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Original Article

The NEK1 gene regulates the JAK-STAT signaling pathway and promotes the proliferation of glioma cells

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Abstract: Objective: To investigate the regulatory mechanisms of the NIMA-related kinase 1 (NEK1) gene on the JAK-STAT signaling pathway in glioma cells. Methods: Human glioma cell U251 was cultured and cell passage was carried out. Glioma cell line U251 was divided into five groups and transfected. The groups included the blank group, the NC group, the si-NEK1 group, the AG490 group (a JAK-STAT signaling pathway inhibitor), and the si-NEK1 + AG490 group. Quantitative real-time PCR (qRT PCR) and Western blot (WB) were used to determine the mRNA and protein expression levels of NEK1, the JAK-STAT signaling pathway, the proliferating cell nuclear antigen (PCNA), the N-myc downstream regulated gene 1 (NDRG1), and the apoptosis factors Bcl-2 and Bax. Cell proliferation, cell apoptosis, and cell invasion were evaluated using MTT assays, flow cytometry, and Transwell assays, respectively. A BALB/c nude mice tumorigenesis assay was conducted to evaluate the tumor volume growth curve and the survival rate of the mice with the transfected U251 cell line. Results: Compared with the blank group, in the si-NEK1, AG490, and si-NEK1 + AG490 groups we found significantly lower mRNA and protein expression levels of NEK1, JAK, STAT, PCNA, NDRG1, and Bcl-2, higher Bax mRNA and protein expression levels, decreased cell proliferation and invasion abilities, increased apoptosis abilities, a slower tumor growth rate, and an increased survival rate in the mice (all $P<0.05$). There were no significant differences between the blank and NC groups (all $P>0.05$). Compared with the si-NEK1 group, the NEK1 mRNA and protein expression levels were significantly higher in the AG490 group (both $P<0.05$), with no differences in the other indicators (all $P>0.05$). Similarly, in the si-NEK1 + AG490 group, we found lower mRNA and protein expression levels of JAK, STAT, PCNA, NDRG1, and Bcl-2, and higher mRNA and protein expressions of Bax, decreased cell proliferation and invasion abilities, an increased cell apoptosis ability, a slower tumor growth rate, and an increased survival rate (all $P<0.05$). Conclusion: Interfering with the NEK1 gene can inhibit the JAK-STAT signaling pathway, thereby inhibiting the proliferation of glioma cells and promoting apoptosis.

Keywords: NIMA-related kinase 1, JAK-STAT signaling pathway, glioma cells, proliferation, differentiation, apoptosis

Introduction

A gliomas is a type of tumor that grows in neuroepithelial sites and is one of the most common intracranial malignancies [1]. Gliomas are highly invasive, and delayed treatment often results in a transition from low to high grade, leading to a poor prognosis [2, 3]. Current clinical treatments for glioma include surgical resection, radiotherapy, chemotherapy, gene therapy, targeted therapy, and immunotherapy. These treatments play a positive role in delaying the recurrence of the disease and prolonging the survival time [4, 5]. However, because of their highly invasive growth, except for small, early onset tumors, the tumor tissues in most patients are difficult to effectively removed, resulting in high recurrence rates and poor prognoses [6]. Progress in molecular biology and histopathology have allowed for deeper research on the pathological features and the gene and signal pathways in gliomas, and the search for new and effective molecular therapeutic targets is the main focus [7].

The NIMA-related kinase 1 (NEK1) gene is an important member of the NEK protein kinase family. Previous studies have confirmed that the abnormal expression of the NEK gene family is highly correlated with the occurrence of many tumors [8]. NEK1 gene activity has been found to be significantly enhanced during cell

