

Expression of Nemo-like kinase in cervical squamous cell carcinoma: a clinicopathological study

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Objective: Nemo-like kinase (NLK) has been reported to play different roles in tumors. However, the role of NLK in cervical squamous cell carcinoma (CSCC) remains unknown. In this study, we explored the clinical significance including survival of NLK protein expression in CSCCs.

Patients and methods: Immunohistochemical method was performed using tissues from 130 patients with CSCC. The associations between NLK expression and the clinicopathological factors and prognosis of CSCCs were evaluated. Statistical analyses were performed using the chi-square test, the multivariate Cox proportional hazard model, and the Kaplan–Meier method.

Results: Immunohistochemical staining analysis showed that NLK was localized predominately in the nucleus of the tumor cells, and increased NLK expression was detected in 71 (54.6%) of 130 patients. NLK overexpression significantly correlated with higher histological grade ($P=0.001$), vascular/lymphatic invasion ($P=0.010$), lymph node metastasis ($P=0.012$), and recurrence ($P=0.022$). Patients with elevated NLK expression had poorer overall survival (OS) and disease-free survival (DFS) ($P=0.006$ and $P=0.004$, respectively) compared with patients with decreased NLK expression. Multivariate Cox analysis demonstrated that NLK overexpression was an independent factor for OS and DFS ($P=0.034$ and $P=0.025$, respectively).

Conclusion: NLK may be a valuable biomarker for predicting the prognosis of CSCC patients and may serve as a potential target for cancer therapy.

Keywords: Nemo-like kinase, cervical squamous cell carcinoma, prognosis, progression, lymph node metastasis, survival, biomarker

Introduction

Almost 90% of cervical cancer-related deaths occurred in the developing countries, and the highest cervical cancer death rates are in Asia, with an estimated 144,400 deaths occurring in 2012.¹ Invasive cancer accounts for >70% of patients diagnosed with cervical cancer, among which squamous cell carcinoma is the most frequent type.^{2,3} Early-stage cervical squamous cell carcinoma (CSCC) cases have a greater possibility of being cured and are associated with excellent survival rates.^{4,5} Since early detection results in a better prognosis, there is an urgent need to identify more efficient early diagnostic biomarkers and therapeutic targets for CSCC.

The Nemo-like kinase (NLK), originally identified as a vertebrate ortholog of *Drosophila nemo*,⁶ is an evolutionarily conserved serine/threonine protein kinase and belongs to the extracellular signal-regulated kinases/microtubule-associated protein kinases family.^{7,8} NLK was proved to be important for tumor cell proliferation, migration, and apoptosis.^{9–13} Increased NLK expression has been reported in several cancers,^{14–17} and variation in NLK may be associated with the risk of invasive

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epithelial ovarian cancer.¹⁸ However, the status of NLK protein expression in CSCCs remains unknown. This study was performed to explore NLK expression in CSCCs and to analyze its relationship with various clinicopathological features, including patient outcome.

Patients and methods

Patients and surgical specimens

This study was approved by the ethical committee of the Harbin Medical University, and all tissue samples were obtained from patients (n=130) at the Department of Gynecology, Harbin Medical University Cancer Hospital, China, between January 2010 and December 2011. All patients with CSCC underwent radical hysterectomy and pelvic lymphadenectomy. None of the patients received chemotherapy, immunotherapy, or radiotherapy before surgery. Patients with high-risk factors underwent postoperative radiotherapy. The tumor stages were assessed according to the International Federation of Gynecology and Obstetrics staging system.¹⁹ The histological grades were classified according to the World Health Organization criteria.

Written informed consent was obtained from the patients for the purpose of research. The patients with CSCC were followed up for clinical evaluation either in the hospital or by telephone. All patients with CSCC were followed up periodically for survival analysis until death or until the study closing date (December 2016). The median follow-up time was 64 months, with a range of 13–88 months. Overall survival (OS) was defined as the period from the date of surgery until death or to the time of the most recent follow-up, and disease-free survival (DFS) was defined as the time interval in months between completion of therapy and recurrence, respectively. Recurrence was either radiologically or histologically confirmed.

