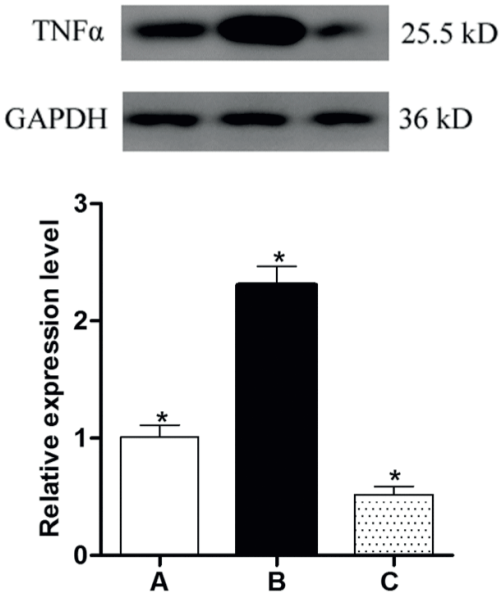

Figure 1. The expression of TNFα protein in U251 cells (**A:** control group, **B:** overexpression group, **C:** knockdown group). * Indicates p<0.05 compared to control group. The relative expression level of TNFα was normalized by that of GAPDH. These data were analyzed by one-way ANOVA.

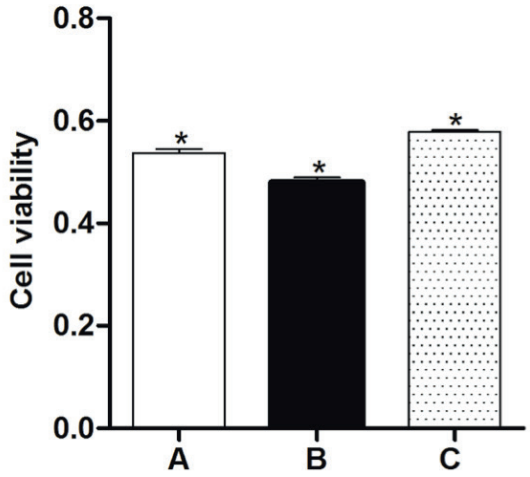

Figure 2. The effect of the TNFα on U251 cells viability (**A:** control group, **B:** overexpression group **C:** knockdown group). * Indicates p<0.05 compared to control group.

of the same number from each group were inoculated and assigned to the corresponding treatment (TNFα overexpressed or depleted or only treated with Lipofectamine™ 2000) and subjected to MTT assay. We found that U251 cells viability in the overexpression group was significantly lower than that in the control group (p< 0.05) and that U251 cells viability in the knockdown group was significantly higher than that in the control group (p< 0.05) (Figure 2). These results suggested that overexpression of TNFα might be related to the decrease in U251 cell viability.

Effect of TNFa on the cell cycle of U251 cells

Cell cycle analysis revealed that overexpression and knockdown groups had less U251 cells in G0/G1 phase compared with the control group (p< 0.05). More U251 cells were identified in S phase in the knockdown group compared to the overexpression and the control group (p< 0.05). Furthermore, the overexpression group and knockdown group had more U251 cells in G2/M phase in relation to the control group (p<0.05) (Figure 3). These results indicated that upregulation of TNFα might lead to U251 cell cycle arrest in G2/M phase and downregulation of TNFα promoted U251 proliferation proved by the increase of ratio of S phase.

Effect of TNFa on apoptosis of U251 cells

Flow cytometry analysis of U251 cell apoptosis showed that the number of apoptotic cells in the overexpression group was significantly higher than that in the control group and that the number of apoptotic cells in the knockdown group was significantly lower than that in the control group (p< 0.05) (Figure 4). These data suggested that the upregulation of TNFα might enhance U251 cell apoptosis and that the knockdown of TNFα expression might suppress U251 cell apoptosis.

Evaluation of TNFa on the activity of caspase3

According to the manufacture's instruction, the caspase3 activity was detected and the results indicated that caspase3 activity in the overexpression group was markedly higher than in the control group (p<0.05) and caspase3 activity in the knockdown group was significantly reduced compared to the control group (p<0.05) (Figure 5). The results suggested that the upregulation of TNFα could increase the caspase3 expression and downregulation of TNFα could decrease the caspase3 expression.

