Research Article

Wenjun Liu#, Min Du#, Hongping Wan, Hao Yang*, Xiaorong Deng*, Yu Chen, Qian Zhang

Serpin family A member 1 is an oncogene in glioma and its translation is enhanced by NAD(P)H quinone dehydrogenase 1 through RNA-binding activity

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Abstract: Serpin family A member 1 (SERPINA1) is expressed abundantly in gliomas and can predict unfavorable prognosis of patients with glioma. Studies have shown that nicotinamide adenine dinucleotide phosphate quinone dehydrogenase 1 (NQO1) can promote the proliferation of glioblastoma multiforme cells and enhance the expression of SERPINA1, but its effects on glioma cells remain unknown. In this study, we explored the functions of SERPINA1 in glioma tumorigenesis in vitro and then investigated whether NQO1 affects the protein expression of SERPINA1 and its mRNA level. The results showed that the translation of SERPINA1 was suppressed while its mRNA level had no significant changes under the condition of NQO1 silencing. Luciferase reporter assay and biotin pull-down assay further indicated that NQO1 bond with SERPINA1 3′ untranslated region. miR-1321 was also identified to target SERPINA1, repressing its mRNA and protein levels. SERPINA1 and NQO1 promoted glioma cell proliferation and suppressed cell apoptosis. Moreover, SERPINA1 rescued the effects of sh-NQO1 in glioma cell malignant phenotypes. In conclusion, our findings showed that oncogene NQO1 and antioncogene miR-1321 bind to oncogene SERPINA1 to affect proliferation and apoptosis of glioma cells, which can bring new solution of antitumor treatments for glioma in the future.

Keywords: glioma, SERPINA1, NQO1, RNA-binding protein, miR-1321

1 Introduction

Glioma, known as an aggressive malignant cancer in brain, causes nearly 80% of malignant brain cancers in the world with a high rate of relapse [1–3]. It is characterized by infiltrative growth of malignant glioma cells into the surrounding brain parenchyma, which makes it hard for operative treatment. Although treatment for neuronal oncology has been improved rapidly, the prognosis of glioma is unsatisfactory [4]. Novel treatment strategies for malignant gliomas should focus on improving tumor debulking and enhancing tumor cell killing [5]. Glioblastoma multiforme (GBM) is a highly immunosuppressive tumor, and immunotherapy can achieve long-lasting tumor remission by manipulating the immune system [6]. However, immunotherapy may cause over-inflammation, and its efficacy is not high [7]. Therefore, it is important to develop new strategies for the treatment of glioma.

Serpin family A member 1 (SERPINA1), identified in 1981 [8], serves as a serine protease inhibitor gene. It was reported that in patients with carcinomas, SERPINA1 enhances the invasive and metastatic abilities of carcinomas in gastric cancer, colorectal carcinoma, and lung cancer [9–13] and indicates poor prognosis of patients with colorectal cancer [14]. The expression of SERPINA1 was found in the spindle cells and pleomorphic cells from the sarcomatous area of GBM [15,16]. An immunostaining result demonstrates the expression of SERPINA1...
in gliomas [17]. In this study, we used short hairpin RNAs (shRNAs) to determine the effects of SERPINA1 on the proliferation and apoptosis of glioma cells.

Nicotinamide adenine dinucleotide phosphate quinone oxidoreductase (NQO1), one of the major cytosolic quinone reductases, induces a two-electron reduction of quinones substrate to hydroquinones. Previous studies showed that NQO1 increases glioma cell proliferation [18,19]. Meanwhile, new evidence suggested that the expression of SERPINA1 is upregulated by NQO1 through RNA-binding activity [20]. However, the property of the binding of NQO1 to SERPINA1 in glioma cells remains unknown.

We made a hypothesis that NQO1 binds to SERPINA1 to play an oncogenic role in glioma cells. A noncoding RNA miR-1321 targeting SERPINA1 was subsequently investigated. Influences of NQO1, SERPINA1, and miR-1321 on the proliferation and apoptosis of glioma cells were assessed.

