Original Article
Roles of MED27 in regulating glioma cells via NF-κB iNOS

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Abstract: Neuroglioma is one major challenge in neurosurgery, due to its complicated pathogenesis mechanism, which has not been fully resolved yet. The identification of effective molecular targets for pathogenesis of neuroglioma thus can benefit the treatment efficacy of glioma. Previous study found that mediator complex 27 (MED27) was involved in mediating tumor occurrence and progression. The role of MED27 in glioma and related mechanism, however, has not been illustrated. In vitro cultured glioma U87 cells were randomly divided into control group, scramble group, which received negative controlled siRNA, and MED27 siRNA transfection group. Real-time PCR was used to detect the expression of MED27 mRNA, while Western blotting was used for analyzing MED27 protein. MTT assay and invasion assay were used to detect the effect of MED27 on U87 cell proliferation and invasion. Caspase-3 activity assay was used to reveal U87 apoptosis, followed by Western blotting to detect NF-κB/iNOS protein expression. After transfecting MED27 siRNA, expression of MED27 protein was decreased, leading to inhibition of U87 cell proliferation or invasion, in addition to enhanced caspase3 activity (P<0.05 compared to control group). The transfection of MED27 siRNA significantly down-regulated the expression of NF-κB/iNOS protein in glioma cells (P<0.05 compared to control group). The inhibition of MED27 facilitates glioma cell apoptosis, inhibits their proliferation or invasion, mainly via suppressing NF-κB/iNOS signal pathway.

Keywords: MED27, glioma, NF-κB, iNOS, cell proliferation

Introduction

Neuroglioma is characterized by high invasiveness and unfavorable prognosis, making it one major challenge in neurosurgery. As one of the most common malignant tumors, neuroglioma mainly derives from neural ectoderm, as one primary brain tumor in central nervous system [1, 2]. The incidence of neuroglioma is keeping at high levels, with increasing trends and younger populations [3]. Neuroglioma is most frequently occurred in cerebral hemisphere, with multifocal glioma as the dominant pathological type [4, 5]. In China, neuroglioma gradually occupies the most popular tumor in central nervous system, accompanied with elevated incidence [6]. With the advancement of medical science, treatment for brain glioma is becoming various. However, due to the unillustrated pathogenesis mechanism, the prognosis of patients is still unfavorable [7, 8]. The residual tumor cells after surgery may lead to reoccurrence, accompanied with glioma invasion and metastasis, thus severely compromising patient survival quality and rate, and causing heavy mental and economic burdens for patients and their families, making it one major challenge in tumor treatment [9, 10]. Therefore the study for related regulatory mechanism of neuroglioma benefits both prevention and diagnosis of glioma.

Mediator complex is one complex molecule composed of at least 33 proteins, and is widely distributed in plant, fungus and animals, although both number and subtypes of mediator complex in different species were different [11]. All members of mediator complex family have highly conserved sequence during evolution. Mediator complex has four domains, namely head, center, tail and enzymatic part, with different composition across species [12].

Table 1. Primer sequence

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
MED27	ACCTCTTCCCCTAGAATCTG	TAGACCTCTTAATGGATGTT

Previous studies demonstrated that mediator complex was one important co-factor in RNA polymerase II transcription cassette, as it is one bridge for transmitting information between transcription factor and RNA polymerase II [13, 14]. Mediator complex 27 (MED27) is widely distributed in human tissue/organ, without any tissue-specificity regarding the distribution of mRNA or protein, indicating its role as one important factor for transcription initiation [15]. Recent study reported the participation of MED27 in regulating tumor progression, such as the development of melanoma [16]. The regulatory role of MED in glioma, or related mechanisms, however, is still unknown yet.