Effect of TNFa on the expression of miR-9, miR-17 and miR-31

RT-PCR indicated that the miR-9 expression (including miR-9-3p and miR-9-5p) in the overexpression group was markedly upregulated compared to the control group (p<0.05) and that the miR-9 expression in the knockdown group was

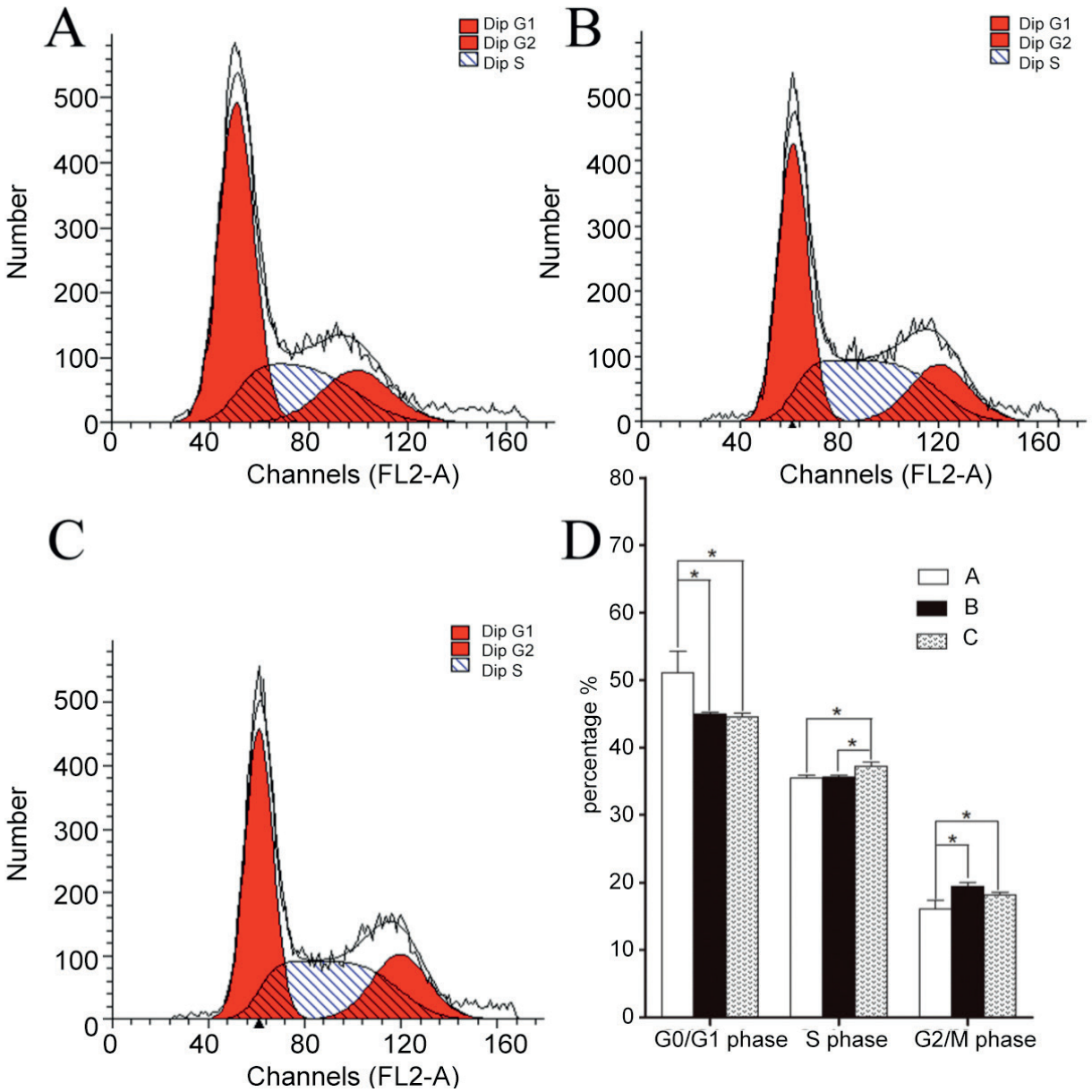

Figure 3. The effect of TNFα on cell cycle of U251 cells (**A:** control group, **B:** overexpression group, **C:** knockdown group, **D:** the relative proportion of various phases of the cell cycle). *Indicates p<0.05 compared to control group.