Table 1. Primer sequences

Name	Sequence (5'-3')
NEK1	F: GCTCCCTTCTTTTAAGGGTTCAA
	R: ACCAGTAACTGTGACTCCGGT
JAK2	F: TTCTACATGGGGGGATAG
	R: CATGGAGTTGACCTCGGAGTG
STAT3	F: CATGGAGTTGACCTCGGAGTG
	R: TGGCAGAATGCAGGTAGGC
PCNA	F: AGGTGTTGGAGGCACTCAAG
	R: AGGTATCCGCGTTATCTTCG
NDRG1	F: GCCTGTCATCCTCACCTACCA
	R: CAGGAAGCATTTCAGCCAGC
Bcl-2	F: GTGGAGGAGCTCTTCAGGGA
	R: AGGCACCCAGGGTGATGCAA
Bax	F: GCCCACCAGCTCTGAGCAGATCAT
	R: CGGCAAT-CATCCTCTGCAGC
GAPDH	F: GACTCATGACCACAGTCCATGC
	R: AGAGGCAGGGATGATGTTCTG

Note: NEK1: NIMA-related kinase 1; PCNA: proliferating cell nuclear antigen; NDRG1: N-myc downstream regulated gene 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

mitosis [9]. However, other studies have found that the overexpression of NEK1 is an important cause of abnormalities in chromosome segregation and cell apoptosis [10]. Because cell mitosis and apoptosis are important and influential factors in the pathophysiology of cancer, this study focused on the effect of the NEK1 gene on the biological characteristics of glioma cells. The JAK-STAT signaling pathway has been shown to be involved in brain development and has important effects on neural differentiation and regeneration [11, 12]. Tu et al. reported that JAK-STAT signaling intervenes in the proliferation and invasion of glioma cells [13]. However, it is not clear whether it can be regulated by the upstream factors.

The purpose of this study was to investigate the effects of the NEK1 gene and the JAK-STAT signaling pathway on the biological characteristics of glioma cells and their specific roles.

Materials and methods

Cell culture

Human glioma U251 cells (purchased from ATCC, USA; BH0836) were cultured in a DMEM medium containing 10% fetal bovine serum and incubated at 37°C in a 5% CO_2 incubator (Thermo Scientific 8000, USA). A cell passage culture was performed when the cell monolayer had grown and covered 90% of the culture flask.

Plasmid construction

The primer sequence of si-NEK1 (sense strand 5'-CCTATGATCTCCGCAGTTT-3', and antisense strand 5'-GGATACTAGAGGCGTCAAA-3', with BamH I and EcoR I restriction sites in both ends of the sequence) was synthesized by Invitrogen (USA) was used to anneal and prepare the DNA double strands. These were ligated into BamH I and EcoR I double-digested pRNA-u6.1/Neo vector (Invitrogen, USA). The vector was then transformed into DH5α competent cells. These were plated on a PLB culture plate of kanamycin. Then, single colonies were selected and plasmids were extracted for sequencing verification. After verification, the si-NEK1 vector was obtained.

Cell grouping and transfection

Third-generation passage cells were digested by trypsin and seeded on a 24-well plate. After the cell monolayer growth, the culture medium was discarded and the cells were divided into 5 groups: a blank group (U251 cells which were not transfected with any sequence), an NC group (U251 cells transfected with blank plasmids), an si-NEK1 group (transfected with si-NEK1 plasmids), an AG490 group (with a JAK-STAT signaling pathway inhibitor), and an si-NEK1 + AG490 group (transfected with si-NEK1 plasmid and the JAK-STAT signaling pathway inhibitor). Each group of cells was transfected when cell density reached 30%-50%, and the transfection process was performed according to the Lipofectamine 2000 instruction manual (Invitrogen, USA).

qRT-PCR

Total RNA was extracted from each group using an miRNeasy Mini Kit (217004, QIAGEN, Germany), with primers synthesized by Takara, Japan (**Table 1**). cDNA was synthesized by reverse transcription using the PrimeScript RT kit (Takara, Japan). The reverse transcription system had a volume of 10 μL. The reaction conditions were set to 37°C for 15 min, 3 times for the reverse transcription reaction, and 85°C for 5 s for the reverse transcriptase

inactivation reaction. The reaction solution was subjected to qRT-PCR, and the procedure was performed according to the instruction manual of the SYBR® Premix Ex Taq™ II Kit (TaKaRa, Japan). A 50 μL reaction system was selected: SYBR® Premix Ex Taq™ II (2×) 25 μL, PCR upstream primer 2 μL, PCR downstream primer 2 μL, ROX Reference Dye (50×) 1 μL, DNA template 4 μL, and ddH_2O 16 μL. Real-time fluorescence quantitative PCR was performed using an ABI 7500 quantitative PCR machine (7500, ABI, USA). The reaction conditions were set as follows: Pre-denatura-tion at 95°C for 30 s, denaturation at 95°C for 5 s, and annealing at 60°C for 30 s, for a total of 40 cycles. Then, 2 μg of total RNA were taken as a template and GAPDH as an internal reference.

