European Review for Medical and Pharmacological Sciences 2019; 23: 6226-6233

Effect of NLK on the proliferation and invasion of laryngeal carcinoma cells by regulating CDCP1

N. SHEN[1], X.-H. DUAN[2], X.-L. WANG[2], Q.-Y. YANG[2], Y. FENG[1], J.-X. ZHANG[1]

[1]Department of Otorinolaringoiatra Head and Neck, The 4th Central Hospital of Tianjin, Tianjin, China
[2]Department of Otorhinolaryngology Head and Neck, Affiliated Hospital of Hebei Engineering University, Handan, Hebei, China

Na Shen and Xiaohui Duan contributed equally to this article

Abstract. – OBJECTIVE: The morbidity and mortality of laryngeal cancer are increasing rapidly, seriously threatening human health. There are several causes of laryngeal cancer, but the exact molecular mechanism is unclear. Finding the molecular targets of laryngeal cancer has become an emerging hot spot. Nemo-like kinase (NLK) is abnormally expressed in tumors, but its role in laryngeal cancer has not been reported.

PATIENTS AND METHODS: Real Time PCR and Western blot were used to detect NLK mRNA and protein expression in cancer tissues and adjacent tissues of laryngeal cancer patients. The laryngeal carcinoma cell line Hep-2 cells were cultured *in vitro* and randomly divided into three groups: control group, NC group, and NLK siRNA group followed by the analysis of cell proliferation by MTT assay, Caspase3 activity, and cell invasion by the transwell chamber. MMP-9 and CDCP1 expression was measured by Western blot.

RESULTS: NLK mRNA and protein expression was significantly increased in laryngeal carcinoma tissues compared with those in adjacent tissues ($p<0.05$). NLK siRNA transfection into Hep-2 cells significantly down-regulated NLK expression, inhibited Hep-2 cell proliferation and invasion, increased Caspase-3 activity with statistical differences compared to control group ($p<0.05$). Down-regulation of NLK expression in Hep-2 cells inhibited MMP-9 expression and decreased CDCP1 expression.

CONCLUSIONS: NLK is expressed in tumor tissues of patients with laryngeal cancer. The down-regulation of NLK expression may play a role in the proliferation, apoptosis, and invasion of laryngeal carcinoma cells and it is possible by regulating MMP-9 and CDCP1 expression.

Key Words
NLK, Laryngeal cancer, CDCP1, Proliferation, Invasion, MMP-9.

Introduction

Throat squamous cell carcinoma (LSCC), a common malignant tumor in the otolaryngology department, is also known as laryngeal cancer. It is a highly dangerous malignant tumor with a high incidence and mortality rate, which represents a serious threat to human health[1,2]. The population of laryngeal cancer is mainly middle-aged and elderly, with male patients being predominant[3]. Due to changes in people's habits and lifestyle, several pathogenic factors of laryngeal cancer have been identified, such as genetic factors, eating habits, smoking, drinking, viral infections, etc. These cause an increase of laryngeal cancer patients year by year, accounting for about 5% of new tumors worldwide[3,4]. Laryngeal cancer is a multi-gene hereditary disease involving several factors, but its exact mechanism remains poorly understood[5]. The early symptoms of laryngeal cancer are not significant, and most of them are hoarseness, difficulty in breathing, cough, difficulty in swallowing, lymph node metastasis in the neck, etc. Symptoms are significantly different according to the different tumor sites, leading to poor prognosis of patients in the middle and late stages[6,7]. Although the current treatment for laryngeal cancer is mainly surgery in combination with adjuvant radiotherapy and chemotherapy, including immunotherapy, individualized treatment, etc., the current treatment efficacy has not been significantly improved, without improving the prognosis, leading to low survival rate and increased postoperative complications such as cough and loss of sound. In addition, due to the prone tumor metastasis, the patient's recurrence rate is high, seriously affecting the quality of life of patients, bringing greater pressure on the

Corresponding Author: Xiaohui Duan, MD; e-mail: zhangshuiliang7591@126.com

social economy[8,9]. Tumor invasion and metastasis are important causes of laryngeal cancer recurrence. Also, they are an important cause of increased mortality in patients with laryngeal cancer[10]. Therefore, finding and clarifying the molecular targets of the incidence and development of laryngeal cancer will be conducive to tumor treatment and inhibit the metastasis of laryngeal cancer.