Materials and methods

Reagent and equipment

Human glioma U87 cell line was purchased from ATCC cell bank (US). DMEM medium, fetal bovine serum (FBS) and streptomycin-penicillin were purchased from Hyclone (US). DMSO and MTT powders were purchased from Gibco (US). Trypsin-EDTA lysis buffer was purchased from Sigma (US). Caspase3 activity assay kit and PVDF membrane were purchased from Pall Life Sciences (US). EDTA was purchased from Hyclone (US). Western blotting reagent was purchased from Beyotime (China). ECL reagent was purchased from Amersham Biosciences (US). Rabbit anti-human NF-κB monoclonal antibody, rabbit anti-human MED27 monoclonal antibody, rabbit anti-human iNOS monoclonal antibody and mouse anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody were all purchased from Cell Signaling (US). Transwell chamber was purchased from Corning (US). MED27 siRNA and negative controlled oligonucleotides were synthesized by GenePharma (China). RNA extraction kit, reverse transcription kit was purchased from Axygen (US). Other common reagents were purchased from Sangon (China). Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad (US).

Glioma U87 cell culture and grouping

U87 cells were resuscitated in 37°C water-bath until fully thawing. Cells were centrifuged at 1000 rpm for 3 min, and were re-suspended in 1 ml fresh medium and were removed to 5 ml culture flask which contained 3 ml fresh culture medium. Cells were kept in a humidified chamber with 5% CO_2 at 37°C for 24~48 h. U87 cells were seeded in 6-well plate at 1×10^5 per cm^2. Cells at log-phase with 2nd to 8th generation were randomly divided into control group, which received normal culture, scramble group, which were transfected with MED27 siRNA negative controlled oligonucleotide, and MED27 siRNA group, which were transfected with MED27 siRNA.

Liposome transfection

MED27 siRNA (5'-GGCUC CAAUU UGUCU AUAAT T-3' and 5'-ACAAG GGUUC GCGUA UAGGU G-3') or MED27 siRNA control (5'-AUGCA UCCGG UAGGC AUGAU G-3' and 5'-ACAAGG GUUCG CGUAU AGGUG-3') oligonucleotides were transfected into U87 cells. In brief, cells were cultured in 6-well plate until reaching 70%~80% confluence. MED27 siRNA or negative control sequences were mixed with liposome in 200 μl serum-free medium for 15-min room temperature incubation. Lipo2000 reagent was then mixed with MED27 siRNA or MED27 siRNA controlled dilutions for 30-min room temperature incubation. Serum was removed from cells, followed by PBS rinsing and the addition of 1.6 ml serum-free culture medium. Cells were then kept in a humidified chamber with 5% CO_2 at 37°C for 6 h, followed by the application of serum-containing medium in 48 h continuous incubation for further experiments.

Real-time PCR for MED27 mRNA expression

Trizol reagent was used to extract RNA from all groups for cells. Reverse transcription was performed according to the manual instruction, using primers designed by Primer6.0 and synthesized by Invitrogen, Shanghai (China) as shown in **Table 1**. Real-time PCR was performed on target genes under the following conditions: 56°C for 1 min, followed by 35 cycles each containing 92°C for 30 s, 58°C for

45 s and 72°C for 35 s. Data were collected and calculated for CT values of all samples and standards based on fluorescent quantification using GAPDH as the baseline. Standard curve was firstly plotted using CT values of standards, followed by semi-quantitative analysis by 2-ΔCt method.

Western blotting for protein levels of MED27, NF-κB and iNOS

U87 cell proteins were firstly extracted. In brief, RIPA lysis buffer containing proteinase inhibitor was used to lyse cells on ice for 15~30 min, followed by ultrasound rupture (5 s × 4) and centrifugation (4°C, 10 000 g, 15 min). Supernatants were saved and quantified for protein contents, and were stored at -20°C for further Western blotting. Proteins were then separated using 10% SDS-PAGE gel, and were transferred to PVDF membrane using semi-dry method. Non-specific background was removed by 5% defatted milk powder at room temperature for 2 h, followed by the addition of anti-MED27 monoclonal antibody (1:1000 dilution), anti-NF-κB monoclonal antibody (1:2000) or anti-iNOS monoclonal antibody (1:1500) in 4°C overnight incubation. On the next day, the membrane was rinsed in PBST, and incubated with 1:2000 goat anti-rabbit secondary antibody for 30 min incubation. After PBST rinsing, ECL reagent was used to develop the membrane, which was exposed under X-ray for observing results. Protein imaging analysis system and Quantity One software were used to scan X-ray films for observing band density. Each experiment was repeated for four times (N=4) for further analysis.

