

## Original Article

# Regulatory effect of Nemo-like kinase on nasopharyngeal carcinoma

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**Abstract:** Nasopharyngeal carcinoma (NPC) is the leading malignant tumor in ear-nose-throat. It has high incidence in China with regional distribution. Multiple factors underlie the pathogenesis of NPS. Nemo-like kinase (NLK) is one novel tumor marker identified recently and exerts pro- or anti-tumor functions. Previous study has indicated up-regulation of NLK in NPC, but with unclear mechanism so far. Cultured CNE2 cells were transfected with small interference RNA (siRNA) of NLK. Real-time PCR and Western blotting described the expressional profile of NLK in cells. The proliferation was quantified by MTT assay, while the invasion ability was determined by Transwell chamber invasion assay. Further studies tested caspase-3 activity and expression of Bcl-2 and Bax proteins in CNE2 cells. SiRNA transfection significantly depressed mRNA and protein levels of NLK compared to scramble RNA group ( $P<0.05$ ). Such down-regulation of NLK inhibited proliferation of CNE2 cells and depressed tumor invasion. In those NLK-knock down cells, caspase-3 activity was potentiated, along with lower Bcl-2 protein and higher Bax protein levels ( $P<0.05$ ). Down-regulating NLK in NPC cells can facilitate tumor cell apoptosis, and inhibit the survival and progression of tumors.

**Keywords:** Nasopharyngeal carcinoma, nemo-like kinase, cell proliferation, tumor invasion, cell apoptosis

### Introduction

Nasopharyngeal carcinoma (NPC) is the leading malignant tumor in ear-nose-throat [1]. Most of NPC patients are distributed in Southern China, with race and familial traits, and regional distribution patterns [2, 3]. Due to the complex structure of nasopharyngeal tissues and deep location of tumor region, treatment of NPC is relatively difficult. The lack of individualized treatment and effective targeting drugs limits the overall survival rate of patients. The sensitivity to chemo- or radio-therapy is variable due to different clinical features and body immune functions, causing differential treatment efficacy. In general, those NPC patients at advanced stage with local metastasis had unfavorable prognosis [4, 5]. The pathogenesis of NPC is complicated as it is one poly-genetic disease and is accompanied with EB viral infection, diet habit and environmental factors [6, 7]. The precise pathogenesis mechanism, however, still remains unclear.

NPC has an insidious onset and slow progress at early stage. The special onset location often leads to misdiagnosis or rejection of biopsy/

early treatment. Therefore the screening of specific molecular marker for early diagnosis and treatment is of critical importance [8]. Nemo-like kinase is firstly identified in 5'-noncoding region of neurofibroma type 1 (NF1) gene, and was so named due to its sequence homology with Nemo gene [9, 10]. NLK has certain homology with mitogen activating protein kinases (MAPKs), and is one novel biomarker for tumors [11, 12]. Studies have revealed the dual role of NLK in tumor progression as its tumor-inhibitory function for ovary cancer, prostate cancer and colorectal carcinoma but tumor-facilitating role in liver cancer and oral squamous adenoma [13, 14]. This study thus investigated the expression and regulatory function of NLK in NPC cells, in order to illustrate the significance of NLK in NPC pathogenesis, and to provide evidences for revealing the pathogenesis of NPC.

### Materials and methods

#### Reagents

NPC cell line CNE2 was purchased from ATCC cell bank (US). DMEM medium, fetal bovine

**Table 1.** Primer sequence

| Gene  | Forward primer (5'-3') | Reverse primer (5'-3') |
|-------|------------------------|------------------------|
| GADPH | ACCAGGTATCTGCTGGTTG    | TAACCATGATGTCAGCGTGGT  |
| NLK   | GACTCTCTCCACAGT        | GCCTGGGCATTAGCTATT     |

serum (FBS) and EDTA were purchased from Hyclone (US). MTT and DMSO were produced by Gibco (US). Trypsin-EDTA lysis buffer was a product of Sigma (US). PVDF membrane and caspase-3 activity kit were purchased from Pall Life Sciences (US). Other reagents used in Western blotting were purchased from Beyotime (China) while ECL kit was a product of Amersham Biosciences (US). Rabbit anti-human NLK monoclonal antibody, anti-Bax monoclonal antibody, anti-Bcl-2 monoclonal antibody and mouse anti-rabbit horseradish peroxidase (HRP) labelled IgG secondary antibody were purchased from Cell signaling (US). Transwell chamber was provided by Corning (US). RNA extraction kit and reverse transcription kit were purchased from Axygen (US).