Nemo-like kinase (NLK), a member of the silk/threonine protein kinase family, is highly conserved and can be widely expressed in many tissues and organs of the body, participating in embryonic development, bone formation and growth, as well as in cells apoptosis, proliferation, differentiation, and metabolism[11,12]. The abnormal expression of NLK is involved in the incidence and development of various diseases such as inflammation, tumor, and cardiovascular disorders[13,14]. NLK plays a biphasic role in tumors and in suppressing tumors mainly as a tumor suppressor in prostate cancer, ovarian cancer, breast cancer, and colorectal cancer. Whereas, it plays a role in promoting tumor progression in liver cancer, gallbladder cancer, and oral squamous cell carcinoma[15,16]. However, the role and related mechanisms of NLK in laryngeal cancer have not been fully elucidated.

Patients and Methods

Patients

Clinical data and tissue samples from 52 patients (average age: 51.8 ± 5.5 years old; ranging 39-63) with laryngeal squamous cell carcinoma confirmed by histopathological examination from January 2018 to December 2018 were selected. All patients were treated with surgery. Inclusion and exclusion criteria included[17]: inclusion criteria included the first-time diagnosis of laryngeal cancer patients, first surgery, no chemotherapy, radiotherapy, etc. before surgery; exclusion criteria included recurrent laryngeal cancer; previous surgical treatment; previous radiation or chemotherapy treatment; complicated with other diseases such as infectious diseases, malignant tumors, severe diabetes, and other organ-depleting diseases, systemic immune diseases, and malignant tumor complications. Tumor tissue and adjacent tumor tissues (≥3 cm around the tumor tissue) were collected during surgery and stored in liquid nitrogen. The research was approved by the Medical Ethics Committee of the hospital. Informed consent was obtained from all the selected subjects.

Reagents and Equipment

The laryngeal cancer cell line Hep-2 cell line (product number: HB-8065) was purchased from the American Type Culture Collection (ATCC) cell bank of the United States (Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) medium, fetal bovine serum (FBS), green and chain double antibody were purchased from Hyclone (San Angelo, TX, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, Thiazolyl Blue Tetrazolium Bromide (MTT) powder were purchased from Gibco (Great Island, NY, USA); trypsin-EDTA digest was purchased from Sigma-Aldrich (St. Louis, MO, USA). polyvinylidene difluoride (PVDF) membrane was purchased from Pall Life Sciences (Port Washington, NY, USA), Western blot related chemical reagent was purchased from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China), enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Pittsburgh, PA, USA), rabbit anti-human NLK monoclonal antibody, rabbit anti-human matrix metalloprotein (MMP) -9 monoclonal antibody, rabbit anti- Human CUB domain-containing protein-1 (CDCP1) monoclonal antibody, goat anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody was purchased from Cell Signaling Corporation (Danvers, MA, USA). The RNA extraction kit and the reverse transcription kit were purchased from Axygen (Union City, CA, USA). The Caspase-3 activity assay kit was purchased from R&D (Minneapolis, MN, USA). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation (Hercules, CA, USA). The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI (Waltham, MA, USA). The ultra-clean workbench was purchased from Suzhou Sutai Purification Equipment Engineering Co., Ltd (Suzhou, China). The transwell chamber was purchased from Corning (Corning, NY, USA). The DNA amplification instrument was purchased from the US PE Gene Amp PCR System 2400. Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd (Shanghai, China).

Hep-2 Cell Culture and Grouping

The liquid nitrogen-preserved Hep-2 cells were resuscitated and passaged, and then inoculated in a culture dish at a density of 1×10^7 cells/cm^2 in culture medium including 10% FBS, 90% high glucose DMEM medium (containing 100 U/

ml penicillin, 100 μg/ml streptomycin) at 37°C with 5% CO2. Cells were randomly divided into 3 groups, control group, normal control (NC) group (transfection of NLK negative control) and NLK siRNA group (transfection of NLK siRNA).

Transfection of NLK siRNA into Hep-2 Cells

NLK siRNA and siRNA negative controls were transfected into Hep-2 cells. The NLK siRNA sequence was 5'-CGCGTCCCCGCAAGAGGCTAGGCT-3'. The siRNA-NC sequence was 5'-CAAAGCAAGATTAGTACCTGGACC-3'. The cell density was fused to 70-80% in a 6-well plate. NLK siRNA and siRNA-NC negative control liposomes were separately added into 200 μl of serum-free DMEM medium, mixed well, and incubated at room temperature for 15 min. The mixed lipo2000 was separately mixed with the corresponding dilution and incubated for 30 min at room temperature followed by removal of serum, rinsing with phosphate-buffered saline (PBS), and subsequent addition of 1.6 mL serum-free DMEM medium. After culture in a 5% CO2 incubator at 37°C for 6 h, the serum DMEM medium was replaced and cultured for 48 h for experiments.

