Nemo-like kinase expression predicts poor survival in colorectal cancer

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Abstract. Nemo-like kinase (NLK), a serine/threonine protein kinase, was previously reported to be associated with tumor proliferation and invasion. The present study aimed to evaluate whether NLK participates in the tumorigenesis and progression of colorectal cancer (CRC). NLK expression was examined using reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analysis in 50 paired CRC tissues as well as immunohistochemical analysis of 406 cases of primary CRC tissues and paired non-cancerous tissues. Correlations between NLK expression, the clinicopathological features of CRC patients and clinical outcome were then analyzed. NLK expression was found to be significantly higher in CRC tissues as well as associated with the depth of tumor invasion, lymph node metastasis, distant metastasis, histological differentiation, vascular invasion and advanced tumor stage. Patients with NLK-positive tumors demonstrated higher rates of recurrence and mortality than patients with NLK-negative tumors. Multivariate analyses revealed that NLK expression was an independent factor for overall survival [hazard ratio (HR)=0.035; 95% confidence interval (CI)=0.02-0.19; P<0.001] and disease-free survival (HR=0.033; 95% CI=0.007-0.09; P<0.001) in CRC patients. In conclusion, the results of the present study indicated that NLK may serve as a novel biomarker for tumor recurrence and survival for CRC patients.

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Introduction

Colorectal cancer (CRC) is a prevalent type of cancer, which has a high mortality rate worldwide (1). In Europe and the USA, CRC is the third most common type of human cancer and the second leading cause of cancer-associated mortality (2,3). In China, the incidence of CRC has risen steadily over the last few decades, with increasing morbidities in younger patients (<50 years) (4). Recent cancer statistics have indicated that CRC accounts for ~9% of all cancer-associated mortalities (5). The survival rate of CRC is higher with earlier diagnoses followed by treatment with surgical resection; however, the long-term survival and prognosis of the patients at stages III and IV remain poor (6). Genes associated with mutations in *TP53* (7), *KRAS* (8,9) and *BRAF* (9,10) as well as defective DNA mismatch repair (11) have been investigated for their prognostic and predictive value in CRC; however, the application of these markers requires validation in clinical practice and further evaluation. Sensitive biomarkers enable an early diagnosis and prognosis prediction; therefore, novel factors for predicting tumor recurrence and prognosis following surgery are urgently required.

The Wnt signaling pathway and its downstream components have a role in the regulation of cancer progression through numerous processes, including tumor initiation, tumor growth, cell senescence, cell death, differentiation and metastasis (12). The Wnt signaling pathway molecule Nemo-like kinase (NLK) is a member of the extracellular-signal regulated kinase/mitogen-activated protein kinase (MAPK) and cyclin-dependent kinase families (13). NLK was reported to induce apoptosis and inhibit androgen receptor-mediated transcriptional activity in prostate cancer cells (14). However, NLK also contributes to tumor growth via the activation of cell cycle proteins cyclin D1 and cyclin-dependent kinase 2 in human hepatocellular carcinoma (15). NLK was also demonstrated to induce apoptosis in glioma cells via activation of caspases (16). These previous studies have indicated that NLK may be a critical regulator of tumor growth and development. In the present study, reverse transcription quantitative polymerase chain reaction (RT-qPCR) and immunohistochemical

analysis were used to determine whether there was an association between NLK expression and the clinical outcome of CRC patients.

Materials and methods

Tissue specimens and patient information. A total of 406 clinical specimens were collected from the medical records of patients with CRC who underwent surgery at the Department of Gastrointestinal Surgery of Qianfoshan Hospital of Shandong Province and the Department of Digestive Diseases of Shandong Provincial Hospital Affiliated to Shandong University (Shandong, China). All specimens were archived under protocols approved by the institutional review boards of Shandong University and written informed consent was obtained from the patients. The group was composed of 172 males and 234 females with a mean±standard error of the mean age of 64.8±17.1 (range, 23-91) years. The diagnoses were confirmed by two pathologists and based on the tumor, node, metastasis classification system: 48 cases at stage I, 162 cases at stage II, 160 cases at stage III and 36 cases at stage IV. Among these patients, 132 had lymph node metastases (LNM). The follow-up of CRC patients post-surgery was performed according to the National Comprehensive Cancer Network Practice guidelines. Overall survival (OS) and disease-free survival (DFS) rates were defined as the interval from the initial surgery to clinically or radiologically proven recurrence/metastasis and mortality, respectively.