MTT assay for cell proliferation

U87 cells at log-phase were seeded into 96-well plate which contained DMEM medium with 10% FBS at 5×10^3 density. After 24 h incubation, the supernatant was removed. Cells were randomly divided into control, scramble and MED27 siRNA groups, which were treated as abovementioned. In brief, 20 μl sterile MTT was added into each test well in triplicates after 48 h cell culture. After 4 h continuous culture, the supernatant was completely removed, with the addition of 150 μl DMSO for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values was measured at 570 nm in a microplate reader. The proliferation rate was

calculated in each group. Each experiment was repeated for more than three times.

Transwell chamber assay for cell proliferation

Following the manual instruction, serum-free culture medium was used for 24 h cell culture. Transwell chamber was pre-coated using 1:5 50 mg/L Matrigel dilutions on the bottom and upper layer of the membrane, followed by 4°C air-dry. 500 μl DMEM culture medium containing 10% FBS was then added into inner and outer surface of the chamber, which contained 100 μl tumor cell suspensions prepared by serum-free culture medium. The chamber was placed in a 24-well plate in triplicates for each group. Control cells were cultured in Transwell chamber without Matrigel. After 48 h, PBS was used to rinse Transwell chamber, with the removal of membrane-fixed cells, which were then fixed in cold ethanol and stained by crystal violet. The number of cells at the lower surface of the micro-pore membrane was then counted in triplicates (N=3).

Caspase 3 activity assay

Caspase 3 activity in each group of cells was assayed following the manual instruction of test kits. In brief, trypsin was used to digest cells, followed by 600 g centrifugation for 5 min at 4°C. The supernatant was discarded, with the addition of lysis buffer for 15 min iced incubation. The mixture was then centrifuged at 20000 g for 5 min at 4°C, with the addition of 2 mM Ac-DEVD-pNA. OD values were measured at 405 nm wavelength for calculating caspase3 activity.

Statistical analysis

All data were presented as mean ± standard deviation (SD). Student t-test was used to compare means between two groups. SPSS11.5 software was used in statistical analysis. Analysis of variance (ANOVA) was used for between-group analysis. A statistical significance was identified when P<0.05.

Results

Expression of MED27 mRNA in glioma cells

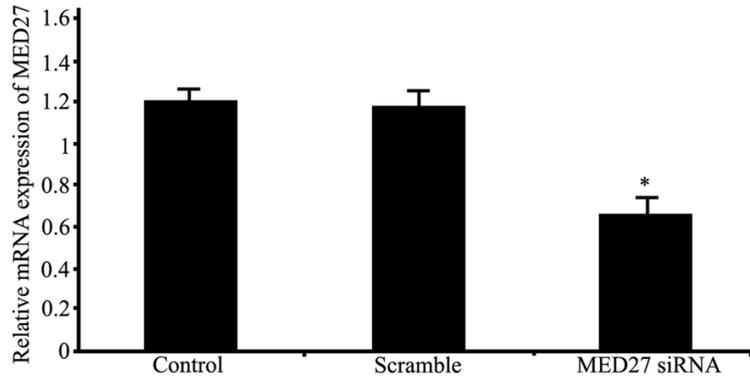We employed real-time PCR to check the effect of siRNA on MED27 mRNA expression level in

Figure 1. Expression of MED27 mRNA in glioma cells. *, P<0.05 compared to control group.
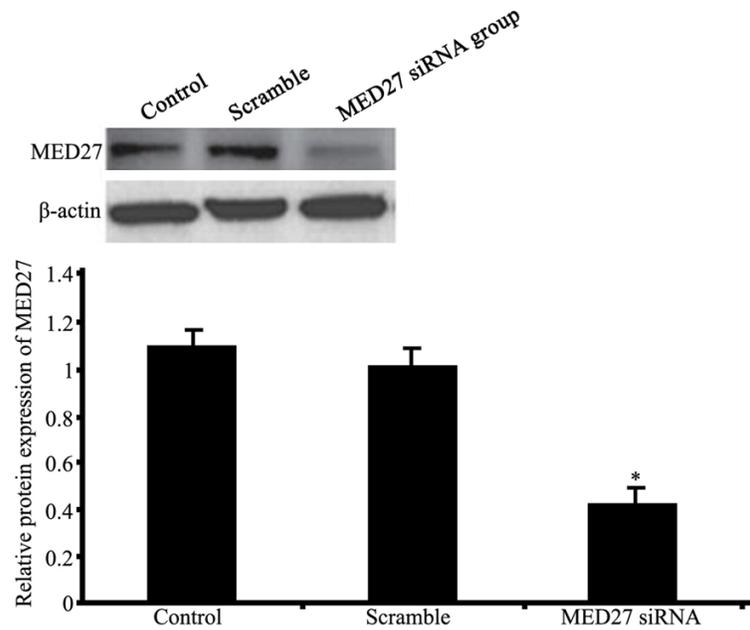