#### CNE2 cell culture

CNE2 cells were resuscitated in 37°C until complete thawing, and were centrifuged at 1 000 g for 3 min. Cells were then re-suspended in 1 mL fresh medium and were removed into 50-mL culture flask. Cells were cultured in a humidified chamber at 37°C with 5% CO<sub>2</sub> for 48 hours. Cells were then seeded in 6-well plate at 1×10<sup>5</sup> per cm<sup>2</sup> density. The culture medium consists of 90% high glucose DMEM (containing 100 U/mL penicillin and 100 µg/mL streptomycin) and 10% FBS. Cells at log-phase after 3~8 generations of passages were randomly divided into control, scramble siRNA, and NLK siRNA groups.

#### Cell transfection

NLK siRNA (5'-GATAG ACCTA ATGGA TAG-3') and scramble siRNA (5'-ACCTA CTTCC GTAAT GT-3') were mixed with 0.2 mL serum-free medium. After 15 min room-temperature incubation, Lipo2000 reagent was mixed with siRNA dilutions for 30-min incubation. Cells at 70%~80% confluences were mixed with transfection mixture. After removing culture medium cells were rinsed in PBS and were mixed with 1.6 mL serum-free medium. Cells were incubated in a humidified chamber at 37°C with 5% CO<sub>2</sub> for 6 hours. After changing for serum-con-

taining medium, cells were continuously cultured for 48 hours.

#### Real-time PCR

Trizol reagent was used to extract total RNA from all cells. Reverse-transcription was performed to synthesize DNA. Real-time PCR was performed to detect NLK expression level using specific primers that were designed by Primer 6.0 software (Table 1). Amplification conditions were: 92°C 30 sec, 58°C 45 sec and 72°C 35 sec, repeating for 35 cycles. Using GAPDH as the internal reference, CT values of all standards and samples were calculated. Semi-quantitative analysis was performed by 2<sup>-ΔCt</sup> method.

#### MTT assay

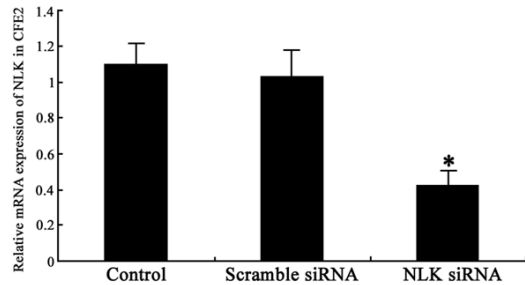
CNE2 cells at log-phase were seeded into 96-well plate at 3000 cells per well and were randomly divided into scramble RNA, NLK siRNA and control group (N=5 each). After incubation for 48 hours, 20 µL MTT solution (5 g/L) was then added for further 4-hour incubation. Supernatants were removed, followed by the addition of 0.15 mL DMSO into each well for vortex until complete resolving of violet crystals. Absorbance (A) values at 570 nm was measured by a microplate reader to calculate cell proliferation rate. All experiments were performed in triplicates.

#### Caspase-3 activity assay

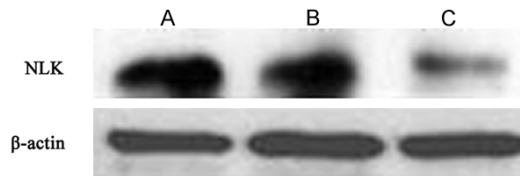
Using test kits, intracellular activity of caspase-3 was determined following manual instruction. In brief, cells were digested in trypsin, and were centrifuged at 600 g for 5 min. The supernatant was discarded followed by cell lysis buffer on ice for 15 min. The mixture was centrifuged at 20000 g for 5 min. After adding 2 mM Ac-DECD-pNA, OD values at 405 nm was measured to reflect caspase-3 activity.