Western blot

Cells in the logarithmic phase of growth were lysed on ice by adding 100 μL of lysate and 1 μL of enzyme inhibitor (Roche, Beijing Jiamey Nuno Biotechnology Co., Ltd., China) per 20 μL of packed cell volume for 30 min, and centrifuged at 12,000 r/min for 10 min at low temperature. Next, the supernatant protein was taken for protein quantification, with samples of 50 μg protein being taken, dissolved in a 2× SDS loading buffer and boiled at 100°C for 5 min. Each of the above samples was then subjected to gel electrophoresis on a mass concentration of 10% SDS-PAGE and transferred to a PVDF film. After blocking with a mass concentration of 5% skim milk for 1 h at room temperature, the PVDF film was rinsed with PBS for 2 min, and the film was added to the diluted murine clone antibodies for NEK1 (1:100, Shanghai Fanke Biotechnology Co., Ltd., China), proliferating cell nuclear antigen (PCNA) (1 μg/mL, Abcam, UK), JAK2 (1:500, Abcam, UK), STAT3 (1:5,000, Abcam, UK), Bcl-2 (1:500, Abcam, UK), and Bax (1 μg/mL, UK), respectively. After sitting overnight at 4°C, the film was washed three times with TBST for 5 min and incubated with a 1:100 dilution of HRP-labeled secondary goat anti-mouse IgG antibody (HA1003, Shanghai Yanhui Biotechnology Co., Ltd.) for 1 h. The film was then treated for reaction with an ECL solution (ECL 808-25, Biomiga, USA) for 1 min at room temperature. The liquid was absorbed, the preservation film was covered, and the x-ray film (Qianfeng Biotechnology Co., Ltd., China) was taken for observation. The ratio of the gray value of the target band to the internal reference band was used as the relative expression of protein, with GAPDH as the internal reference. The experiment in each group was repeated 3 times.

MTT assay for cell proliferation in each group

The cells in the flask were seeded into 96-well plates and cultured for 24, 48, and 72 h in a complete medium containing 1.0 mM ATP. The activity of the human U251 glioma cells was tested at each time point. In parallel, 10 μL MTT of working solution was added to each well and then cultured in an incubator for 4 h. In the end, 150 μL of dimethyl sulfoxide (DMSO) solution was added to each well, and the incubation was repeated. After the blue crystals were dissolved in the DMSO, the culture was stopped. The absorbance value at wavelength 490 nm was measured using a microplate reader and recorded.

Flow cytometry for determining the apoptosis rate of each group

After 48 h of continuous cell transfection, the cells of each group were collected, and the supernatant was discarded after the centrifugation. The apoptotic quantification was determined according to the instructions of the Annexin-V-FITC kit (Biovision, USA).

Transwell cell invasion assay

Matrigel (Cat. No.: 356234, BD, USA) was incubated overnight at 4°C and then diluted on ice using a 4°C pre-cooled serum-free RPMI-1640 medium to reach a final concentration of 1 mg/mL. Diluted Matrigel was added to the upper compartment of the Transwell chamber (8 μm diameter) at 80 μL per well. The Matrigel was evenly distributed and then incubated at 37°C for 4 h. Then, the digested cells, after 48 h of transfection, were washed with PBS and a serum-free RPMI-1640 medium. The cells were resuspended and counted in the serum-free RPMI-1640 medium, and the concentration was adjusted to $1 * 10^6$/mL. Next, 700 μL of a RPMI-1640 medium containing 10% fetal bovine serum was added to the lower chamber of a 24-well plate, the cell suspension was added to the upper chamber, and the plate was cultured in an incubator for 24 h. The chamber was removed with forceps, the upper chamber liquid was dried, and the chamber was fixed at room temperature for 30 min with 4% parafor-