The patients were enrolled in the present study between 2006 and 2009. The follow-up for all cases was terminated in February 2012. During survival analysis, cases were regarded as censored data when patients were lost to follow-up or succumbed to their disease.

RNA extraction and RT-qPCR. Total RNA extraction of 50 paired freshly frozen primary tumor and adjacent normal mucosa (10 cm away from the original tumor site) of CRC specimens were performed according to the manufacturer's instructions (Qiagen, Shanghai, China). A Reverse Transcription kit (Qiagen) was used to reverse transcribe total RNA according to the manufacturer's instructions. Quantitative PCR was performed using a SYBR Green PCR kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The human NLK gene was amplified using a commercial NLK qPCR Primer Pair (NM_016231; OriGene, Rockville, MD, USA) and β-actin (HP204660; OriGene) was used as the internal control. Cycling conditions were as follows: Denaturation (5 min at 93˚C) followed by 40 cycles of denaturation (30 sec at 93˚C), annealing (15 sec at 58˚C) and elongation (1 min at 72˚C). Each reaction was performed in triplicate and the $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression.

Western blot analysis. Western blot analysis was performed as previously described (17). Monoclonal human anti-NLK antibodies (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) and monoclonal anti-β-actin antibodies (1:2,000; Beyotime Biotechnology, Jiangsu, China) were used as primary antibodies. Immunoreactive bands were detected using a Phototope-horseradish peroxidase western blot detection kit (Cell Signaling Technology, Inc.). For densitometric analysis, NLK protein bands on the blots were measured using Image J software (National Institutes of Health, Bethesda, MD, USA) following normalization to the corresponding β-actin expression levels.

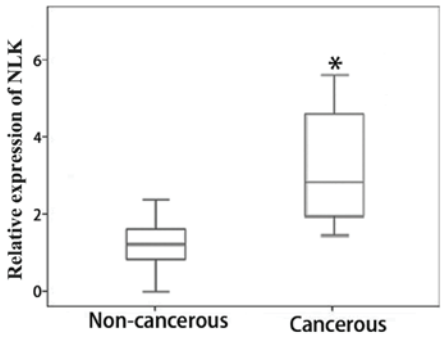

Figure 1. Reverse transcription polymerase chain reaction was performed in order to quantify the differences in nemo-like kinase expression between cancerous and adjacent non-cancerous control tissues (n=50). *P<0.01 vs. non-cancerous tissue. Ct, cycle threshold. Boxes represent percentiles. Bars within represents the median. Error bars represent standard error.

Immunohistochemical analysis. Paraffin-embedded sections fixed in formalin were deparaffinized, rehydrated and incubated with 3% hydrogen peroxidase (Qiagen). The sections were then heated in a microwave oven (1,000 Watts 8503; Kenmore, Chicago, IL, USA) for 3 min at 100˚C for antigen retrieval. Slides were incubated with blocking serum (Qiagen) and primary antibodies for NLK (1:100) overnight at 4˚C. The immunohistochemical reaction was visualized using 0.05% diaminobenzidine followed by counterstaining with hematoxylin. Sections were then examined and analyzed using a microscope (Leica M80; Leica Microsystems, Wetzlar, Germany). Negative control sections were incubated with preimmune rabbit serum (Qiagen) instead of the primary antibodies.

Immunostaining was defined independently using two pathologists blinded to the clinical data and scored by multiplying the staining intensity and the percentage of the stained tumor cells. Staining intensity was graded from 0-3 and the percentage of the stained tumor cells was graded as follows: 0, <5%; 1, 5-25%; 2, 26-50%; 3, 51-75%; and 4, >75%. Final scores ranged from 0 to 12. Samples with overall scores from 0-4 were defined as negative expression, while the samples with scores 5-12 were grouped and defined as positive expression (18). Specimens with inconsistent scores were re-evaluated by two pathologists until an agreement was reached.