#### Western blotting

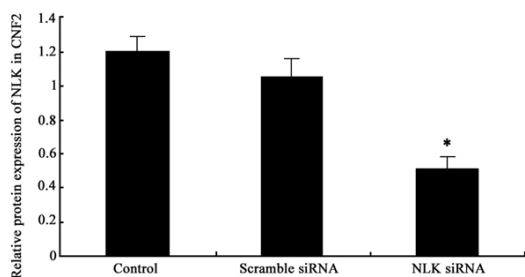
Cells from all groups were lysed in lysis buffer on ice for 30 min. Cells were ruptured under ultrasonic processing for 5 sec. After homogenization, the lysate was centrifuged at 10000 g for 15 min. The supernatant was saved. Protein samples were separated by 10% SDS-PAGE, and transferred to PVDF membrane using semi-dry method. After blocking in 5% defatted milk



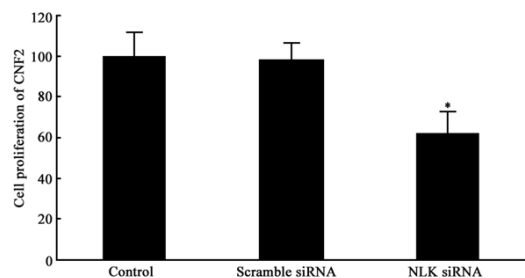
**Figure 1.** mRNA level of NLK gene in CNE2 cells. \*,  $P < 0.05$  compared to control group.



**Figure 2.** Western blotting bands of NLK. A: Control; B: Scramble siRNA; C: NLK siRNA.



**Figure 3.** Protein level of NLK gene in CNE2 cells. \*,  $P < 0.05$  compared to control group.



**Figure 4.** CNE2 cell proliferations. \*,  $P < 0.05$  compared to control group.

powder for 2 hours, primary antibody against NLK, Bcl-2, Bax or beta-actin was used for overnight incubation. On the next day, the membrane was rinsed in PBST, followed by the addition of secondary antibody and further 30-min incubation. ECL reagents were used to develop the membrane, which was then exposed under

X-ray. Quantity One software determined the optical density of each protein band.

## Transwell invasion assay

Transwell chamber was firstly pre-coated with Matrigel (1:5, 50 mg/L) on its membrane. The chamber was dried in a 4°C. 0.5 mL DMEM containing 10% FBS was added inside the chamber. 0.1 mL cell suspensions with serum-free medium were filled in the chamber. The whole chamber was placed into 24-well plate in triplicates, with parallel control group using Transwell chamber without Matrigel-precoating. After 48-hour incubation, the membrane was cleaned to remove remaining cells. The chamber was fixed in cold ethanol and stained in hematoxylin. Under an inverted microscope, the number of perforated cells in each membrane was calculated.

## Statistical analysis

SPSS 19.0 software was used to process all collected data. Measurement data were presented as mean  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) was employed to compare multiple groups. A statistical significance was defined when  $P < 0.05$ .