2 Methods and materials

2.1 Bioinformatics analysis

Interactive Analysis of Gene Expression Profiles, abbreviated as GEPIA (http://geopia.cancer-pku.cn/), is a web server to analyze the expression of SERPINA1 in glioma and normal tissues from the TCGA and GTEx projects. Association of NQO1 and SERPINA1 with the prognosis of glioma patients as well as the expression correlation between NQO1 and SERPINA1 in glioma tissues were also obtained from GEPIA.

2.2 Cell culture

The normal human astrocyte (NHA) (Cloneticsss Astrocyte Cell Systems, Cambrex BioScience, Wokingham, UK) and four glioma cell lines (U251, T98G, LN-229, and A172) (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) were used. The GBM cells were first stored in 5% CO2 at 37°C in a humidified chamber and then cultured with 10% fetal bovine serum [21]. DMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM Glutamax (Invitrogen) was used to culture cells under standard cell culture conditions. Primary glioma cells were purchased from PriCells (HUM-TUM-0018, Wuhan, China) and cultured in special basal medium for primary glial cells (MED-0014, PriCells) supplemented with PBS and antibiotics.

2.3 Cell transfection

A SERPINA1 complementary DNA (cDNA) was cloned into the pcDNA3.1 vector (Genechem, Shanghai, China) to produce the SERPINA1 overexpressing vector. The shRNAs used for targeting SERPINA1 and NQO1 were synthesized by Genechem. MiR-1321 inhibitor for silencing miR-1321 expression and miR-1321 mimics for enhancing miR-1321 expression were synthesized by GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for the transfection of abovementioned oligonucleotides or vectors into T98G and A172 cells for 24 h.

2.4 Colony formation assay

Cells were plated in six-well plates (1,000 cells per well) for cell culture for 2 weeks. Next, 75% alcohol and 1% crystal violet were used to fix and stain the colonies.

2.5 Flow cytometry

The evaluation of apoptosis was taken using flow cytometry with Annexin V (PE)/7AAD (BD Biosciences, San Jose, CA, USA) double staining. Annexin V/7AAD solution was used to incubate cells for 15 min at room temperature in the dark after transfection. A FACSCanto™ system (BD Biosciences) was used to analyze the samples. Annexin V and 7AAD positive cells were late apoptotic cells. Annexin V positive and 7AAD-negative cells were early apoptotic cells. Apoptotic rate was calculated as the percentage of early apoptotic cells + percentage of late apoptotic cells.

2.6 RNA isolation and RT-qPCR analysis

TRIZol was used to isolate RNA from cell extracts according to manufacturer’s procedures. An iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad) was used for reverse transcription. iTaq™ Universal SYBR® Green Supermix (Bio-Rad) was used for real-time quantitative (q)PCR analysis. GAPDH was used as a reference gene. The primer used for amplification are listed as the following:

SERPINA1: F: 5’-ATCATAGGCACTGTCGG-3’; R: 5’-TCTTAAAGGCAATGGGAGG-3’.

NQO1: F: 5’-ACATCACAGTTAAGCTGAGG-3’; R: 5’-TCAGATGCGCTTTATGAC-3’.
2.7 Luciferase reporter assay

The psiCHECK2 Luciferase reporter vector (Promega, Madison, WI, USA) was used to load the 3’-UTR, coding regions, and 5’-UTR of SERPINA1. A172 and T98G cells were seeded into 24-well plates. Then, sh-DQO1 plasmid and luciferase reporter vectors were co-transfected into the cells for 24 h using the Effectene transfection reagent (Qiagen, Hilden, Germany). After that, a Dual-Luciferase assay system (Promega, Madison, WI, USA) was used to measure the Firefly luciferase (FL) activity and Renilla luciferase (RL) activity. The results were shown as the normalized value of FL to RL.