Statistical analysis. For categorical variables, values are expressed as the numerical count and the χ^2 test or Fisher's exact test were used to determine the statistical significance of differences between NLK and clinicopathological variables. Kaplan-Meier curves with log-rank tests represented the cumulative survival rate for OS and DFS using NLK expression levels. The Cox proportional hazards model was used to calculate univariate and multivariate hazard ratios

Table I. NLK expression in adjacent normal mucosa, cancerous tissues and LNM tissues.

Tissue sample	n	Expression of NLK		P-value
		Negative, n (%)	Positive, n (%)	
Normal mucosa	406	364 (89.7)	42 (9.9)	<0.001[a]
Cancerous	406	172 (42.4)	234 (57.6)	<0.001[a]
LNM	132	12 (9.1)	120 (90.9)	<0.001[a]

[a]P<0.01 vs. negative NLK expression. NLK, nemo-like kinase; LNM, lymph node metastasis.

Table II. NLK expression and clinicopathological characteristics in colorectal cancer.

Variable	NLK protein expression		P-value
	Negative (n=172)	Positive (n=234)	
Age			
<65	76	86	0.586
≥65	96	148	
Gender			
Male	72	100	0.374
Female	100	134	
pT stage			
pT1	8	8	<0.001[a]
pT2	34	12	
pT3	72	80	
pT4	58	134	
pN stage			
pN0	166	50	0.001[a]
pN1	2	120	
pN2	4	64	
M stage			
M0	170	50	<0.001[a]
M1	2	15	
Vessel invasion			
No	168	200	0.018[a]
Yes	4	34	
Differentiation			
Good	102	96	0.0015[a]
Moderate/poor	70	140	

[a]P<0.01 vs. negative NLK expression. NLK, nemo-like kinase; pT, tumor invasion depth; pN, lymph node metastasis; M, distant metastasis.

for the study variables. P<0.01 was considered to indicate a statistically significant difference between values. All statistical analyses were performed using the SPSS 17.0 statistical software package (SPSS, Inc., Chicago, IL, USA).

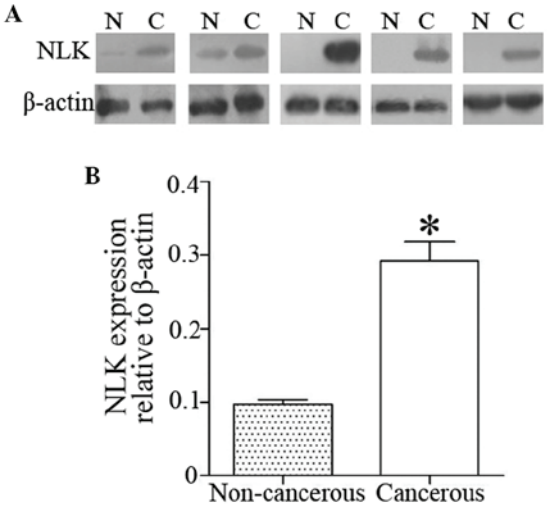

Figure 2. Western blot analysis of NLK in cancerous and adjacent non-cancerous tissues. (A) Representative western blots and (B) relative expression of NLK levels in cancerous and adjacent non-cancerous paired colorectal tissues of patients. β-actin was used as internal control and the χ^2 test was used for statistical analyses. *P<0.01 vs. non-cancerous tissue. NLK, nemo-like kinase; N, non-cancerous; C, cancerous.

Results

NLK upregulation in CRC tissues. Among the 50 paired specimens available for RT-qPCR analysis, the relative expression levels of NLK mRNA showed a minimum of a two-fold increase in 78.0% of tumor tissues compared to those of the adjacent normal mucosa (Fig. 1). This therefore suggested that NLK expression was upregulated in CRC tissues.