## Results

### NLK mRNA expression

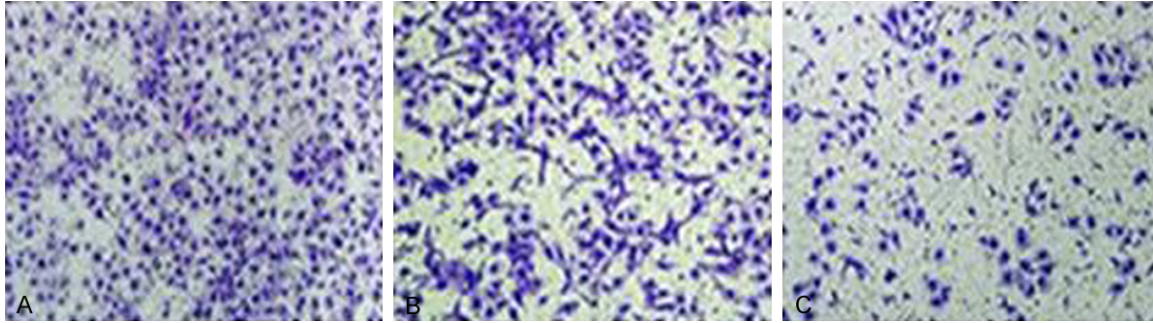
Using real-time PCR to detect the expression of NLK mRNA in CNE2 cells, results showed decreased NLK expression after siRNA transfection as compared to control group ( $P < 0.05$ , **Figure 1**).

### NLK protein expression

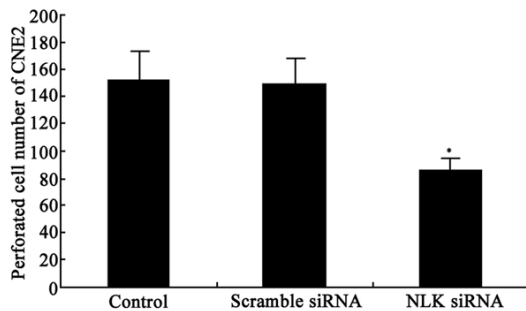
Western blotting was used to detect NLK protein level. Results are consistent with those from RT-PCR. After siRNA transfection, NLK protein expression was also decreased compared to control group ( $P < 0.05$ , **Figures 2, 3**). These results suggested the successful knock-down of NLK expression by RNA interference.

### NLK knock-down and CNE2 cell proliferation

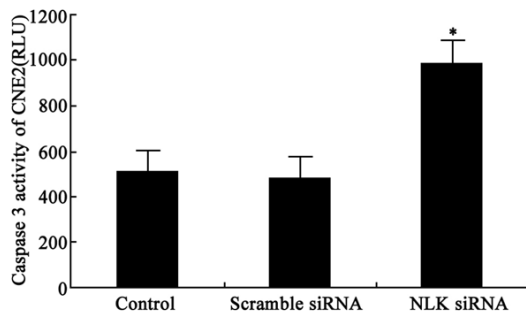
MTT assay showed that, after siRNA interference of NLK expression, tumor cell's proliferation was significantly inhibited ( $P < 0.05$ , **Figure 4**), suggesting the role of NLK in tumor cell proliferation.



**Figure 5.** NLK and invasiveness of NPC cells. A: Control; B: Scramble siRNA; C: NLK siRNA.



**Figure 6.** Effect of NLK down-regulation on cell invasion. \*,  $P < 0.05$  compared to control group.



**Figure 7.** Caspase-3 activity and NLK expression. \*,  $P < 0.05$  compared to control group.

#### NLK down-regulation and tumor invasion

Transwell chamber assay showed, after siRNA transfection, the invasion ability of tumor cells was significantly suppressed compared to control group ( $P < 0.05$ , **Figures 5, 6**). These results further suggested the alternation of tumor cell invasion by NLK expression.

#### Caspase-3 activity

The transfection of NLK-siRNA significantly increased intracellular activity of caspase-3 in CNE2 cells as compared to control group

( $P < 0.05$ , **Figure 7**), suggesting the role of NLK in modulating cell apoptosis in NPC tissues.