2.8 Immunofluorescence staining

Four percent paraformaldehyde was used to fix cells for 30 min. Five percent blocking solution was used for permeabilization. An anti-SERPINA1 antibody (ab207303, 1:1,000; Abcam, Cambridge, UK) was used to incubate cells overnight at 4°C followed by washing with phosphate-buffered saline. A goat anti-rabbit IgG Alexa Fluor® 647 antibody (ab150075, 1:1,000; Abcam) was used as second antibody and used to incubate the membrane for 2 h. 4’,6-Diamidino-2-phenylindole (DAPI) was used to counterstain cell nuclei. At last, a fluorescence microscope (IX71, Olympus Corporation, Tokyo, Japan) was used to capture images.

2.9 Western blot

RIPA buffer was used to prepare whole-cell lysates. Five micrograms of proteins were isolated with 4–15% Criterion™ TGX™ precast gels (Bio-Rad) by electrophoresis and transferred to nitrocellulose membrane using Trans-Blot Turbo Transfer System (Bio-Rad). After that, primary antibodies against NQO1 (ab80588, 1:10,000; Abcam), SERPINA1 (ab207303, 1:5,000; Abcam), and GAPDH (ab8245, 1:1,000, Abcam) were added for incubation overnight at 4°C. Next, appropriate HRP-labeled secondary antibody IgG (ab7090, 1:1,000; Abcam) was added. Enhanced chemiluminescence (Amersham) was used to detect immunocomplexes. At last, quantitative densitometry of the protein bands was performed with the ImageJ software (National Institutes of Health).

2.10 Biotin pull-down assay

Primers containing the T7 RNA polymerase promoter sequence were used as templates for PCR. In vitro transcription was performed with a MegaScript T7 kit (Ambion). Purification was taken using Nuc-Away Spin Columns (Applied Biosystems). Cytoplasmic lysates from A172 and T98G cells (150 μg lysate, 1 μg biotinylated RNA) were incubated with biotinylated transcripts for 30 min at room temperature. Streptavidin-coated magnetic Dynabeads (Dynal) were used to isolate complexes. NQO1 protein was detected by western blot. The primer used to prepare biotinylated SERPINA1 3’UTR is listed as follows: forward, 5’-AGTAATACGACTCATATAGGGCCCAGAACTGCCTGATCGT-3’; reverse, 5’-GCCATTCTGGTAGAGA CGG-3’.

2.11 Statistical analysis

Statistical differences were determined by unpaired Student’s t test between two groups or one-way analysis of variance (ANOVA) among three or more groups. GraphPad Prism 6.0 was used to generate p values (p < 0.05 indicates significance). Error bar represents standard error of the mean (SEM).

3 Results

3.1 SERPINA1 is Upregulated in Glioma

Using the GEPIA dataset, we obtained SERPINA1 expression in glioma tumors and normal tissues. The findings revealed that glioblastoma has higher SERPINA1 expression level than normal tissue (p < 0.05) (Figure 1a). The survival analysis suggested that glioma patients with high level of SERPINA1 expression had poor survival (Figure 1b and c). The expression of SERPINA1 at the messenger RNA (mRNA) and protein levels was also measured in NHA and four glioma cell lines, which showed that the expression of SERPINA1 was increased remarkably in glioma cells (Figure 1d and e). Higher expression
of SERPINA1 in T98G and A172 cells than NHA was confirmed by immunofluorescence staining, which also indicates the cytoplasmic location of SERPINA1 (Figure 1f). These results suggest that SERPINA1 might indicate poor prognosis in gliomas.