Real Time-PCR Detection of NLK mRNA Expression

The laryngeal cancer tumor tissues and adjacent tissues and the Hep-2 tumor cell mRNAs of each group were extracted using TRIzol reagent and reversely transcripted into cDNA according to the kit instructions. The primers were designed according to each gene sequence by Primer Premier 6.0 and synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Table I). Real Time PCR was performed with the conditions as follows: 55°C 1 min, 92°C 30 s, 58-60°C 45 s, 72°C 35 s, a total of 35 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference. According to the fluorescence quantification, starting cycle number (CT) of all samples and standards was calculated. Based on the standard CT value, a standard curve was drawn, and then the semi-quantitative analysis was carried out using the $2^{-\Delta Ct}$ method.

MTT Assay Analysis of the Growth of Hep-2 Cells

The log phase-grown Hep-2 cells were digested and counted, and seeded into 96-well plates at 3000 cells/well. They were randomly divided into 3 groups and each group was treated for 48 h followed by addition of 20 μl MTT solution (5 g/L) into each well and subsequent culture for 4 h. After complete removal of the supernatant, dimethyl sulfoxide (DMSO;150 μl/well) was added followed by shake for 10 min until the purple crystals were fully dissolved. The absorbance value was measured at a wavelength of 570 nm, and the cell proliferation rate was calculated.

Caspase 3 Activity Assay

The changes in Caspase3 activity were examined according to the kit instructions. Trypsin digested cells were centrifuged at 600 g at 4°C for 5 min, followed by discarding the supernatant and placing the cell lysate on ice for 15 min. After centrifuging at 20000 g at 4°C for 5 min, 2 mM Ac-DEVD-pNA was added and the OD value was measured at a wavelength of 405 nm.

Transwell Chamber Analysis of Cell Invasion

According to the kit instructions, 200 μl of serum-free DMEM medium was replaced. After 24 h, the bottom and membrane upper chamber of the transwell chamber was coated with a 1:5 50 mg/L Matrigel dilution and air dried at 4°C. 100 μl of tumor cell suspension prepared by adding 500 μl 10% FBS DMEM medium and 200 μl serum-free DMEM medium was added into the inside and outside the chamber, respectively with 3 replicate wells in each group and the chamber was placed in a 24-well plate. Each control group was cultured in a transwell chamber without Matrigel. After 48 h of culture, the transwell chamber was washed with PBS, the cells on the membrane were removed, fixed in ice-ethanol. After staining with crystal violet, the cells in the lower layer of the microporous membrane were counted. The experiment was repeated three times.

Table I. Primer sequences for real-time PCR.

Gene	Forward 5′-3′	Reverse 5′-3′
GAPDH	AGTAGTCACCTGTTGCTGG	TAATACGGAGACCTGTCTGGT
NLK	ATAATGACTCCGGTCCATGG	TCAAGC ACCGT ACCGGTA

Western Blot

Radioimmunoprecipitation (RIPA) lysis buffer was added into tissues or cells followed by being lysed on ice for 15-30 min, the cells were disrupted by sonication for 5 s × 4 times, centrifuged at 4°C at 10 000 × g for 15 min, and the supernatant was transferred to a new Eppendorf (EP) tube (Hamburg, Germany). The extracted protein was quantified using the bicinchoninic acid (BCA) method and stored at –20°C for Western blot analysis. The isolated protein was separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) transferred to a PVDF membrane by semi-dry transfer, 100 mA, 1.5 h, and blocked with 5% skim milk powder for 2 h. NLK (1:1000), MMP-9 (1:1000), and CDCP1 (1:1500) mAb diluted in different ratios were incubated with the membrane at 4°C overnight. After washing with PBST, the corresponding secondary antibodies diluted in different proportions were added, incubated for 30 min, washed with PBST, and developed by chemiluminescence for 1 min. X-ray exposure imaging was performed to observe the results. X-film and strip density measurements were separately scanned using protein image processing system software and Quantity one software. The experiment was repeated four times.

Statistical Analysis

All data are expressed as mean ± standard deviation (SD) and processed using SPSS 11.5 statistical software. The mean values of the two groups were compared by the Student's *t*-test and the differences between groups were analyzed by analysis of variance (ANOVA) with Bonferroni as Post-Hoc analysis. $p<0.05$ indicated a statistical difference.