Western blot analysis

Proteins from 15 frozen CSCC and 5 frozen normal cervical tissue samples were extracted by suspending them in RIPA buffer consisting of 1% protease inhibitor mixture. The mixture was centrifuged at 12,000×g for 15 minutes at 4°C, and the supernatant was collected. Protein concentrations were quantified by the bicinchoninic acid protein assay kit, and 30 µg of protein extracts was separated by sodium dodecyl sulfate polyacrylamide gel and transferred to methanol-activated nitrocellulose filter membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA). Membranes were blocked with 5% non-fat dry milk before immunodetection. Primary antibodies, NLK (1:1,000 dilution; Abcam, Cambridge, MA,

USA), and β-actin were diluted in the buffer and incubated at 4°C overnight. After standard washing, membranes were incubated with a secondary antibody (horseradish peroxidase conjugated) for 1 hour at room temperature. The experiment was accomplished in triplicate.

Immunohistochemistry

Paraffin-embedded CSCC specimens were cut into 4 µm sections, and then the slides were blocked with 0.3% hydrogen peroxide at 37°C for 30 minutes. To increase specificity and sensitivity, samples were placed in 10 mmol/L citrate buffer (pH 6.0) in a pressure cooker for 2 minutes. The mouse monoclonal antibody against NLK antibodies (1:100 dilution; Abcam) was added to the sections for overnight at 4°C, followed by incubation with mouse secondary antibody (Zhong Shan Golden Bridge Biological Technology Beijing, China) for 20 minutes at room temperature. The sections were immersed in a solution of 3,3-diaminobenzidine tetrahydrochloride (Dako Denmark A/S, Glostrup, Denmark) and then counterstained with hematoxylin. The negative ones were stained with phosphate-buffered saline instead of primary antibodies.

Assessment of immunohistochemical staining

The NLK immunoreactivity was semiquantitatively estimated by combining intensity and percentage of positive tumor cells under the microscope as described earlier.¹⁷ The immunostaining results were optimized and judged by 2 independent senior pathologists according to the following standards. The percentage was classified as follows: 0 (0%), 1 (<5%), 2 (5%–50%), and 3 (>50%). The intensity was also scored as follows: 0 (negative staining), 1 (weak staining), 2 (moderate staining), and 3 (intense staining). The final score of NLK expression, ranging from 0 to 6, was the sum of the percentage of positive cells and the intensity score. Patients with a final score of <4 were classified as the low expression group and vice versa.

Statistical analysis

Student's *t*-test was used to compare continuous variables, and chi-square test was performed to assess differences in clinicopathological variables. OS and DFS were evaluated using the Kaplan–Meier method, and the differences among the levels of possible prognostic factors were compared by the log-rank test in the univariate analyses. The Cox proportional hazards model was used for the multivariate analysis of the independent prognostic factors for OS and DFS. All analyses were performed using statistical software SPSS 13.0 (SPSS

Inc., Chicago, IL, USA), and the findings were considered statistically significant at $P < 0.05$.

Results

Patients' characteristics and NLK protein expression in CSCC tissues

The expression of NLK was analyzed using specimens from 130 untreated CSCC patients. The median age of the patients was 43 years (range: 27–70). Of 130 patients, 72 patients (55.4%) had CSCC classified as the Federation of Gynecology and Obstetrics (FIGO) Stage I and 58 (44.6%) had FIGO Stage II. On the basis of the analysis of histological grade, 21 cases (16.2%) were well differentiated (G1), 75 (57.1%) were moderately differentiated (G2), and 34 (26.7%) were poorly differentiated (G3). Of these patients, lymph node metastases were present in 31 patients (23.8%) and absent in 99 patients (76.2%). A total of 96 patients (73.8%) were shown to have tumors <4 cm in size and 34 patients (26.2%) with tumors at least 4 cm in size. Parametrial involvement and vascular/lymphatic invasion occurred in 20 (15.4%) and 39 (30.0%) patients, respectively. Deep stromal involvement occurred in 53 (40.8%) of 130 patients, and recurrence was observed in 16 (12.3%) patients (Table 1).