### 3.2 SERPINA1 affects the proliferation and apoptosis of glioma cells

The relative SERPINA1 expression was reduced with transfection of sh-SERPINA1#1/2/3 (Figure 2a) and increased significantly with transfection of pcDNA SERPINA1 (Figure 2b). Then, colony formation assay was taken to analyze the impact of SERPINA1 silence on the proliferation of glioma cells. The proliferation was significantly suppressed by sh-SERPINA1 and promoted by pcDNA SERPINA1 (Figure 2c and d). To explore whether sh-SERPINA1 inhibits cell proliferation by enhancing apoptosis, a flow cytometry assay (Annexin V/7AAD staining) was conducted. The results showed that in glioma cells, apoptosis rate was increased when SERPINA1 was silenced (Figure 2e and f). These findings showed that SERPINA1 promotes the proliferative ability of glioma cells and suppresses apoptosis.

### 3.3 NQO1 enhanced SERPINA1 mRNA translation through binding to the 3′UTR

The survival analysis showed that high level of NQO1 is associated with the low survival rates of glioma patients (Figure 3a). SERPINA1 expression had positive correlation with NQO1 expression in glioblastoma (Figure 3b). The relative mRNA level of NQO1 was suppressed in T98G and A172 cells after transfection with sh-NQO1 (Figure 3c). Silence of NQO1 reduced the protein expression of SERPINA1 without causing significant changes in its mRNA level (Figure 3d–e). To explore NQO1 binds to which region of SERPINA1, a luciferase reporter construct (psiCHECK2) bearing the RL coding region was used to load SERPINA1 3′UTR, coding region, and 5′UTR (Figure 3f). The silence of NQO1 obviously reduced the activity of psiCHECK2-SERPINA1-3′UTR and had no significant effects on plasmids containing SERPINA1 coding region and 5′UTR, indicating

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**Figure 1:** SERPINA1 is upregulated in glioma. (a) Differential expression of SERPINA1 in glioblastoma (box plot) (red: tumor tissue, gray: normal tissue). (b and c) Kaplan–Meier survival curves of disease-free survival and overall survival of patients who had gliomas with differential expression of SERPINA1. (d and e) qRT-PCR analysis of SERPINA1 mRNA expression and western blotting analysis of SERPINA1 protein in NHA, U251, T98G, LN-229, and A172 cell lines. One-way ANOVA was conducted. (f) Co-staining of SERPINA1 (red) with DAPI (blue) in NHA, T98G, and A172 cell lines. One-way ANOVA was conducted. Data are expressed as means ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001, n = 3).
that NQO1 directly binds with SERPINA1 3′UTR (Figure 3g). Binding assays using biotinylated transcripts indicated that in both A172 and T98G lysates, NQO1 has significant interaction with the 3′UTR of SERPINA1 mRNA (Figure 3h).

### 3.4 NQO1 boosts the proliferation of glioma cells by reducing apoptosis

The colony formation ability of glioma cells was suppressed by sh-NQO1 and enhanced by pcDNA-NQO1. pcDNA-SERPINA1 rescued the effects of sh-NQO1 on cell proliferation and sh-SERPINA1 had the same rescue function on pcDNA-NQO1 (Figure 4a). Furthermore, Annexin V/7AAD staining followed by flow cytometry analysis showed that pcDNA-SERPINA1 suppressed the apoptosis rate that is promoted by sh-NQO1, whereas sh-SERPINA1 restored the declined apoptosis rate that is induced by pcDNA-NQO1 (Figure 4b). These findings suggested that NQO1 facilitates the proliferation of glioma cells by suppressing the apoptosis, which depends on the translation of SERPINA1.

### 3.5 NQO1 suppresses SERPINA1 translation and SERPINA1 rescues effects of sh-NQO1 on proliferation and apoptosis of primary glioma cells

sh-NQO1 did not affect SERPINA1 mRNA (Figure 5a) but decreased its protein expression in primary glioma cells (Figure 5b). Silencing of NQO1 reduced the luciferase activity of psiCHECK2-SERPINA1-3′UTR in primary glioma cells (Figure 5c). Moreover, cell proliferation and apoptosis assays were conducted using primary glioma cells. The results revealed that sh-NQO1 reduced colony number and increased cell apoptosis rate, while pcDNA-SERPINA1 had inverse effects. pcDNA-SERPINA1 rescued the effects of sh-NQO1 in the primary glioma cell proliferation and apoptosis (Figure 5d–e).
3.6 miR-1321 is a negative regulator upstream SERPINA1