#### Bcl-2 and Bax protein expression

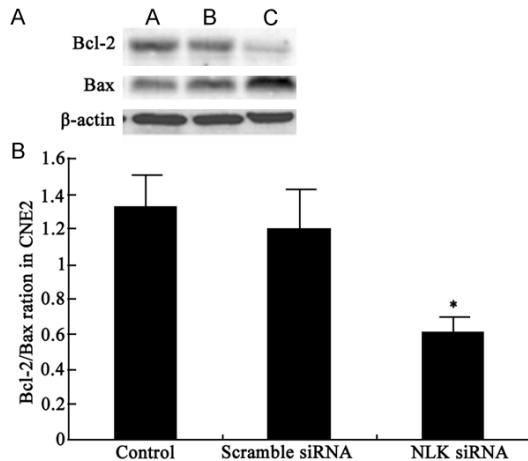
We further employed Western blotting to describe the effect of NLK on apoptotic proteins including Bcl-2 and Bax in CNE2 cells. We found the knock-down of NLK significantly decreased Bcl-2 protein expression while increased Bax level, causing elevated Bcl-2/Bax ratio ( $P < 0.05$ , **Figure 8**). Such results indicated the modulation of NLK on apoptotic/anti-apoptotic homeostasis, for exerting functions on both proliferation and invasion of NPC cells.

#### Discussion

Currently most NPC patients were already at terminal stage at the time of first diagnosis. The late diagnosis of NPC is accompanied with rapid progression of tumors [15]. Due to the special anatomical position and biological features, most NPC tumors are unsuitable for surgical resection. The undifferentiated nature of tumors makes them to be insensitive for chemotherapy. Therefore radio-therapy is often the first choice for NPC treatment. Common reasons impeding the treatment of NPC includes local recurrence and distal metastasis [16]. Therefore the establishment of effective molecular marker can assist evaluating the treatment efficacy and prognosis, achieving individualized treatment [17].

Belong to the serine/threonine kinase family; NLK can affect the function of target proteins via recognizing S/TP domain and phosphorylation [18]. NLK plays various roles within different signal pathways. The dual effect of NLK in multiple tumors has drawn lots of research interests [13, 14]. The elevated NLK expression





**Figure 8.** Apoptotic proteins and NLK expression. A: Representative Western blotting bands (A: Control; B: Scramble siRNA; C: NLK siRNA); B: Relative expression level of Bcl-2/Bax ratio. \*,  $P < 0.05$  compared to control group.

in NPC patients is accompanied with shortening of tumor-free survival period and is more susceptible to recurrence, suggesting the potency of NLK as one independent diagnostic index for NPC [19]. This study knocked down the expression of NLK in cultured CNE2 cells, whose proliferation and invasion abilities were tested. Our results showed the inhibition of cell proliferation and invasion by NLK down-regulation, thus demonstrating the facilitating role of NLK in NPC cells regarding tumor proliferation and invasion. Therefore NLK may work as one drug target for inhibiting tumor progression.

Cell apoptosis is one critical step for body homeostasis. It can also retard the occurrence of tumor via inhibiting over-growth. This study showed the elevated caspase-3 activity and Bax expression, and decreased Bcl-2 protein expression in NLK knock-down cells. As one anti-apoptotic gene, Bcl-2 can elongate cell survival via inhibiting apoptosis. In most of NPC tumor cells and atypical hyperplasia tissues with EBV infection, Bcl-2 expression was potentiated, especially at the early stage. Moreover, differential Bcl-2 expression may suggest the sensitivity of NPC patients during radio-therapy. Therefore Bcl-2 might play critical roles in the occurrence and progression of NPC. Apoptotic protein Bax, on the other hand, may initiated death signal for facilitating cell apoptosis, in addition to inhibit or antagonize Bcl-2 proteins [20, 21]. Caspase-3 has also been

shown to be related with cell apoptosis in NPC [22]. Our results thus illustrated the role of NLK in modulating apoptosis-anti-apoptosis homeostasis in NPC pathogenesis, for inducing tumor cell apoptosis.

In summary, as one novel molecular marker for NPC, NLK may benefit the early-diagnosis, individualized treatment and prognostic prediction of NPC patients in clinics. The down-regulation of NLK in NPC cells may facilitate cell apoptosis and thus inhibit tumor progression. The development of drugs targeting NLK thus may provide new insights regarding next generation treatment of NPC.

#### Disclosure of conflict of interest

None.

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