Western blot analysis was used to assess the different levels of NLK expression in CSCC and normal cervical tissues. In Figure 1, it is shown that NLK protein expression is significantly elevated in CSCC tissues compared with that in normal cervical tissues ($P < 0.05$).

As shown in Figure 2, NLK was localized mainly in the nucleus of the tumor cells. Of the CSCC specimens examined, low NLK expression was detected in 59 (45.4%) of 130 patients, whereas high NLK expression was detected in 71 (54.6%) of 130 patients. Elevated NLK expression was observed in 28 (90.3%) CSCC patients with lymph node metastasis (Figure 2C).

Association of NLK protein expression and clinicopathological features

We analyzed the associations between NLK expression status and a series of clinicopathological characteristics, including age, FIGO stage, histological grade, tumor size, parametrial involvement, depth of stromal involvement, vascular/lymphatic invasion, lymph node metastasis, and recurrence (Table 1). Elevated NLK expression was significantly associated with higher histological grade ($P = 0.001$), vascular/lymphatic invasion ($P = 0.010$), lymph node metastasis ($P = 0.012$), and recurrence ($P = 0.022$). However, no strong association was observed between NLK and age,

Table 1 Relationships of NLK expression status with clinicopathological features of CSCC

Characteristics	No of cases	NLK expression		P-value
		Low (n=59)	High (n=71)	
Age (years)				0.457
<43	64	33	31	
≥43	66	26	40	
FIGO stage				0.909
I	72	33	39	
II	58	26	32	
Histological grade				0.001
G1	21	13	8	
G2	75	36	39	
G3	34	10	24	
Tumor size				0.82
<4 cm	96	43	53	
≥4 cm	34	16	18	
Parametrial involvement				0.599
Yes	20	8	12	
No	110	51	59	
Depth of stromal involvement				0.462
<1/2	77	37	40	
≥1/2	53	22	31	
Vascular/lymphatic invasion				0.01
Yes	39	11	28	
No	91	48	43	
Lymph node metastasis				0.012
Yes	31	8	23	
No	99	51	48	
Recurrence				0.022
Yes	16	3	13	
No	114	56	58	

Notes: G1, well differentiated; G2, moderately differentiated; and G3, poorly differentiated.

Abbreviations: CSCC, cervical squamous cell carcinoma; FIGO, the Federation of Gynecology and Obstetrics; NLK, Nemo-like kinase.

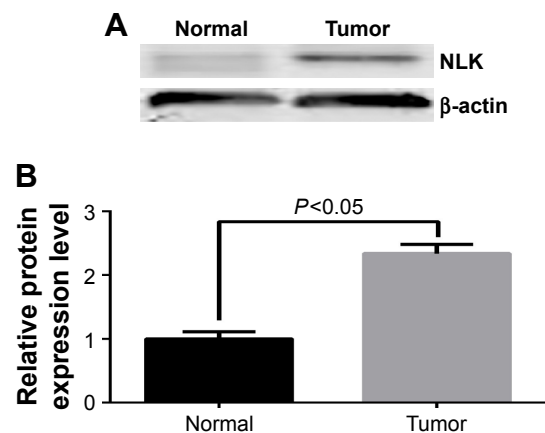


Figure 1 (A) Protein samples obtained from frozen normal cervical (n=5) and CSCC (n=15) tissues were analyzed by Western blot analysis. (B) Histogram of pooled data. There was significant difference in NLK protein expression between normal cervical and CSCC samples ($P < 0.05$).

Abbreviations: CSCC, cervical squamous cell carcinoma; NLK, Nemo-like kinase.