significantly attenuated compared to the control group (p<0.05) (Figure 6). As for miR-17 (miR-17-3p and miR-17-5p), its expression was significantly improved in the overexpression group compared to the control group (p<0.01), while no obvious variation was found between the knockdown group and the control group (p=0.108). In addition, the expression of miR-31-3p was markedly inhibited in the knockdown group compared to the control group (p<0.05) and miR-31-5p was markedly promoted in the knockdown group compared to the control group (p<0.05). However, the expression of miR-31 (miR-31-3p and miR-31-5p) displayed no significant change in the overexpression group related to the control group. These results suggested that miR-9 played a tumor suppressive role in U251 glioma cells.

Effect of miR-9 on the expression of caspase3

The results showed that the miR-9 expression (including miR-9-3p and miR-9-5p) in the miR-9 overexpression group was markedly higher than that in the control group (p<0.05) and that the expression of miR-9 in the miR-9 inhibition group was significantly lower than that in the control group (p<0.05). At the same time, the results also showed that the expression of caspase3 in the miR-9 overexpression group was obviously up-regulated compared to the control group (p<0.05)

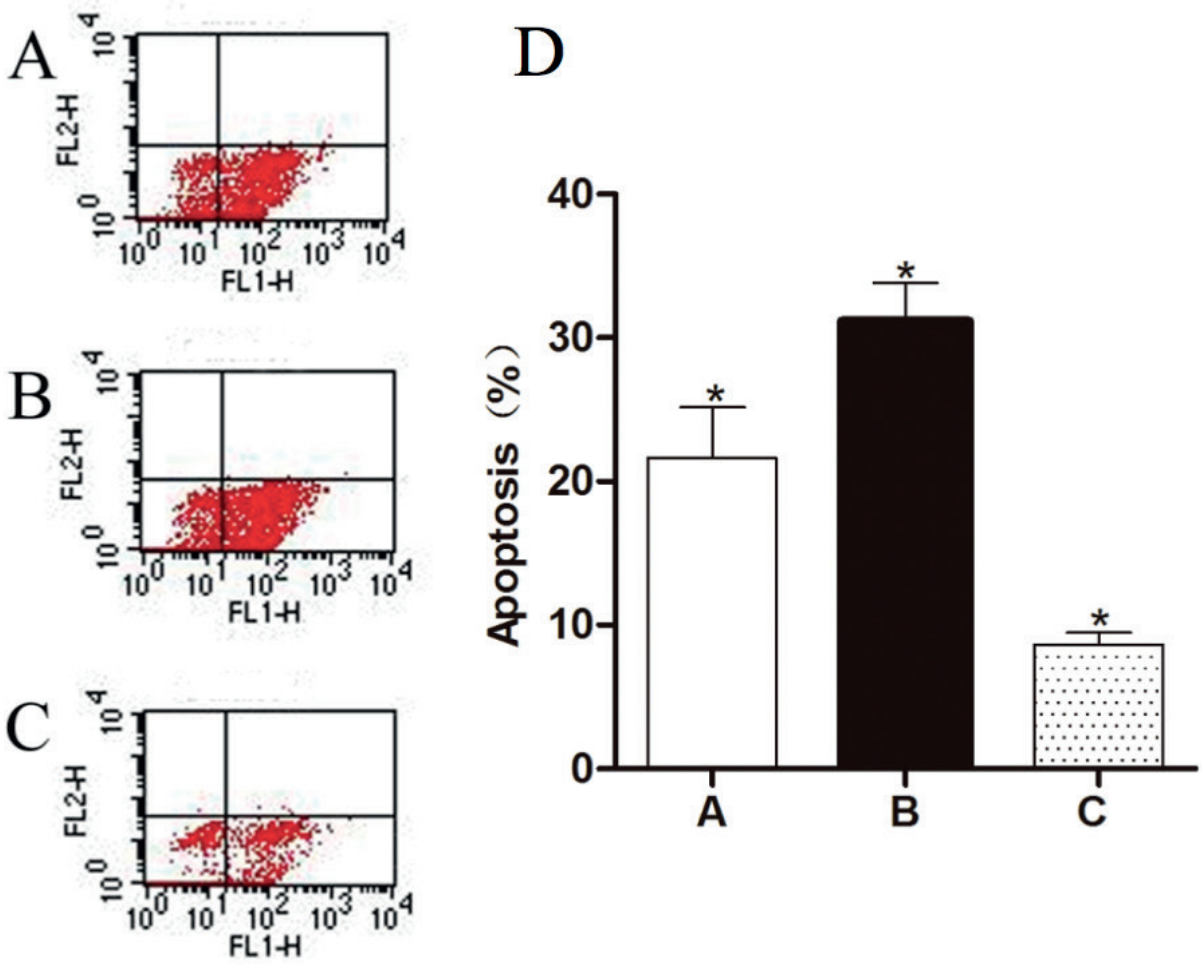

Figure 4. The effect of TNFα on apoptosis of U251 cells (**A:** control group, **B:** overexpression group, **C:** knockdown group, **D:** the relative proportion of the apoptosis cell). *Indicates p<0.05 compared to control group.

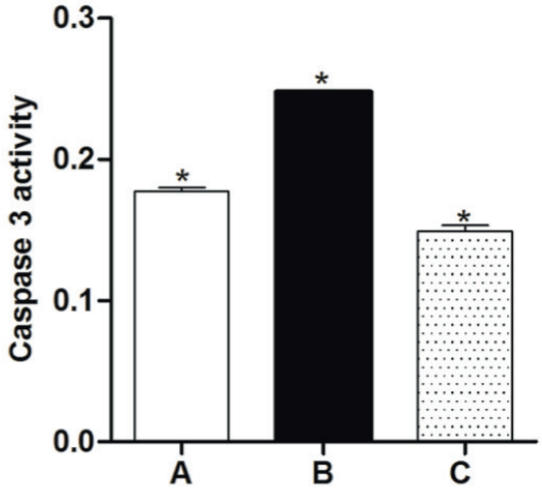

Figure 5. The enzyme activity of caspase3 was analyzed by pNA production (**A:** control group, **B:** overexpression group, **C:** knockdown group). *Indicates p<0.05 compared to control group.

and that in the miR-9 inhibition group the expression was significantly downregulated in relation to the control group (p<0.05) (Figure 7).

Discussion

TNFα is a pleiotropic cytokine produced by a variety of cells and involved in immune modulation, inflammatory reactions, and target cell death in specific pathologic conditions. A study showed that TNFα stimulated cell proliferation, depending on SphK-dependent Akt activation and cyclin D expression [16], however, cell death induced by TNFα has also been reported [2,17]. To date, whether TNFα promotes or inhibits U251 cell apoptosis still remained unclear and, in order to exploit the function of TNFα in U251 cells, we transfected U251 cells with the overexpression and knockdown constructs, respectively. The