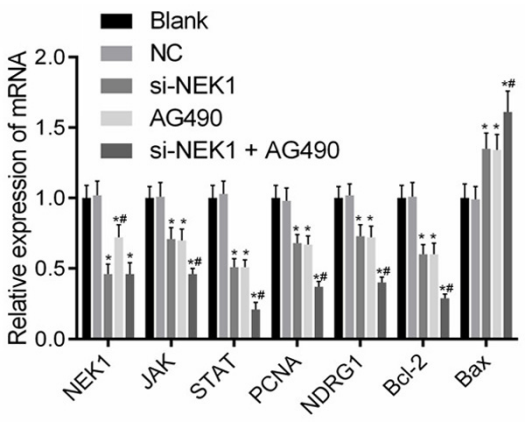

Figure 1. The mRNA expressions of the cell-related genes in each group. Compared with the blank group, *P<0.05; compared with the si-NEK1 group, #P<0.05. NEK1: NIMA-related kinase 1; PCNA: proliferating cell nuclear antigen; NDRG1: N-myc downstream regulated gene 1.

maldehyde, followed by staining with 0.05% crystal violet for 30 min. The chamber was rinsed and soaked with water for 5 times, and the upper chamber cells were wiped off with a wet cotton swab. After drying, an inverted microscope (XSP-8CA, Shanghai Optical Instrument Factory, China) was used to randomly select 10 fields of view for observation at 200× magnification.

BALB/c nude mice tumorigenesis experiment

A total of 50 5-week old male BALB/c nude mice of specific pathogen free (SPF) grade (Beijing Vital River Laboratory Animal Technology Co., Ltd., China) weighing 15-20 g were fed in the SPF grade barrier environment of the Experimental Animal Center of Huazhong Agricultural University. The nude mice were randomly divided into 5 groups: the blank group, the NC group, the si-NEK1 group, the AG490 group, and the si-NEK1 + AG490 group. Each group of U251 cells in a good growth state was digested with 0.25% trypsin and washed three times with PBS. Then, the cells were resuspended with PBS and gently blown into a single cell suspension. 100 μL of this suspension was inoculated subcutaneously in the nude mice's backs. Their body sizes and growth statuses were recorded every 6 days. After 30 days, the nude mice were sacrificed using cervical dislocation and the tumor tissue was removed for measurement. The tumor volume was calculated using the following formula: 0.5 * long diameter (mm) * short diameter^2 (mm^2). The survival rate of each group of nude mice was observed. The experimental protocol was approved by the Ethics Committee of The People's Hospital of China Three Gorges University.

Statistical analysis

All the data were processed using SPSS 21.0 statistical software. The measurement data were expressed as mean ± standard deviation. The t test for independent samples was used for the comparisons between two groups, and one-way analyses of variance (ANOVA) were used for the comparisons between multiple groups. The count data were expressed as percentages and analyzed using chi-squared tests. The results were considered significant when P<0.05.

Results

The expressions of NEK1, JAK, STAT, PCNA, NDRG1, Bcl-2, and Bax mRNA in each group

The results of qRT-PCR (**Figure 1**) showed that, compared with the blank group, the mRNA expression levels of NEK1, JAK, STAT, PCNA, N-myc downstream regulated gene 1 (NDRG1), and Bcl-2 were significantly lower in the si-NEK1, AG490 and si-NEK1 + AG490 groups. The Bax mRNA expression levels were significantly higher in these groups (all P<0.05). There were no significant differences between the blank group and the NC group (all P>0.05). Compared with the si-NEK1 group, the NEK1 mRNA expression level in the AG490 group was significantly higher (P< 0.05), but the other indicators were not significantly different (all P>0.05). Furthermore, compared with the si-NEK1 group, the mRNA expression levels of JAK, STAT, PCNA, NDRG1, and Bcl-2 were lower in the si-NEK1 + AG490 group (all P<0.05), but the Bax mRNA expression levels were significantly higher (P<0.05), with no differences in the expression of NEK1 mRNA (all P>0.05).