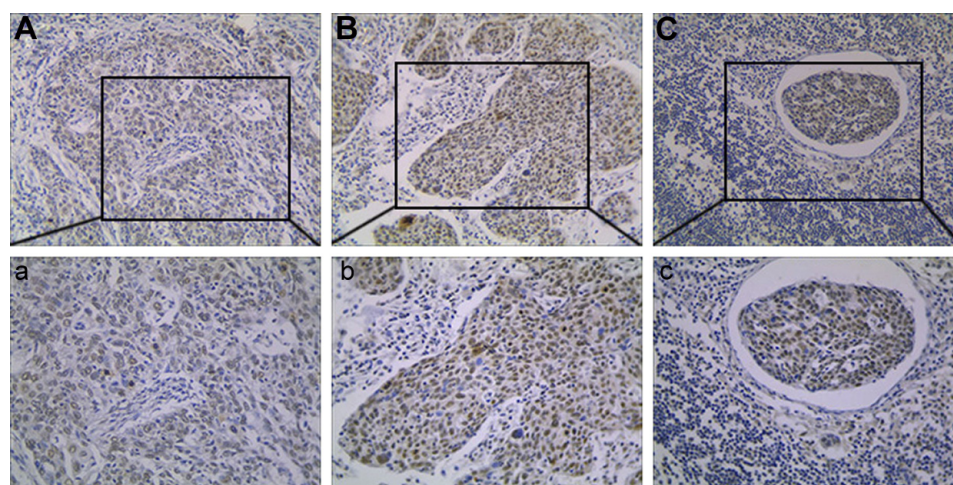


Figure 2 Representative images of NLK immunohistochemical staining.

Notes: (A) Low expression in CSCC tissues ($\times 200$); a, low expression in CSCCs ($\times 400$); (B) high expression in CSCCs ($\times 200$); b, high expression in CSCCs ($\times 400$); (C) high expression in metastatic lymph node tissues ($\times 200$); c, high expression in metastatic lymph node tissues ($\times 400$).

Abbreviations: CSCC, cervical squamous cell carcinoma; NLK, Nemo-like kinase.

FIGO stage, tumor size, parametrial involvement, and deep stromal involvement ($P > 0.05$).

Univariate and multivariate analyses for the prognosis of patients with CSCC

The Kaplan–Meier 5-year survival curves stratified for NLK expression are shown in Figure 3. Both univariate and multivariate survival analyses were used to evaluate the effects of NLK expression and clinicopathological characteristics on prognosis. For OS (Table 2), univariate analyses indicated NLK expression ($P = 0.006$), FIGO stage ($P = 0.011$), tumor size ($P = 0.043$), parametrial involvement ($P = 0.045$), vascular/lymphatic invasion ($P = 0.003$), and lymph node metastasis ($P < 0.001$) as significant prognostic predictors. Other features had no prognostic value. Using multivariate analysis, we found that NLK overexpression

(HR: 2.760; 95% CI: 1.079–7.058; $P = 0.034$), FIGO stage (HR: 2.339; 95% CI: 1.027–5.327; $P = 0.043$), and lymph node metastasis (HR: 3.102; 95% CI: 1.394–6.901; $P = 0.006$) were independently prognostic predictors in CSCC. For DFS (Table 3), univariate analyses showed that NLK expression ($P = 0.004$), FIGO stage ($P = 0.014$), vascular/lymphatic invasion ($P = 0.003$), and lymph node metastasis ($P < 0.001$) were all associated with prognosis. In multivariate Cox regression analysis, the increased expression of NLK (HR: 2.920; 95% CI: 1.143–7.458; $P = 0.025$) and lymph node metastasis (HR: 3.375; 95% CI: 1.512–7.532; $P = 0.003$) were considered as independent prognostic markers.