Results

NLK Expression in Laryngeal Cancer

Real Time PCR and Western blot were used to detect the expression of NLK mRNA and protein in laryngeal squamous cell carcinoma. Results showed that NLK mRNA expression was significantly increased in tumor tissues compared with adjacent tissues ($p<0.05$) (Figure 1A). Meanwhile, NLK protein expression was also significantly increased (Figure 1B).

Effect of NLK siRNA Transfection on NLK Expression

NLK siRNA was transfected into Hep-2 cells and the expression of NLK was detected. The results showed that compared with control group, the expression of NLK mRNA in Hep-2 cells was significantly decreased ($p<0.05$) (Figure 2A). Consistently, NLK siRNA transfected into Hep-2 cells significantly inhibited the expression of NLK protein (Figure 2B).

Down-Regulation of NLK Expression on Hep-2 Cell Proliferation

MTT assay was performed to evaluate the effect of NLK siRNA on Hep-2 cell proliferation and showed that transfection of NLK siRNA for 48 h significantly inhibited the proliferation of Hep-2 cells ($p<0.05$) (Figure 3). This result suggests that the regulation of NLK has a significant inhibitory effect on Hep-2 cell proliferation.

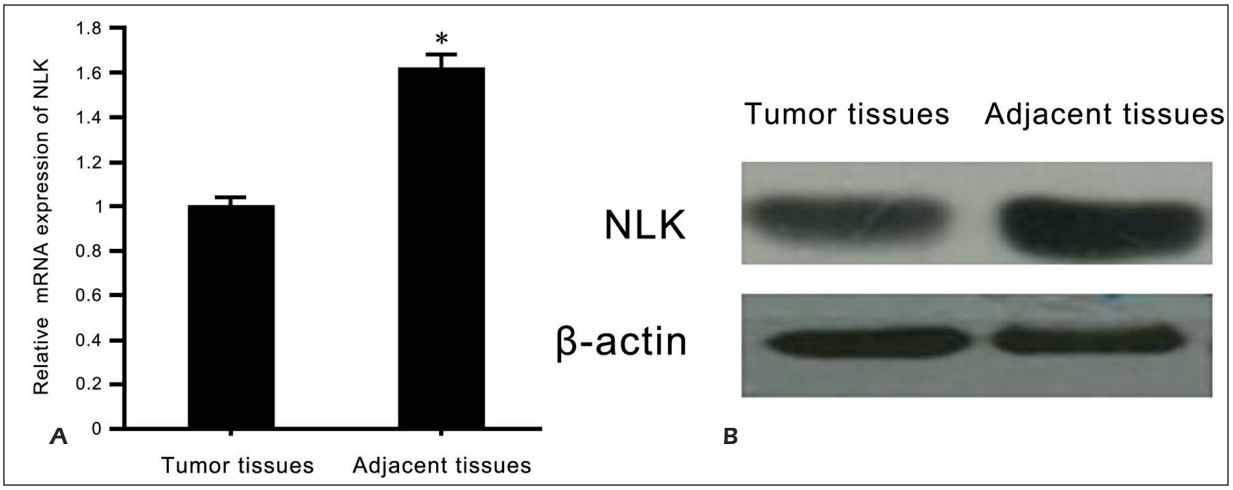

Figure 1. NLK expression in laryngeal cancer. ***A***, NLK mRNA expression in laryngeal carcinoma; Compared with adjacent tissues, *$p<0.05$. ***B***, Western blot analysis of NLK protein expression in laryngeal cancer.