In addition, western blot analysis was used to confirm these results in the examined 50 paired tumors and corresponding normal tissues. The positive rate of NLK expression was 66.0% in CRC tissues and 18.0% in the matched non-cancerous normal tissues; therefore, NLK expression was significantly higher in CRC tissues than that in the matched normal colorectal tissues (P<0.01) (Fig. 2A and B).

Correlation between NLK expression and clinicopathological features in CRC. In order to further analyze the clinical and pathological features of NLK expression, immunohistochemical analysis was used to detect NLK protein expression in 406 cases of CRC and paired adjacent noncancerous tissues (Fig. 3). The

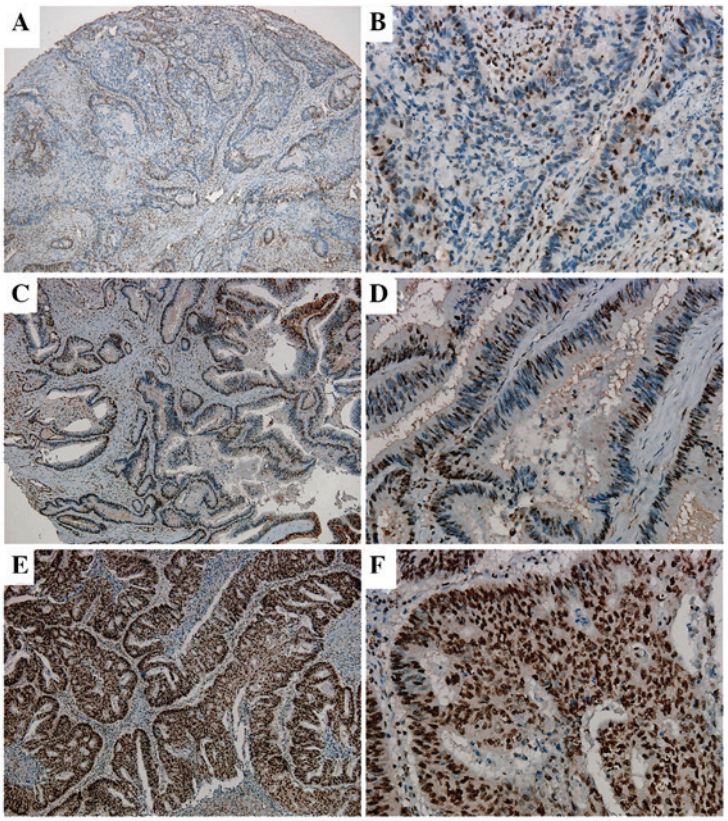

Figure 3. Immunohistochemical analysis of NLK expression in colorectal cancer and adjacent normal colorectal mucosa. (A and B) Weakly positive, 1+; (C and D) moderately positive, 2+; and (E and F) strongly positive, 3+ scoring for nuclear NLK staining [magnification, x100 (A, C, E) and x400 (B, D, F)]. NLK, nemo-like kinase.