Discussion

In this paper, we found that increased NLK expression was significantly associated with higher histological grade,

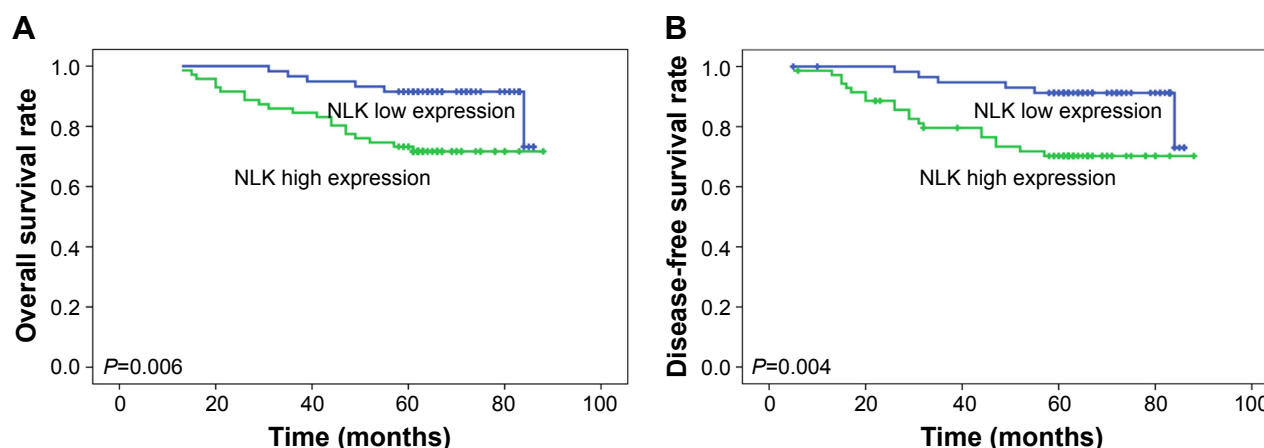


Figure 3 Kaplan–Meier analysis of overall survival (A) and disease-free survival (B) in relation to NLK expression in 130 cervical squamous cell carcinoma patients.

Abbreviation: NLK, Nemo-like kinase.

Table 2 Univariate and multivariate analyses with regard to OS

Variables	Univariate analysis	Multivariate Cox regression		
	P-value	HR	95% CI	P-value
Age (years)	0.658			
<43				
≥43				
FIGO stage	0.011			0.043
I		1.000		
II		2.339	1.027–5.327	
Histological grade	1.000			
G1				
G2				
G3				
Tumor size	0.043			
<4 cm				
≥4 cm				
Parametrial involvement	0.045			
No				
Yes				
Depth of stromal involvement	0.263			
<1/2				
≥1/2				
Vascular/lymphatic invasion	0.003			
No				
Yes				
Lymph node metastasis	<0.001			
No		1.000		0.006
Yes		3.102		
NLK expression status	0.006		1.394–6.901	
Low		1.000		0.034
High		2.760	1.079–7.058	

Notes: G1, well differentiated; G2, moderately differentiated; and G3, poorly differentiated.

Abbreviations: OS, overall survival; FIGO, the Federation of Gynecology and Obstetrics; NLK, Nemo-like kinase.

Table 3 Univariate and multivariate analyses with regard to DFS

Variables	Univariate analysis	Multivariate Cox regression		
	P-value	HR	95% CI	P-value
Age (years)	0.735			
<43				
≥43				
FIGO stage	0.014			
I				
II				
Histological grade	0.096			
G1				
G2				
G3				
Tumor size	0.057			
<4 cm				
≥4 cm				
Parametrial involvement	0.056			
No				
Yes				
Depth of stromal involvement	0.231			
<1/2				
≥1/2				
Vascular/lymphatic invasion	0.003			
No				
Yes				
Lymph node metastasis	<0.001			
No				0.003
Yes		1.000		
NLK expression status	0.004	3.375	1.512–7.532	
Low		1.000		0.025
High		2.920	1.143–7.458	

Notes: G1, well differentiated; G2, moderately differentiated; and G3, poorly differentiated.

Abbreviations: DFS, disease-free survival; FIGO, the Federation of Gynecology and Obstetrics; NLK, Nemo-like kinase.

vascular/lymphatic invasion, lymph node metastasis, and recurrence in CSCCs. In multivariate Cox regression analysis, elevated NLK expression was an independent prognostic factor for both OS and DFS. These findings reveal that NLK may serve a potential function in CSCC progression and may thus be used as a valuable biomarker in predicting prognosis in CSCCs.