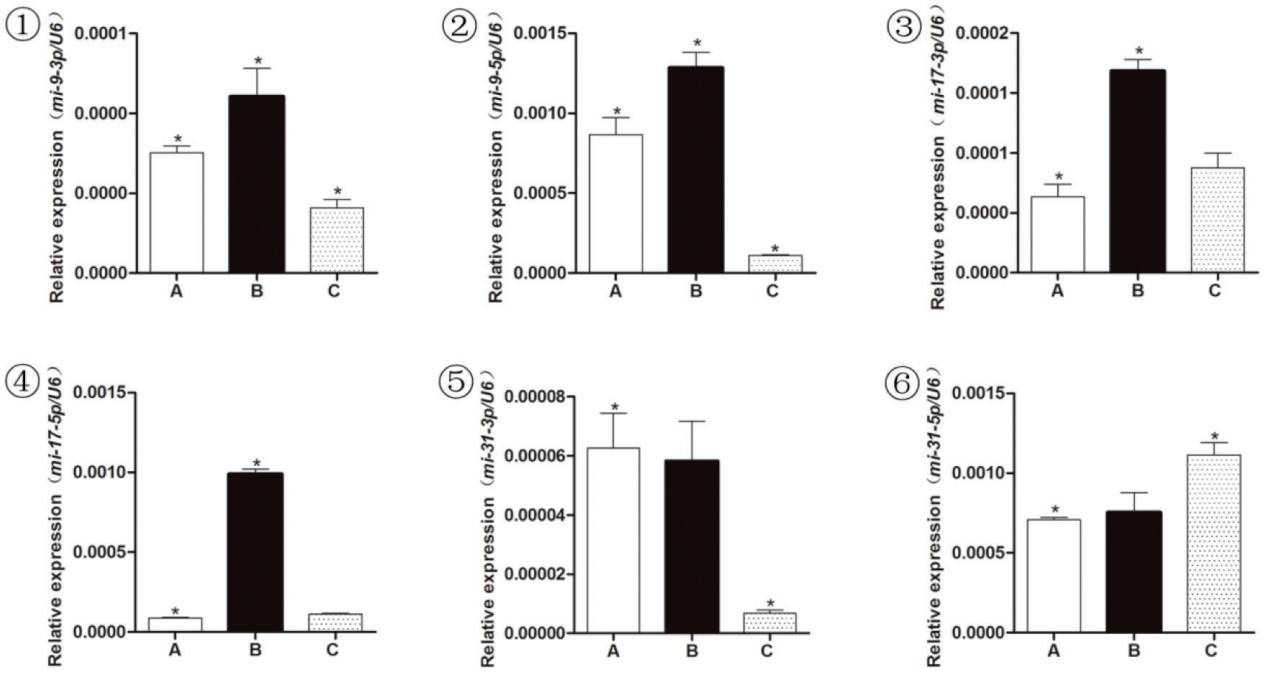

Figure 6. The effects of TNFα on the expression of miR-9-3p (1), miR-9-5p (2), miR-17-3p (3), miR-17-5p (4), miR-31-3p (5), and miR-31-5p (6) in U251 cells. The relative expression level of miR was normalized by that of U6 RNA. (**A:** control group, **B:** overexpression group, **C:** knockdown group). *Indicates $p<0.05$ compared to control group.

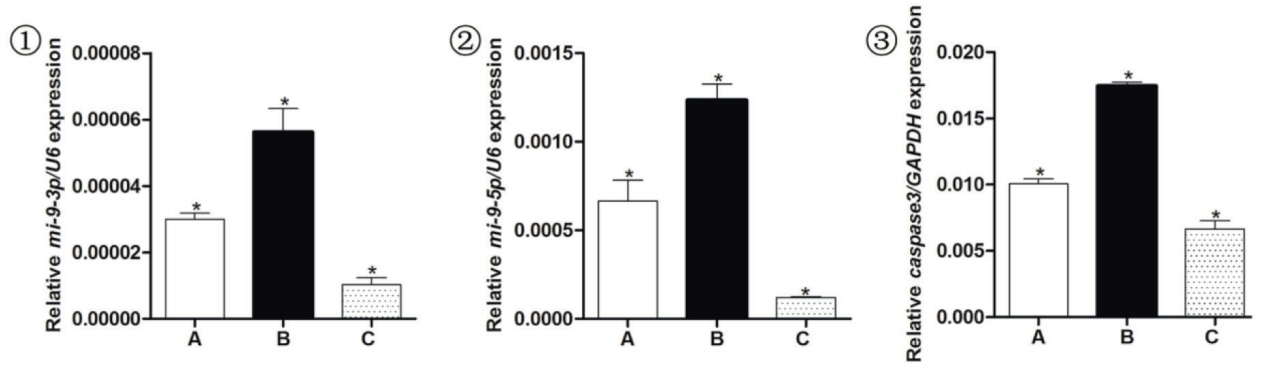

Figure 7. The effect of miR-9 on the expression of caspase3 in U251 cells (**A:** control group, **B:** miR-9 overexpression group, **C:** miR-9 inhibition group). Expression assay: 1 for miR-9, 2 for miR-9-5P, 3 for caspase3. *Indicates $p<0.05$ compared to control group.

results showed that TNFα could promote glioma cell U251 apoptosis compared with the control or knockdown group.