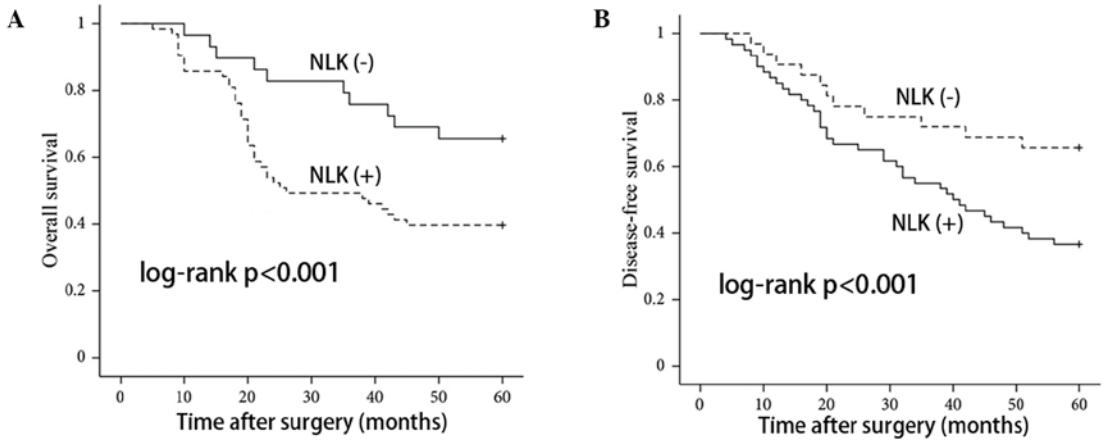

Figure 4. Kaplan-Meier analysis of (A) overall survival and (B) disease-free survival according to NLK expression levels following surgery. P<0.001 betwwn positive and negative NLK expression. NLK, nemo-like kinase.

results demonstrated that 89.7% of non-cancerous specimens were negative for NLK expression; by contrast, 57.6% of CRC specimens exhibited positive NLK expression. In addition, among the 132 LNM tissues, 90.9% displayed positive NLK expression (Table I).

The correlations between NLK protein expression and clinicopathological features are shown in Table II. The positive expression of NLK was significantly correlated with the depth of tumor invasion, LNM, distant metastasis, vascular invasion and histological differentiation. No significant correlations were observed between NLK expression and age or gender. NLK expression levels were found to be significantly higher in the nodal metastasis than those of the CRC and noncancerous tissues (P<0.001). These data indicated that increased NLK expression may correlate with CRC metastasis.

NLK expression and survival analysis. In order to assess the possible associations between NLK expression and

Table III. Univariate and multivariate analysis of survival in 406 colorectal cancer patients.

	OS				DFS			
	Univariate		Multivariate		Univariate		Multivariate	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Age								
<65	1				1			
≥65	1.003 (0.61,1.58)	0.73			0.93 (0.59,1.55)	0.69		
Gender								
Male	1				1			
Female	0.74 (0.43,1.19)	0.46			0.729 (0.44,1.42)	0.53		
pT stage								
pT1	1		1		1		1	
pT2	0.36 (0.07,1.51)	0.18	0.96 (0.21,4.14)	0.96	0.39 (0.09,1.57)	0.16	1.26 (0.22,5.67)	0.88
pT3	0.09 (0.02,0.41)	0.001	0.27 (0.06,1.18)	0.08	0.08 (0.04,0.44)	0.001	0.36 (0.05,1.46)	0.25
pT4	0.34 (0.19,0.56)	<0.001	0.29 (0.15,0.53)	<0.001	0.32 (0.14,0.57)	<0.001	0.42 (0.27,0.84)	0.016
pN stage								
pN0	1		1		1		1	
pN1	0.06 (0.04,0.12)	<0.001	0.68 (0.14,3.09)	0.64	0.06 (0.02,0.18)	<0.001	3.16 (0.67,13.7)	0.19
pN2	0.28 (0.19,0.53)	<0.001	0.38 (0.40,0.69)	<0.001	0.282 (0.14,0.85)	<0.001	0.38 (0.46,0.67)	0.001
M stage								
M0	1		1		1		1	
M1	0.07 (0.04,0.15)	<0.001	0.19 (0.08,0.41)	<0.001	0.05 (0.02,0.08)	<0.001	0.043 (0.01,0.16)	<0.001
Vessel invasion								
No	1		1		1		1	
Yes	0.19 (0.08,0.43)	<0.001	0.72 (0.34,1.42)	0.38	0.01 (0.07,0.26)	<0.001	0.35 (0.17,0.74)	0.006
Differentiation								
Good	1		1		1		1	
Moderate/poor	0.14 (0.07,0.235)	<0.001	0.37 (0.16,0.75)	0.007	0.18 (0.06,0.27)	<0.001	0.355 (0.13,0.78)	0.012
NLK status								
Negative	1		1		1		1	
Positive	0.04 (0.01,0.09)	<0.001	0.035 (0.02,0.19)	<0.001	0.034 (0.02,0.08)	<0.001	0.033 (0.007,0.09)	<0.001

NLK, nemo-like kinase; OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; pT, tumor invasion depth; pN, lymph node metastasis; M, distant metastasis. P<0.01 was considered to indicate a statistically significant difference.