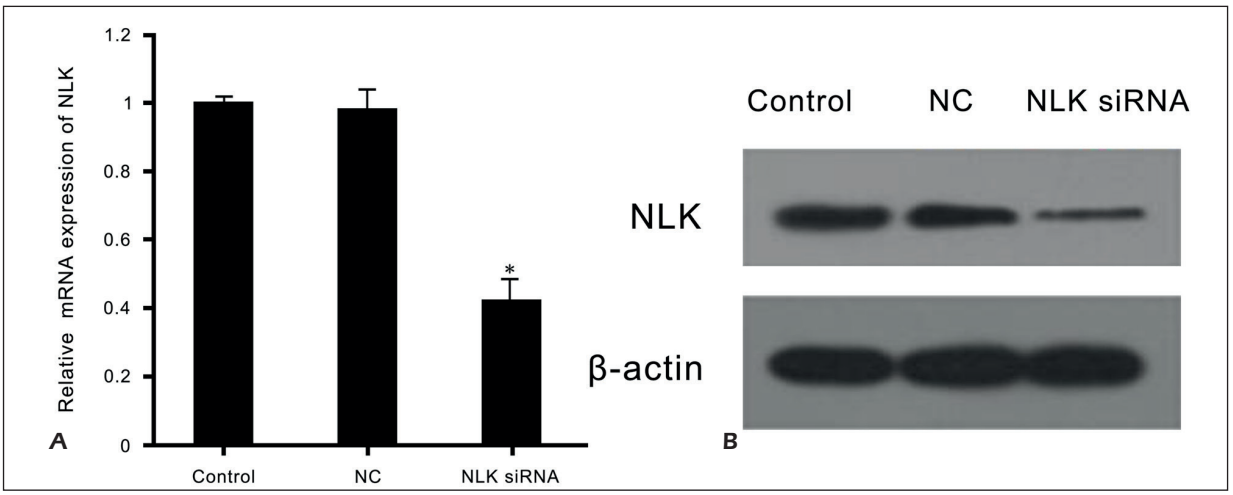

Figure 2. Effect of siRNA transfection on NLK expression in laryngeal carcinoma Hep-2 cells. ***A***, Expression of NLK mRNA in Hep-2 cells; Compared with the control group, *$p<0.05$. ***B***, Western blot analysis of NLK protein expression in Hep-2 cells.

Effect of regulating NLK expression on Caspase 3 activity

The effect of down-regulation of NLK expression on Caspase 3 activity in Hep-2 cells was detected using Caspase 3 activity assay. The results showed that transfection of NLK siRNA into Hep-2 cells significantly increased Caspase 3 activity ($p<0.05$) (Figure 4).

Effect of Regulating NLK Expression on Invasion of Hep-2 Cells

Transwell chamber assays were performed to assess the effects of NLK expression on Hep-2 cell invasion and found that transfection of NLK siRNA into Hep-2 cells significantly inhibited the invasion of Hep-2 cells ($p<0.05$) (Figure 5).

Effects of NLK expression on the expression of MMP-9 and CDCP1

The effect of NLK on the expression of MMP-9 and CDCP1 in Hep-2 cells was detected by Western blot. Transfection of NLK siRNA into Hep-2 cells reduced MMP-9 and CDCP1 expression (Figure 6).

Discussion

Early diagnosis of a tumor is conducive to the treatment, but since the early clinical symptoms of laryngeal cancer are not significant, early screening, diagnosis, and early intervention are very difficult, resulting in poor treatment of laryngeal cancer, se-

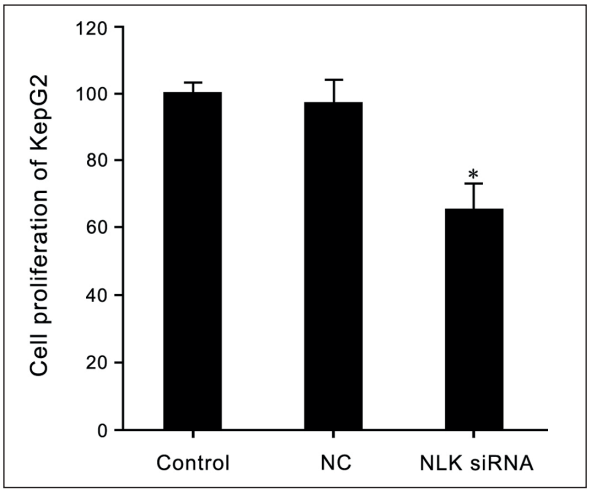

Figure 3. Effect of NLK downregulation on proliferation of Hep-2 cells. Compared with control group, *$p<0.05$.

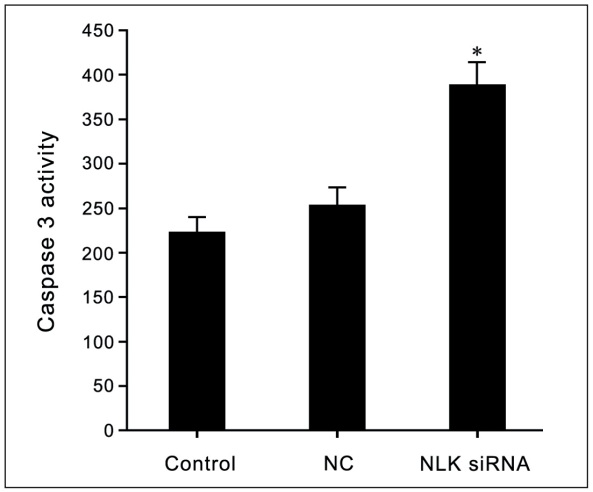

Figure 4. Effect of NLK downregulation on Caspase 3 activity in Hep-2 cells. Compared with control group, *$p<0.05$.