The expressions of the NEK1, JAK, STAT, PCNA, NDRG1, Bcl-2, and Bax proteins in each group

Western blotting showed that, compared with the blank group, the protein expression levels of NEK1, JAK, STAT, PCNA, NDRG1, and Bcl-

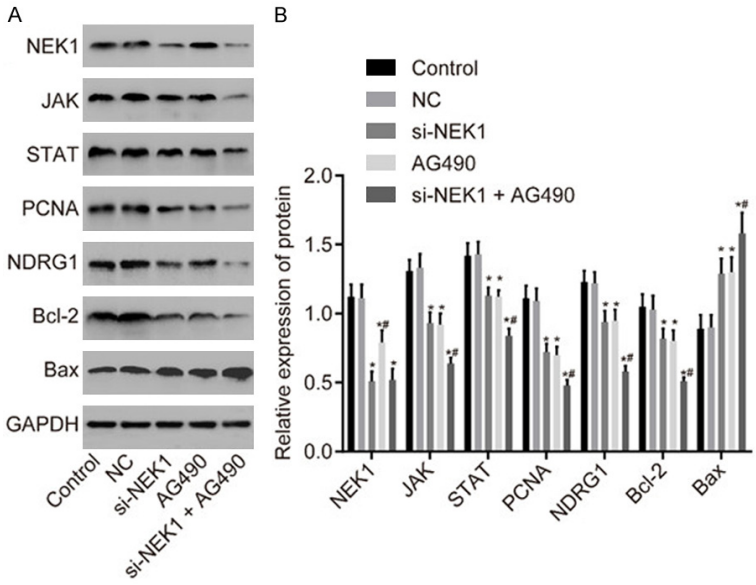

Figure 2. The protein expressions of the cell related genes in each group. A. A protein band diagram showing the related genes in each group of cells. B. A histogram showing the protein levels of the related genes in each group of cells. Compared with the blank group, *P<0.05; compared with the si-NEK1 group, #P<0.05. NEK1: NIMA-related kinase 1; PCNA: proliferating cell nuclear antigen; NDRG1: N-myc downstream regulated gene 1.

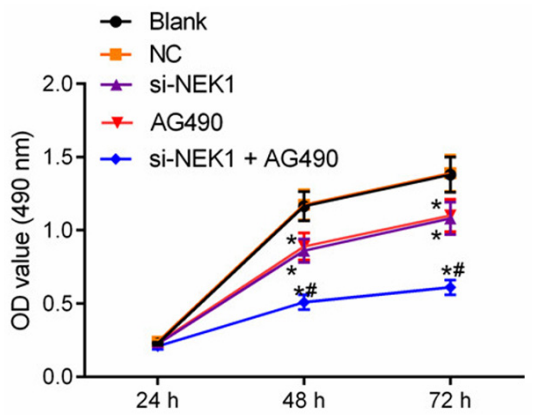

Figure 3. The cell proliferation in each group determined using MTT assays. Compared with the blank group, *P<0.05; compared with the si-NEK1 group, #P<0.05. NEK1: NIMA-related kinase 1.

2 in the si-NEK1, AG490 and si-NEK1 + AG490 groups were significantly down-regulated, but the Bax protein expression levels were significantly up-regulated (all P<0.05) (**Figure 2**). There were no significant differences between the blank group and the NC group (all P>0.05). Compared with the si-NEK1 group, the NEK1 protein expression levels in the AG490 group were significantly up-regulated (P<0.05), without any differences in the other indicators (all P>0.05). The protein expression levels of JAK, STAT, PCNA, NDRG1, and Bcl-2 were significantly lower in the si-NEK1 + AG490 group (all P<0.05), but the Bax expression was significantly up-regulated (P<0.05), without any significant differences in the expression of NEK1 (P>0.05).

MTT assay for the cell proliferation in each group

Figure 3 illustrates the MTT assay, showing that the cell proliferation ability was significantly lower in the si-NEK1, AG490, and si-NEK1 + AG490 groups in comparison with the blank group (all P<0.05). There was no significant difference between the blank and NC groups (P>0.05) or between the AG490 and si-NEK1 groups (P>0.05). But the cell proliferation ability in the si-NEK1 + AG490 group was significantly lower than it was in the si-NEK1 group (P<0.05).