Usually the cell apoptosis pathways triggered by TNFα include the caspase-8/Bid-dependent apoptotic pathway or the caspase independent necrosis pathway. One important mediator of the caspase8/Bid-dependent signal pathway is caspase3, which plays an important role in regulating cell apoptosis [5,18]. In order to confirm whether caspase3 was involved in cell apoptosis induced by TNFα in U251 cells, the activity of caspase3 was assessed separately in three groups. Our results revealed that the overexpression group had more caspase3 activity than the control or the knockdown group. These results suggested that caspase3 was correlated with the apoptosis of U251 cells induced by TNFα.

In addition, a lot of miRs, such as miR-9, miR-17 and miR-31, induced by TNFα could promote cell apoptosis and death [8,19-22]. However, whether these miRs could be induced by TNFα in U251 cells and whether they participated in the apoptosis induced by TNFα remain elusive. Consequently, the expressions of miR-9, miR-17 and miR-31 were detected by RT-PCR in three differ-

ent treatment groups. Among them, the miR-9-3p and miR-9-5p had the same variation tendency in three different treatment groups. The expression level of miR-9-3p and miR-9-5p in the overexpression group was dramatically higher than that in the control or the knockdown group. The results documented that miR-9 could be induced by TNFα in U251 cells and we speculated that miR-9 possibly exerted its apoptosis promotion effect by acting as a tumor suppressor gene in the U251 cells.

Intriguingly, our analysis showed that a different variation trend emerged in the expressions of miR-17-3p and miR-31-3p compared with the corresponding miR-17-5p and miR-31-5p in different experimental groups. Similar to previous studies, we found that miR-17 had a paradoxical role in cell behaviors. For example, miR-17 suppressed cell growth and promoted apoptosis of cervical cancer cells [23]. However, in hepatocellular carcinomas (HCCs) miR-17-5p was significantly upregulated and associated with poor prognosis [24] and inhibition of miR-17 improved lung and heart function [25]. These studies showed that miR-17, as a multifunctional factor, played different roles in different tissues. miR-31 was similar to miR-17, possessing different roles in different tissues or cells [15,26-28]. Based on the previous studies and our results, we speculated that miR-17 and miR-31 might not be correlated with the apoptosis of U251 cells or they did not act as important factors in mediating the apoptosis of U251 cells induced by TNFα.

Our study indicated that the apoptosis of U251 cells induced by TNFα was correlated with miR-9 and caspase3. In order to clarify the relationship between the miR-9 and caspase3 in U251 cells, three groups of U251 cells were examined with miR-9 overexpressed or inhibited or only treated with transfection reagents and the caspase3 expression was also measured in the three groups. Our results showed that the caspase3 expression had the same variation trend with miR-9, therefore, we speculated that the caspase3 perhaps was indirectly regulated by miR-9. miR-9 was reported to be downregulated in human ovarian cancer relative to normal ovary and to suppressed cell growth through inhibition of NF-kB1 expression [29]. The NF-kB signaling pathway mediated by miR-9 was independent of the caspase cascade pathway mediated by caspase3 in promoting cell apoptosis [30,31]. Although our results indicated the caspase3 possibly was regulated by miR-9, the U251 cell apoptosis induced by TNFα through the two molecules in concerted or in independent way still needed to be elucidated by further research in the future.

In view of the above, we inferred that TNFα could be involved in the inhibition of proliferation and the boost of apoptosis in U251 cells, and caspase3 and miR-9 were possibly associated with the apoptosis of U251 cells induced by TNFα, while the caspase3 was perhaps regulated by miR-9 in an indirect way. However, further research is still needed to provide a good understanding of its function and mechanism of action.

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