Using the starBase online database and under the condition of high stringency of CLIP Data and program number ≥3, miR-1321 was identified to potentially target SERPINA1 (Figure 6a). miR-1321 shows downregulated expression in glioma cells than control NHA cell line (Figure 6b). miR-1321 mimics decreased SERPINA1 protein expression, while miR-1321 inhibitor caused the upregulation of SERPINA1 protein expression (Figure 6c–d) and rescued the suppressive effect of sh-NQO1 in SERPINA1 protein expression (Figure 6e). miR-1321 inhibitor increased the expression of psiCHECK2-SERPINA1-3’UTR (Figure 6f) and rescued the inhibitory effect of sh-NQO1 on psiCHECK2-SERPINA1-3’UTR (Figure 6g). Moreover, miR-1321 inhibitor increased colony number of T98G and A172 cells and rescued the effects of sh-NQO1 in cell proliferation (Figure 6h).

4 Discussion

In the present work, we confirmed that NQO1 functions as an RNA-binding protein (RBP) that binds with SERPINA1 mRNA and enhances its translation, facilitating the proliferation of glioma cells by reducing the apoptosis. NQO1 binds with SERPINA1 mRNA and significantly increases its translation, thus boosting glioma tumorigenesis. Moreover, miR-1321 targets SERPINA1 mRNA and represses its translation. MiR-1321 exerts an opposite effect of SERPINA1. The mutual effect of miR-1321 and NQO1 on SERPINA1 will cast new direction for treatment of glioma in the future exploration.

Glioma has been known as a primary malignant carcinoma in central systems nervosum [22]. Many studies about malignant cancers focused only on gene coding proteins, and their interactions are in lack of exploration [23]. Alpha-1 antitrypsin, the protein encoded by SERPINA1, is synthesized mainly by pulmonary alveolar cells,
macrophages, and hepatocytes and modulates protease and corresponding inhibitors to defend glioma cells from host offense \[24\]. It also inhibits the cytotoxic reactions of lymphocytes \[25–28\]. The high expression of SERPINA1 may protect tumor cells from enzymes and immune system \[29\]. We found that high expression of SERPINA1 marks poor overall survival in glioma. The results of function assays showed that SERPINA1 increased glioma cell proliferation by suppressing apoptosis.

NQO1 binds with many mRNAs including SERPINA1 \[30,31\]. We used shRNAs against NQO1 to analyze the effect of NQO1 silence on SERPINA1, which showed the same results as previous work that it downregulated the expression of SERPINA1 at the protein level but not the mRNA level \[30,31\]. Through the luciferase reporter assay, our findings suggested that NQO1 binds to the 3′ UTR of SERPINA1 mRNA. Considering the pro-proliferative and anti-apoptotic effects of SERPINA1 in glioma, we had the hypothesis that by upregulating the expression of SERPINA1, NQO1 would enhance the proliferation of glioma cells and suppress apoptosis, and such hypothesis was confirmed by the rescue assays.

NQO1 may compete with the RBP suppressing SERPINA1 translation or cooperate with RBP promoting SERPINA1 translation. It is also possible that NQO1 competes with certain microRNA (miRNA) that suppresses SERPINA1 translation. In this study, we identified miR-1321 as a negative regulator upstream SERPINA1. MiR-1321 bond with SERPINA1 3′UTR and reduced its protein expression. miR-1321 inhibitor rescued the negative effect of sh-NQO1 on psiCHECK2-SERPINA1-3′UTR activity and SERPINA1 protein expression. Moreover, miR-1321 shows downregulation in glioma cells and its silencing reduced glioma cell proliferation, indicating the tumor suppresser role of miR-1321 in glioma.