Flow cytometry for the apoptosis rate of each group

Flow cytometry showed that, in comparison with the blank group, the apoptotic abilities of the si-NEK1, AG490 and si-NEK1 + AG490 groups were significantly higher (all P<0.05) (**Figure 4**). There was no significant difference between the blank and NC groups (P>0.05). Likewise, there was no significant difference in the apoptotic abilities between the AG490 and si-NEK1 groups (P>0.05). However, the apoptotic ability in the si-NEK1 + AG490 group was significantly higher than it was in the si-NEK1 group (P<0.05).

Transwell cell invasion assay

The Transwell cell invasion assay (**Figure 5**) showed that, compared with the blank group, the invasive abilities of the si-NEK1, AG490, and si-NEK1 + AG490 groups were significantly lower (all P<0.05). There was no significant difference in the cell invasion abilities between the blank and NC groups or between the

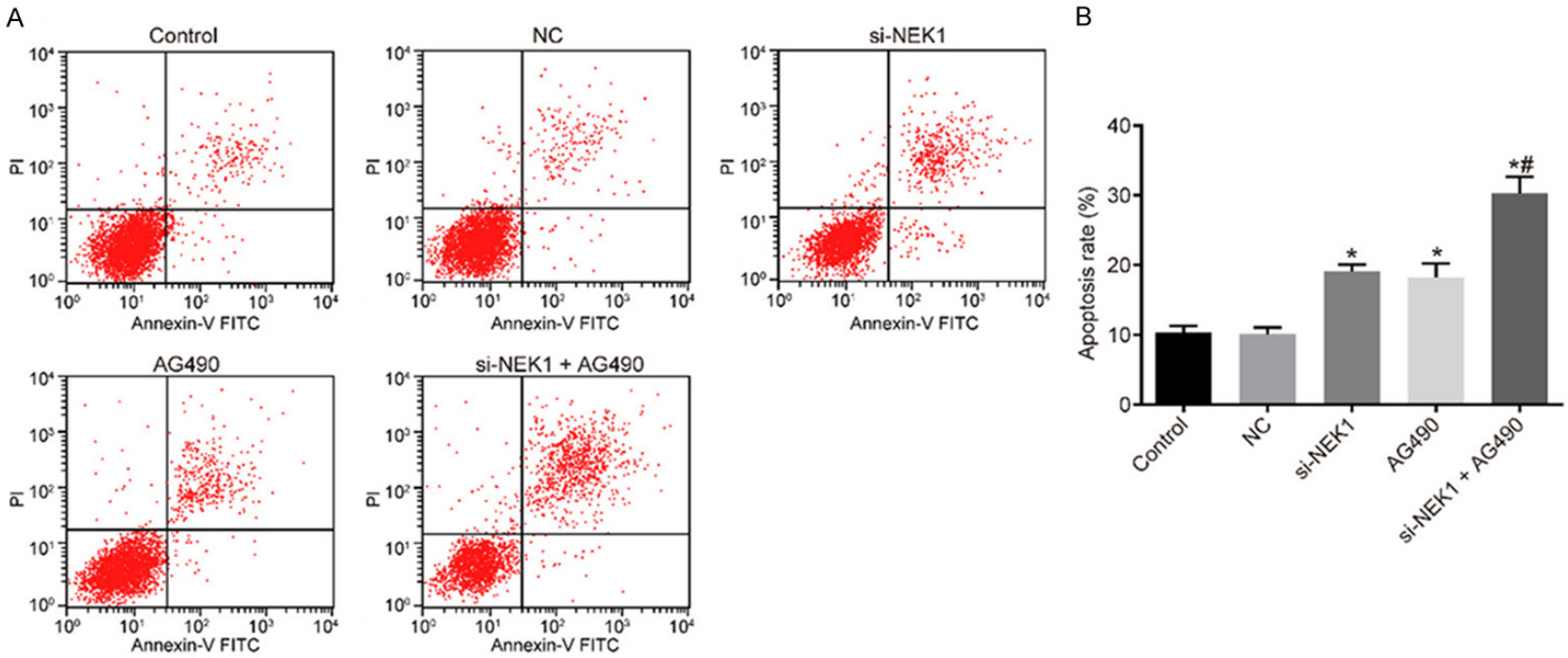