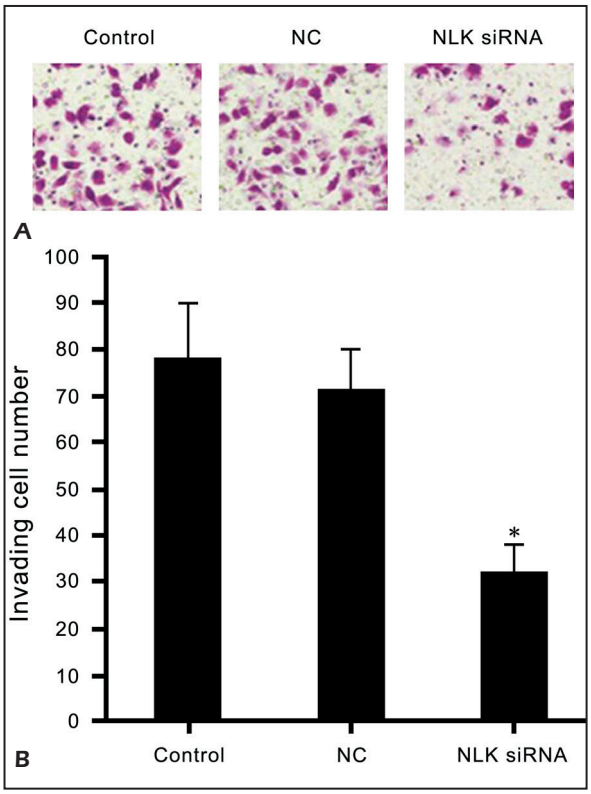

Figure 5. Effect of NLK downregulation on invasion of Hep-2 cells. ***A***, Representative image of transwell chamber assay analysis of Hep-2 cell invasion. ***B***, Quantification of the Hep-2 cell invasion. Compared with control group, *$p<0.05$.

riously affecting the prognosis of laryngeal cancer patients. Therefore, an identification of the molecular targets for the development of laryngeal cancer might be beneficial for early prevention and diagnosis and treatment of laryngeal cancer[18,19]. NLK is widely distributed and belongs to one of the cell cycle factors, playing a dual role in promoting or suppressing cancer in different tumorigenesis and development[15,16]. NLK is closely related to the progression of tumors and the abnormal expression of NLK in tumors is an important marker for tumorigenesis and might be used as an important indicator to evaluate tumor prognosis[20,21]. Therefore, this research first analyzed the expression of NLK in laryngeal carcinoma and the results confirmed that NLK mRNA and protein was increased in laryngeal carcinoma tissue, suggesting that NLK might participate in the regulation of laryngeal cancer. Furthermore, siRNA technology was used to interfere with the expression of NLK in laryngeal carcinoma cell line Hep-2 and confirmed that down-regulation of NLK inhibits tumor cell proliferation, promotes apoptosis, and inhibits invasion, indicating that NLK plays a role in the development of laryngeal carcinoma and it might be

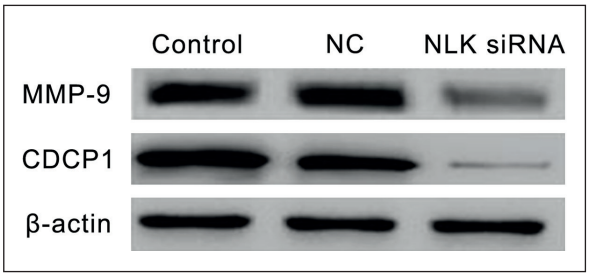

Figure 6. Effects of NLK downregulation on the expression of MMP-9 and CDCP1 proteins in Hep-2 cells.

used as a potential molecular target for the development and diagnosis of laryngeal cancer.