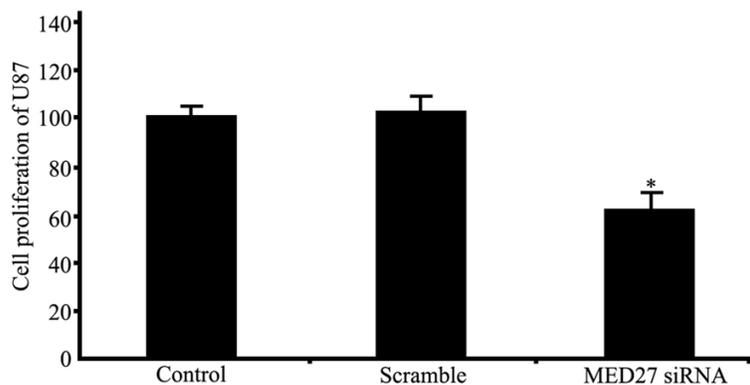**Figure 2.** Regulation of MED27 protein expression in glioma cells. *, P<0.05.

Figure 3. Regulation of U87 cell proliferation by MED27. *, P<0.05 compared to control group.

glioma cells. Results showed that the transfection of MED27 siRNA on U87 cells significantly inhibited the expression of MED27 mRNA in U87 cells (P<0.05 compared to control group). The transfection of MED27 siRNA negative control oligonucleotide did not affect MED27 mRNA expression (P>0.05 compared to control group, **Figure 1**).

MED27 protein expression in glioma cells

Western blotting was used to test the effect of siRNA on MED27 protein expression in glioma cells. Results showed that, similar to those in real-time PCR, the transfection of MED27 siRNA in U87 cells significantly inhibited MED27 protein in U87 cells (P<0.05 compared to control group). The transfection of siRNA negative controlled sequence did not affect MED27 protein expression (P>0.05 compared to control group, **Figure 2**).

MED27 level and U87 cell proliferation

MTT assay was used to detect the effect of siRNA on U87 cell proliferation. Results showed that after transfecting MED27 siRNA, the proliferation of U87 cells was significantly inhibited (P<0.05 compared to control group, **Figure 3**). These results suggested that the alteration of MED27 expression benefited the regulation of abnormal proliferation of glioma cells.

Effect of MED27 on U87 cell invasion

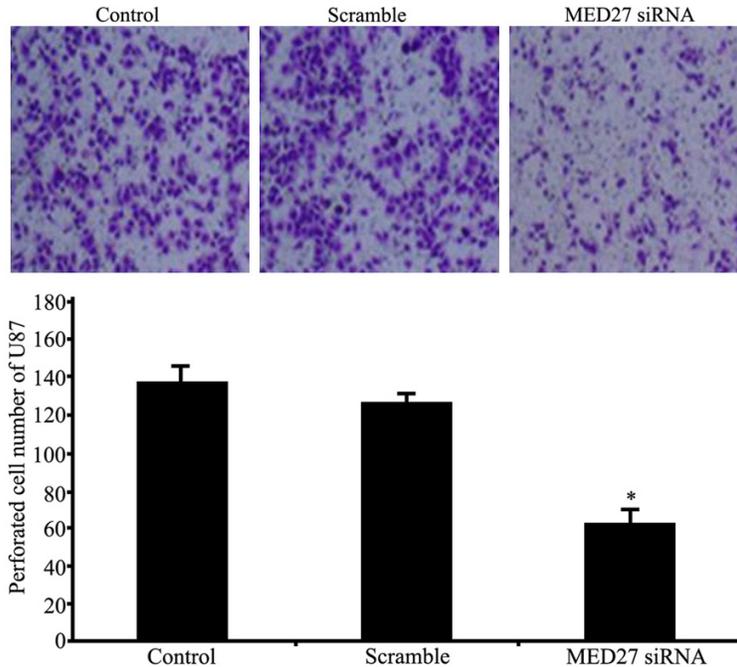Transwell chamber assay was employed to detect the effect of siRNA on U87 cell invasion.