CRC patient survival, Kaplan-Meier curves using log-rank tests for OS and DFS were performed. As shown in Fig. 4, patients with positive NLK expression showed decreased rates of OS and DFS, respectively. In addition, patients with positive NLK expression had a higher recurrence rate than patients with negative expression (P<0.001; data not shown). As shown in Table III, univariate analysis revealed that OS as well as DFS were significantly associated with advanced tumor stage, lymph node metastasis, distant metastasis, histological differentiation, vascular invasion and NLK expression. Multivariate analysis was performed using the Cox proportional hazards model, and the results demonstrated that positive NLK expression remained a significant independent prognostic factor for OS [hazard ratio (HR)=0.035; 95% confidence interval (CI)=0.02-0.19; P<0.001] and DFS (HR=0.033; 95% CI=0.007-0.09; P<0.001).

Discussion

The results of the present study have demonstrated the correlation between elevated NLK expression and human CRC prognosis; of note, correlations were observed between positive NLK expression and aggressive features of CRC, including tumor depth, lymph node metastasis and distant metastasis. This therefore indicated the potential use of NLK as a tumor biomarker for CRC; furthermore, these results suggested that NLK may be used as a novel prognostic marker for more aggressive phenotypes of CRC patients following surgical resection.

The NLK gene, which encodes a proline-directed MAPK family member, was identified in 1994 (19). Functional analyses have demonstrated that NLK contributed to numerous signaling pathways via its ability to phosphorylate diverse transcription factors (20). NLK was reported to be a pivotal regulator of the Wnt/β-catenin signaling pathway (21). In addition, NLK has also been shown to regulate the activity of multiple transcription factors, including NF-κB, Smads and p53 (22). Therefore, the reported involvement of NLK in numerous signaling pathways has demonstrated its vital role in mediating cell signals. Studies of NLK function in human cancers have also confirmed the role of NLK in cell growth and proliferation. In addition, NLK was reported to function as a tumor suppressor gene or an oncogene in different types of cancers; for example, data have demonstrated that NLK expression was decreased during prostate cancer progression and indicated that NLK inhibited androgen receptor (AR) expression and subsequent AR-mediated transcription as well as promoted apoptosis in prostate cancer cell lines (14). In a previous study, clinicopathological analysis revealed that NLK expression levels were significantly higher in human glioma tissues compared with those of lower grade tumors and the survival rate of glioma patients expressing low levels of NLK was significantly decreased compared to that of patients with gliomas expressing high levels of NLK (16). Conversely, NLK was reported to act as an oncogene in certain types of tumors. A previous study demonstrated the mitogenic potential of NLK in hepatocellular carcinomas through siRNA-mediated disruption of NLK, which was shown to inhibit proliferation of Hep3B cells and arrest cell cycle transition (15). These discrepancies may be due to the different pathologies of the types of tumors being studied. In the present study, NLK expression was shown to be significantly upregulated in CRC tissues compared to that of the paired non-cancerous samples, indicating the involvement of NLK in CRC progression.

A recent study reported that NLK overexpression was associated with the progression of gallbladder cancer and that NLK may have potential for use as a prognostic marker (23). Therefore, the present study aimed to analyze the correlations between NLK expression and the clinicopathological features of CRC patients as well as clinical outcome. These results revealed that positive NLK expression was significantly correlated with the depth of tumor invasion, lymph node metastasis, distant metastasis, histological differentiation, vascular invasion and advanced tumor stage. Kaplan-Meier survival analysis demonstrated that positive NLK expression was negatively correlated with the decreased overall survival rate of CRC patients. Of note, Cox multivariate analysis revealed that NLK expression was an independent factor in predicting OS and DFS for CRC patients. In conclusion, the results of the present study indicated that NLK may have a crucial role in promoting the aggressive phenotypes of CRC and therefore may have the potential for use as a prognostic marker of CRC.

Acknowledgements

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