MMP is a family of metalloproteinases, which can destroy the histological barrier of tumor cell invasion by degrading various protein components in the extracellular matrix, playing a key role in tumor invasion and metastasis. MMP-9 is a key member and can participate in the decomposing extracellular matrix and promoting tumor neovascularization[22,23]. Therefore, the regulation of MMP-9 has become one of the therapeutic targets for inhibiting the progression of laryngeal cancer. CDCP1 is expressed on a variety of cell surfaces and belongs to the transmembrane glycoprotein and its intracellular portion interacts with various proteins in the cell[24]. Uekita et al[25] have shown that CDCP1 is expressed in a variety of tumor cells such as lung cancer and is involved in the regulation of tumor cell proliferation, growth, survival, and angiogenesis. Also, it is associated with tumor metastasis. This study showed that down-regulation of NLK expression inhibited the expression of MMP-9 and CDCP1, suggesting that the role of NLK in laryngeal cancer is closely related to the regulation of MMP-9 and CDCP1 expression. However, the exact molecular mechanism and specific targets of NLK in the regulation of laryngeal cancer proliferation remains unclear and requires further investigations to provide guidance on the clinical selection of molecular targets for the treatment of laryngeal cancer.

Conclusions

We observed that NLK was expressed in tumor tissues of patients with laryngeal cancer. The down-regulation of NLK expression may play a role in the proliferation, apoptosis, and invasion of laryngeal carcinoma cells possibly by regulating MMP-9 and CDCP1, indicating that NLK

might be used as a potential molecular target for laryngeal cancer treatment, which provides an important theoretical reference for the diagnosis and treatment of laryngeal cancer.

Acknowledgments

This work was supported by the Key Science and Technology Research Project of Health Commission of Hebei Province (No. 20180794).

Conflict of Interests

The Authors declared that they have no conflict of interests.