Subcellular fractionation from COS7 cells and HEK293 cells verifies that ~60%–70% of NLK is localized in the nucleus and 30%–40% is localized in the cytoplasm.²⁰ NLK was predominantly located in the nucleus of tumor cells in nasopharyngeal carcinoma, hepatocellular carcinoma, glioma, and gallbladder cancer;^{10,14,15,17} however, NLK was expressed mainly in the cytoplasm of tumor cells in colorectal cancer.²¹ NLK-positive staining was confined mainly to the nucleus and cytoplasm in non-small-cell lung cancer

(NSCLC).²² In our study, we found that NLK was localized mainly in the nucleus of the tumor cells. The abovementioned findings suggested that different cytological localizations of NLK in different tumor types might contribute to different biological mechanisms by various signal pathways.

Evidence shows that NLK can serve as an oncogene or tumor suppressor gene dependent on the organ/cell-type specificity. On one hand, NLK acts as an oncogene, and downregulation of this gene may prevent the progression of cancers. Jung et al⁹ showed that silencing of NLK decreases cell growth and positively regulates the expression of cyclinD1, a core component of cell cycle regulation in hepatocellular carcinoma cells: Hep3B and SNU-423. Furthermore, low NLK expression decreased significantly the proliferation and migration abilities of GBC-SD and SGC-996 cells in gallbladder carcinoma.¹⁰ On the other

hand, NLK functions as a tumor suppressor and elevated expression of NLK may prevent the progression of cancers by inhibiting cell proliferation, migration, and invasion or inducing cell apoptosis. Upregulated NLK results in an obvious decrease in the number of cells and a sharp increase in apoptosis in prostate cancer cell PC-3 by inhibiting androgen receptor-mediated transcription.²³ In human colon cancer, overexpression of NLK in DLD-1 human colon cancer cell line induced cell apoptosis and inhibited cell proliferation.²⁴ Overexpression of NLK prevents proliferation and elicits apoptosis in breast cancer cell MCF-7.²⁵ In glioblastoma cell U87MG, upregulated NLK increases cell apoptosis.¹⁶ In addition, suppression of NLK expression resulted in significant promotion of proliferation in NSCLC cells by facilitating S-phase and mitotic entry, and the activity of β -catenin/TCF in A549 cells was remarkably enhanced.²⁶ Our results indicated that NLK played as oncogene; however, we still need to conduct cytological experiments to further verify that NLK gene functions as carcinogenic factor or suppressor in cervical cancer cells.

NLK has been demonstrated to correlate with histological grade, advanced stage, and lymph node metastasis.^{14,15,17,21} Similarly, our study suggests that tumors with high NLK expression had greater invasive potential including vascular/lymphatic invasion. NLK overexpression was also observed in high-invasive regions such as metastatic lymph node. As regards NSCLC, NLK has been found to be inversely correlated with NSCLC histological differentiation, clinical stage, lymph node status, and Ki-67.²⁶ Besides, NLK expression was reported to be inversely correlated with glioma grade.¹⁶ These different results may attribute to different cytological functions.

Furthermore, high NLK expression can be a strong predictor of poor prognosis.^{14,15,17,21} To date, this report is the first to show in detail an association between clinicopathological variables and the prognostic significance of NLK in CSCCs by using clinical samples. Yet, Lv et al²⁶ reported that low NLK expression was an independent prognostic factor for NSCLC patients' low survival rate by multivariate analysis. However, Lv et al²⁷ revealed that lentivirus-mediated knock-down of NLK inhibits small-cell lung cancer growth and metastasis. These suggest that the same gene NLK may function differently in the location of histology and cytology.

Conclusion

NLK is overexpressed in the nucleus of the tumor cells in CSCC, and high NLK expression can be correlated with progression, metastasis, and poor survival. These results

suggest that NLK may be a potentially novel biomarker for prediction of CSCC. However, these findings remain to be confirmed by a larger future study.

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Disclosure

The authors report no conflicts of interest in this work.

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