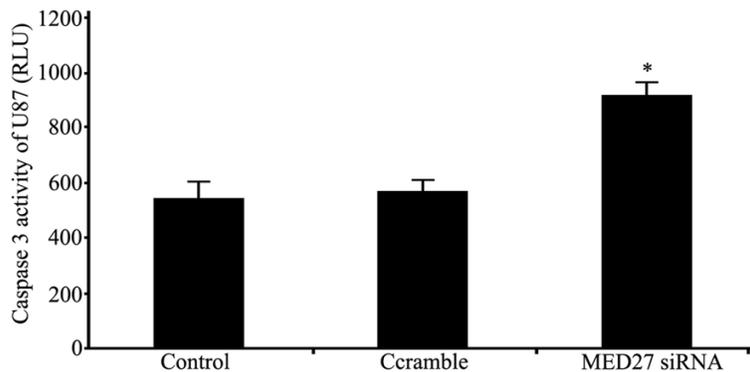Figure 4. Analysis of the effect of MED27 on U87 cell invasion. *, P<0.05 compared to control group.

Figure 5. Caspase3 activity of U87 cells and MED27 expression. *, P<0.05 compared to control group.

Result showed that the transfection of MED27 siRNA significantly inhibited U87 cell invasion potency (P<0.05 compared to control group, **Figure 4**). These results suggested that MED27 expression alternation could affect invasiveness of U87 glioma cells.

Caspase3 activity in U87 cells by MED27

We employed caspase3 activity assay kit to analyze the effect of MED27 on caspase3 activity of U87 cells. Results showed that the transfection of MED27 siRNA significantly ele-

vated caspase3 activity (P< 0.05 compared to control group, **Figure 5**). These results indicated that the decrease of MED27 expression in U87 cells could facilitate caspase3 activity, further facilitating the apoptosis of neuroglioma cells.

MED27 level in U87 cells and NF-κB expression

Western blotting was further replenished to detect the effect of MED27 on NF-κB in U87 cells. As shown in **Figure 6**, the transfection of MED27 siRNA in U87 cells significantly depressed NF-κB expression (P<0.05 compared to control group).

MED27 regulation on iNOS level in U87 cells

We further utilized Western blotting to detect the effect of MED27 on iNOS level in U87 cells under the effect of MED27. Results showed that the transfection of MED27 siRNA depressed MED27 expression, significantly inhibited iNOS expression (P<0.05 compared to control group, **Figure 7**). These results clearly suggested that MED27 in U87 cell could affect neuroglioma cell proliferation and apoptosis via modulating NF-κB/iNOS signal pathway.