Figure 4. The cell apoptosis in each group determined using flow cytometry. A. The apoptosis flow chart for each group. B. A histogram showing the apoptosis rate in each group. Compared with the blank group, *P<0.05; compared with the si-NEK1 group, #P<0.05. NEK1: NIMA-related kinase 1.

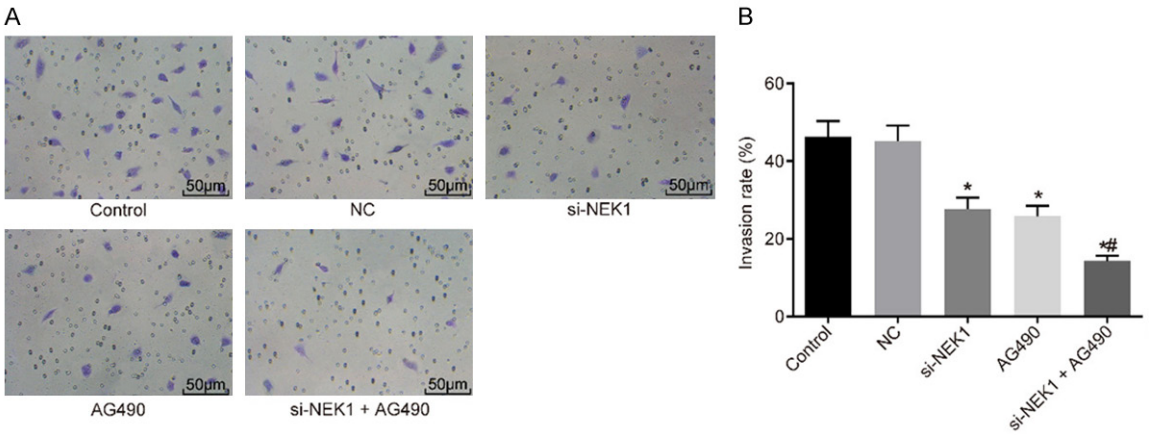

Figure 5. The cell invasion ability in each group determined using Transwell cell invasion assays. A. The cell invasion map (200×). B. The rate of the invading cells. Compared with the blank group, *P<0.05; compared with the si-NEK1 group, #P<0.05. NEK1: NIMA-related kinase 1.

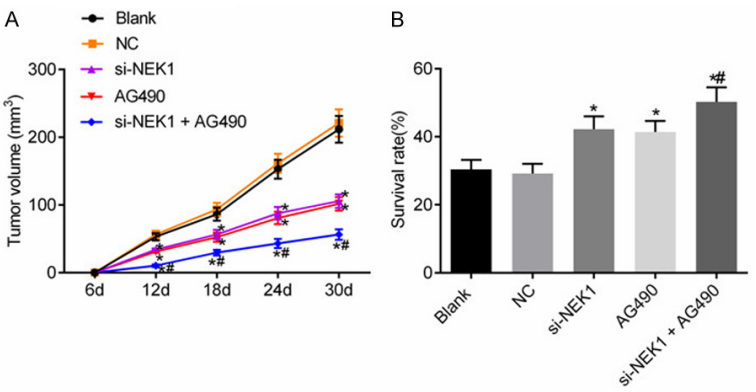

Figure 6. The tumor volumes and survival rates in each group of mice. A. The change in the transplanted tumor volume in each group of mice. B. The survival rate in each group of mice. Compared with the blank group, *P<0.05; compared with the si-NEK1 group, #P<0.05. NEK1: NIMA-related kinas 1.

AG490 and si-NEK1 groups (both P>0.05). However, the cell invasion ability of the si-NEK1 + AG490 group was significantly lower than it was in the si-NEK1 group (P<0.05).