Figure 4: NQO1 boosts the proliferation of glioma cells by reducing apoptosis. (a) Colony formation assay and quantitative analysis of T98G and A172 cells transfected with different plasmids (control, sh-NQO1, sh-NQO1 + pcDNA-SERPINA1, pcDNA-NQO1, pcDNA-NQO1 + sh-SERPINA1). One-way ANOVA was conducted. (b) Annexin V/7AAD double staining assay was used to show cell apoptosis rate in T98G and A172 cells. One-way ANOVA was conducted. Data are expressed as means ± SEM (***p < 0.001 vs control, ##p < 0.01, ###p < 0.001 vs sh-NQO1, ##p < 0.01, ###p < 0.001 vs pcDNA-NQO1, n = 3).
What are known until now based on abovementioned studies include: (1) SERPINA1 is expressed in human glioma tissues; (2) NQO1 binds with SERPINA1 3'UTR in human hepatoma HepG2; and (3) NQO1 increases the proliferation of glioma cell lines. The novel findings of this study are listed as follows: (1) demonstrating the pro-proliferative and anti-apoptotic roles of SERPINA1 in both glioma cell lines and primary glioma cells as well as the same function of NQO1 in primary glioma cells; (2) confirming the binding of NQO1 and SERPINA1 3' UTR in glioma cells on the basis of a previous study showing their binding in another cancer cell line; (3)

Figure 5: NQO1 binds to the 3'UTR of SERPINA1 mRNA and suppresses its translation, and SERPINA1 rescues effects of sh-NQO1 on proliferation and apoptosis of primary glioma cells. (a) Expression of SERPINA1 in sh-NQO1#1/2 transfected primary glioma cells was assessed by qRT-PCR. One-way ANOVA was conducted. (b) SERPINA1 protein levels in sh-NQO1#1/2 transfected primary glioma cells. One-way ANOVA was conducted. (c) The ratios of FL/RL activity in sh-NQO1 transfected primary glioma cells. Student's t test was conducted. (d) Colony formation assay results and quantitative analysis of primary glioma cells after transfection with sh-NQO1, pcDNA-SERPINA1, sh-NQO1 + pcDNA-SERPINA1. One-way ANOVA was conducted. (e) Annexin V/7AAD double staining assay results and quantitative analysis of primary glioma cells after indicated transfections. One-way ANOVA was conducted. Data are expressed as means ± SEM (**p < 0.01, ***p < 0.001 vs sh-NC or control, **** p < 0.001 vs sh-NQO1, n = 3).
revealing that miR-1321 binds to SERPINA1 3′UTR to suppress SERPINA1 translation, indicating that NQO1 may compete with miR-1321 to enhance SERPINA1 expression; and (4) showing the antiproliferative function of miR-1321 in glioma cells.

In conclusion, our work demonstrates that in glioma cells, NQO1 enhances the translation of SERPINA1 mRNA by binding with 3′UTR and thus promotes the function of SERPINA1 by suppressing apoptosis and enhancing the proliferation. Moreover, miR-1321 is a negative regulator of SERPINA1. NQO1 may compete with miR-1321 to bind with SERPINA1 3′UTR and thus enhances its translation. This study suggested that NQO1 may have similar effects on other target mRNAs and brings novel solution of anti-tumor treatments for glioma in the future work.

Abbreviations

**SERPINA1** serpin family A member 1  
**NQO1** nicotinamide adenine dinucleotide phosphate quinone oxidoreductase  
mRNA messenger RNA  
shRNA short hairpin RNA  
SEM standard error of the mean  
GBM glioblastoma multiforme  
cDNA complementary DNA  
DAPI 4',6-diamidino-2-phenylindole  
RBP RNA-binding protein  
RL Renilla luciferase  
FL Firefly luciferase

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**Conflict of interest:** The authors declare that there are no conflicts of interest in this study.

**Data availability statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

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