Discussion

Neuroglioma has high incidence and unfavorable prognosis among all malignant tumors in nervous system. Although multiple treatment approaches existed currently, the survival rate of patients has not been significantly improved, making it one major challenge in clinics [17, 18]. In mammalian cells, certain component protein in MED complex can activate their specific transcription factors via various signal transduction pathways for interaction, thus mediating downstream gene expression [19].
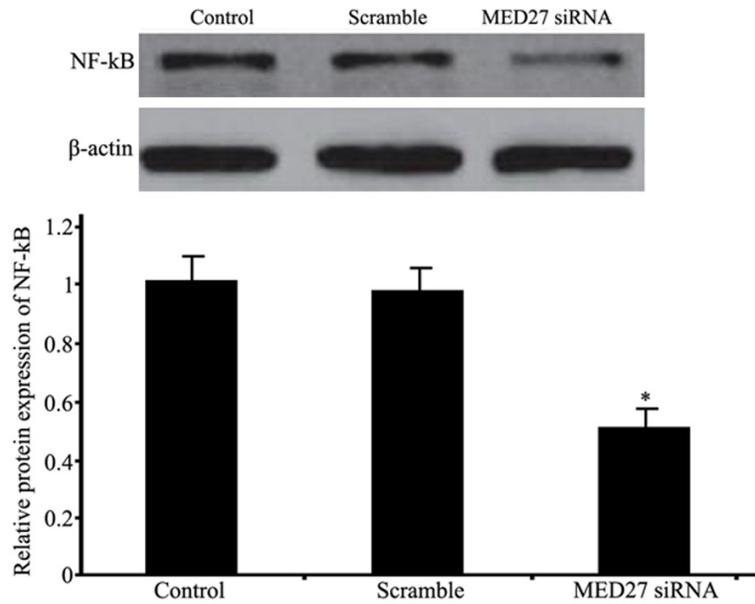
Figure 6. Analysis of the regulation of MED27 on NF-κB in U87 cells. *, P<0.05 compared to control group.
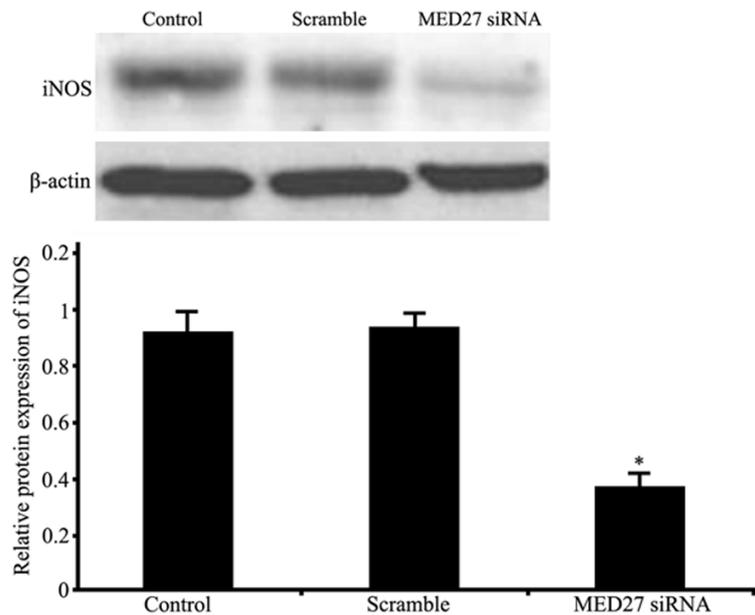

Figure 7. Analysis of MED27 on iNOS in U87 cells. *, P<0.05 compared to control group.

Moreover, MED complex can further regulate cell proliferation, differentiation and various pathological function via mediating signal pathways [20]. MED27 is one member of MED family that is widely distributed in multiple human organs/tissues. Previous study has found the abnormal expression of MED27 in melanoma tissues, and its participation in NF-κB and iNOS downstream signal pathways for regulatory effects [16]. The role of MED27 in glioma mediation, however, has not been reported. This study thus utilized siRNA approach to interfere with MED27 expression, and found down-regulation of mRNA and protein of MED27, accompanied with significant inhibition of glioma cell proliferation or invasion, plus up-regulated caspase3 activity. As one of the most potent member in apoptotic factor family, caspase3 can positively induce tumor cell apoptosis [21]. As on nuclear transcription factor, NF-κB plays a role in inflammatory or immune responses, as it can activate related genes including iNOS [22]. Some studies have indicated significant increase of iNOS expression in glioma cell, as it can facilitate tumor angiogenesis and resist glioma cell apoptosis, plus the facilitation of tumor cell proliferation [23]. This study confirmed suppressed expression of MED-27 mRNA and protein in glioma cells via interfering with MED27 gene expression, and further inhibition of cell proliferation and induction of apoptosis probably via down-regulating NF-κB/iNOS signal pathway.

In summary, inhibition of MED27 can facilitate glioma cell apoptosis and inhibit cell proliferation or invasion via down-regulating NF-κB/iNOS, indicating the potency of MED27 as novel molecular targets for clinical treatment against glioma. This study provides evidences illustrating pathogenesis of glioma.

Acknowledgements

Disclosure of conflict of interest

None.

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