BALB/c nude mice tumorigenesis experiment

The tumorigenesis experiment in the BALB/c nude mice (**Figure 6**) found a slower growth rate of tumors in the si-NEK1, AG490 and si-NEK1 + AG490 groups than in the blank group, along with an increased survival rate (all P<0.05). No significant differences were found between the blank and NC groups (P>0.05) or between the AG490 and si-NEK1 groups regarding tumor growth or survival rates (both P>0.05). The tumor growth rate of the si-NEK1 + AG490 group was slower than it was in the si-NEK1 group, and the survival rate of the mice was higher than it was in the si-NEK1 group (both P<0.05).

Discussion

Glioma is a common malignant brain tumor. Due to its highly invasive growth pattern, surgical resection is not effective, and its prognosis is often poor [14]. Much research has confirmed that the progression of glioma is closely related to variations in many genes and signaling pathways. Therefore, these pathogenic aspects should be key focuses of study in order to improve the treatment strategies [15].

The NEK gene family has been shown to play an important role in cell cycle regulation [16]. In recent years, studies have confirmed that the NEK gene family is also involved in the regulation of tumor progression and cell apoptosis [17]. In particular, NEK2 has been observed to be up-regulated in liver cancer [18]. NEK8 also appears to be up-regulated in breast cancer cells [19]. Mutations of the NEK1 gene have been shown to be related to chromosomal and mitotic abnormalities and the induction of apoptosis [20]. Zhu et al. reported that

the expression of NEK1 is significantly upregulated in human glioma, and its expression level is related to tumor grades and patient prognosis. Therefore, NEK1 may be a key target for the treatment of glioma and the assessment of patient prognosis in these cases [21]. Based on these findings, we further explored the specific molecular mechanisms related to NEK1 on glioma cells. We found the regulation of glioma progression by NEK1 is mainly mediated through the regulation of the downstream JAK/STAT signaling pathway.

PCNA, a regulator of the cell cycle as well as DNA replication and methylation, is mainly found in proliferative cells and can combined with many replication-associated proteins [22]. The expression of PCNA has been previously linked to the differentiation of hepatoma carcinoma cells (HCC) [23]. Moreover, PCNA appears to reflect the proliferation and malignancy of human glioma cells, and an increased expression of PCNA is associated with decreased tumor differentiation and increased malignancy [24]. The NDRG1 gene is in the human chromosome 8q24.3, and is expressed when N-myc levels are decreased and cells are differentiated [25]. Studies have shown NDRG1 expression is low in normal tissues, yet it is significantly increased in many tumor tissues including melanoma, breast cancer, and liver cancer [26]. In this study, the PCNA and NDRG1 expressions were significantly reduced after we silenced the NEK1 gene or inhibited the JAK/STAT signaling pathway, further confirming that silencing NEK1 or inhibiting JAK/STAT activity reduces the proliferation and differentiation of glioma cells. Bcl-2 and Bax play important roles in tumor progression by inhibiting or promoting apoptosis, respectively [27]. The Bax/bcl-2 ratio has been closely related to cell survival under apoptotic signaling [28]. In this study, the expressions of the pro-apoptotic proteins in tumor cells in the si-NEK1, AG490 and si-NEK1 + AG490 groups were significantly enhanced. MTT, flow cytometry and Transwell experiments further confirmed NEK1 silencing can significantly inhibit the activa-tion of the JAK/STAT pathway, thereby inhibiting the proliferation and differentiation of glioma cells and promoting the apoptosis of glioma cells. In the animal experiment, the tumorigenesis rates of the si-NEK1 and AG-490 groups were lower, but the combination group had better performance and an increased survival rate in comparison with the other groups, further highlighting the protective effect of NEK1 silencing and its impact on the JAK/STAT pathway in glioma cells.

In this study, we confirmed that NEK1 regulates proliferation, differentiation and apoptosis in glioma cells by modulating the JAK/STAT signaling pathway. Thus, the NEK1 gene may be an important target for the development of new glioma treatments. Further studies using larger patient cohorts are required to corroborate the clinical applicability of these results.

Disclosure of conflict of interest

None.

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