References

1) Chen L, Jia J, Zang Y, Li J, Wan B. MicroRNA-101 regulates autophagy, proliferation and apoptosis via targeting EZH2 in laryngeal squamous cell carcinoma. Neoplasma 2019; Mar 7. pii: 180811N611. doi: 10.4149/neo_2018_180811N611. [Epub ahead of print]
2) Pandiar D, Nayanar SK, Ankalkoti B, Babu S. Laryngeal basaloid squamous cell carcinoma with a substantial spindle cell component: case presentation and updated review of literature. Head Neck Pathol 2018; Jun 5. doi: 10.1007/s12105-018-0942-2. [Epub ahead of print]
3) Yang L, Luo D, Yi J, Li L, Zhao Y, Lin M, Guo W, Hu L, Zhou C. Therapy effects of advanced hypopharyngeal and laryngeal squamous cell carcinoma: evaluated using dual-energy CT quantitative parameters. Sci Rep 2018; 8: 9064.
4) Aghazadeh K, Dabiri Satri S, Sharifi A, Lotfi M, Maraghehpour B, Hashemiaghdam A. Predictors of thyroid gland invasion in laryngeal squamous cell carcinoma. Iran J Otorhinolaryngol 2018; 30: 153-158.
5) Hui L, Zhang J, Guo X. MiR-125b-5p suppressed the glycolysis of laryngeal squamous cell carcinoma by down-regulating hexokinase-2. Biomed Pharmacother 2018; 103: 1194-1201.
6) Yokota T, Kato K, Hamamoto Y, Tsubosa Y, Ogawa H, Ito Y, Hara H, Ura T, Kojima T, Chin K, Hironaka S, Kii T, Kojima Y, Akutsu Y, Matsushita H, Kawakami K, Mori K, Nagai Y, Asami C, Kitagawa Y. Phase II study of chemoselection with docetaxel plus cisplatin and 5-fluorouracil induction chemotherapy and subsequent conversion surgery for locally advanced unresectable oesophageal cancer. Br J Cancer 2016; 115: 1328-1334.
7) Bandhary SK, Shetty V, Saldanha M, Gatti P, Devegowda D, R PS, Shetty AK. Detection of human papilloma virus and risk factors among patients with head and neck squamous cell carcinoma attending a tertiary referral centre in South India. Asian Pac J Cancer Prev 2018; 19: 1325-1330.
8) Lian R, Lu B, Jiao L, Li S, Wang H, Miao W, Yu W. MiR-132 plays an oncogenic role in laryngeal squamous cell carcinoma by targeting FOXO1 and activating the PI3K/AKT pathway. Eur J Pharmacol 2016; 792: 1-6.
9) Thinn MM, Hsueh CT, Hsueh CT. Sustained complete response to erlotinib in squamous cell carcinoma of the head and neck: a case report. World J Clin Cases 2019; 7: 616-622.
10) Zhang D, Zhou J, Tang D, Zhou L, Chou L, Chou KY, Tao L, Lu LM. Neutrophil infiltration mediated by CXCL5 accumulation in the laryngeal squamous cell carcinoma microenvironment: a mechanism by which tumour cells escape immune surveillance. Clin Immunol 2017; 175: 34-40.
11) Qin L, Mo YL, Li L, Wei ZJ, Zhu XD, Yin X, Wei Q. Sleep characteristics and psychological symptoms in patients with locally advanced nasopharyngeal carcinoma before and after intensity-modulated radiotherapy and concurrent chemotherapy. Psychol Health Med 2015; 20: 662-669.
12) Lei L, Wang Y, Zheng YW, Fei LR, Shen HY, Li ZH, Huang WJ, Yu JH, Xu HT. Overexpression of Nemo-like kinase promotes the proliferation and invasion of lung cancer cells and indicates poor prognosis. Curr Cancer Drug Targets 2018; Nov 19. doi: 10.2174/1568009618666181119150521. [Epub ahead of print]
13) Zeng L, Tian YM, Huang Y, Sun XM, Wang FH, Deng XW, Han F, Lu TX. Retrospective analysis of 234 nasopharyngeal carcinoma patients with distant metastasis at initial diagnosis: therapeutic approaches and prognostic factors. PLoS One 2014; 9: e108070.
14) Bian X, Song T, Wu S. Outcomes of xerostomia-related quality of life for nasopharyngeal carcinoma treated by IMRT: based on the EORTC QLQ-C30 and H&N35 questionnaires. Expert Rev Anticancer Ther 2015; 15: 109-119.
15) Yang W, Gu L, Yang C, Liu T. Expression of Nemo-like kinase in cervical squamous cell carcinoma: a clinicopathological study. Onco Targets Ther 2018; 11: 743-749.
16) Li SZ, Zeng F, Li J, Shu QP, Zhang HH, Xu J, Ren JW, Zhang XD, Song XM, Du RL. Nemo-like kinase (NLK) primes colorectal cancer progression by releasing the E2F1 complex from HDAC1. Cancer Lett 2018; 431: 43-53.
17) Chen J, Shen Z, Deng H, Zhou W, Liao Q, Mu Y. Long non-coding RNA biomarker for human laryngeal squamous cell carcinoma prognosis. Gene 2018; 671: 96-102.
18) Yu S, Lin J, Chen C, Lin J, Han Z, Lin W, Kang M. Recurrent laryngeal nerve lymph node dissection may not be suitable for all early stage esophageal squamous cell carcinoma patients: an 8-year experience. J Thorac Dis 2016; 8: 2803-2812.
19) Wu K, Shen B, Jiang F, Xia L, Fan T, Qin M, Yang L, Guo J, Li Y, Zhu M, Du J, Liu Y. TRPP2 enhances metastasis by regulating epithelial-mesenchymal transition in laryngeal squamous cell carcinoma. Cell Physiol Biochem 2016; 39: 2203-2215.
20) Masoumi KC, Daams R, Sime W, Siino V, Ke H, Levander F, Massoumi R. NLK-mediated phosphorylation of HDAC1 negatively regulates Wnt signaling. Mol Biol Cell 2017; 28: 346-355.
21) Lv M, Li Y, Tian X, Dai S, Sun J, Jin G, Jiang S. Lentivirus-mediated knockdown of NLK inhibits small-cell lung cancer growth and metastasis. Drug Des Devel Ther 2016; 10: 3737-3746.

22) Wang G, Liu J, Gao J and Zheng X. Comparison of the effects of sevoflurane and propofol anesthesia on pulmonary function, MMP-9 and postoperative cognition in patients receiving lung cancer resection. Oncol Lett 2019; 17: 3399-3405.
23) Moawed FS, El-Sonbaty SM, Mansour SZ. Gallium nanoparticles along with low-dose gamma radiation modulate TGF-beta/MMP-9 expression in hepatocellular carcinogenesis in rats. Tumour Biol 2019; 41: 1010428319834856.
24) Kurosawa G, Sugiura M, Hattori Y, Tsuda H, Kurosawa Y. Classification of 27 tumor-associated antigens by histochemical analysis of 36 freshly resected lung cancer tissues. Int J Mol Sci 2016; 17. pii: E1862.
25) Uekita T, Fujii S, Miyazawa Y, Iwakawa R, Narisawa-Saito M, Nakashima K, Tsuta K, Tsuda H, Kiyono T, Yokota J, Sakai R. Oncogenic Ras/ERK signaling activates CDCP1 to promote tumor invasion and metastasis. Mol Cancer Res 2014; 12: 1449-1459.