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Overexpression of NEK3 is associated with poor prognosis in patients with gastric cancer

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Abstract

The NIMA-related kinase 3 (NEK3) plays an important role in cell migration, cell proliferation, and cell viability. Recently, NEK3 was reported to enhance the malignancy of breast cancer. However, its role in gastric cancer has not been completely characterized. In this study, we explored the prognostic significance of NEK3 in human gastric cancer. Reverse transcription-polymerase chain reaction and western blot were performed to detect the NEK3 mRNA and protein expression in 6 paired fresh human gastric cancer tissues and surrounding normal tissues. NEK3 levels in gastric cancer and its adjacent normal samples of 168 cases were detected by immunohistochemistry, and the relationships between the NEK3 level and various clinicopathological features were analyzed. NEK3 mRNA and protein were significantly overexpressed in gastric cancer tissues, compared with adjacent normal tissues. Immunohistochemistry staining assay showed the percentage of high NEK3 expression in gastric cancer samples was higher than that in adjacent normal samples. NEK3 overexpression was significantly correlated with pT stage, pathologic TNM stage, lymph node metastasis, and poor prognosis of gastric cancer. Cox multivariate regression analyses suggested that NEK3 was an independent prognostic factor for survival of patients with gastric cancer. The data demonstrate that NEK3 is overexpressed in gastric cancer, which promotes the malignancy of gastric cancer. NEK3 may be as a prognostic biomarker and a potential therapeutic target for gastric cancer.

Abbreviations: AJCC = American Joint Committee on Cancer, DFS = disease-free survival, LOH = loss of heterozygosity, NEK3 = NIMA-related kinase 3, NIMA = never in mitosis gene A, OS = overall survival, PVDF = polyvinylidene difluoride filter, SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Keywords: gastric cancer, NIMA-related kinase 3, prognosis

1. Introduction

Human gastric cancer is one of the leading causes of cancer-related deaths around the world, especially in China and other East Asian countries.[1–3] To date the mechanisms of the pathogenesis in gastric cancer are still not well understood. Although great progress in the diagnosis and treatment of gastric cancer, the outcome of patients with gastric cancer remains poor, with a 5-year survival rate of <25%.[1,4] Currently, therapeutic strategies for gastric cancer involving surgery, chemotherapy and radiotherapy remain unsatisfactory.[5] Furthermore, due to late diagnosis, most patients are diagnosed at an advanced stage, which usually indicates a poor prognosis.[6] Therefore, many researches focus on the prognostic factors for gastric cancer, which can be used as prognostic marker and potential treatment target and improve the prognosis of patients with gastric cancer.[7–10]

It is now known that the never in mitosis gene A (NIMA)-related kinases (NEKs) have been identified in Drosophila, Xenopus, mice, and humans. Eleven genes encoding NEK1 to NEK11 were identified in human cells.[11] Previous studies showed that NEKs were involved in cell cycle, checkpoint control, and cancer.[11,12] The function of NEK3 is still not well characterized, compared with other members of NEK family. NEK3 contains a conserved N-terminal catalytic kinase domain and 2 predicted PEST motifs, which regulate both protein–protein interactions and protein stability.[13] Previous studies indicated that human NEK3 has a similar preference, which was involved in cell migration, cell proliferation, cell viability, and neuronal development.[12,14–16] However, its role in cancer development is still unclear. Recent studies showed that NEK3 was involved in breast cancer and some cancer cell lines.[14,17]

In this study, we studied the expression of NEK3 in human gastric cancer specimens. The relationship between NEK3 expression and clinical features or prognosis of gastric cancer was analyzed. The data demonstrate that NEK3 is overexpressed in gastric cancer, which was significantly correlated with pT stage, pathologic TNM (pTNM) stage, lymph node metastasis, and poor prognosis of gastric cancer. This study may help to better understand the mechanisms of gastric cancer development and to find promising prognostic markers of gastric cancer and potential therapeutic targets for gastric cancer.

2. Materials and methods

2.1. Patients and tissue samples

Paired gastric cancer and its adjacent normal specimen were collected from 168 patients who underwent surgical resection at the Surgery Department of the Affiliated Tumor Hospital of

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Nantong University between 2005 and 2008. All patients have not been treated by systemic chemotherapy or radiotherapy before operation. Specimens were fixed in formalin and then embedded in paraffin for immunohistochemistry after surgical removal. In addition, 3 paired fresh cancer tissue and its adjacent normal tissue were snap-frozen in liquid nitrogen for western blot analysis. Use of tissue for this study was approved by the Institutional Review Board of Nantong University (IRB20050068). All patients provided written informed consent. The follow-up time was 1 to 96 months. The main clinical and pathological features of patients are summarized in Table 1. Tumors were classified according to American Joint Committee on Cancer (AJCC) stage.[18]

2.2. RT-PCR analysis

The total RNA was isolated from cancer and paracancer specimens were analyzed using protocol described previously by Li.[19] The first strand cDNA was synthesized using RevertAidTM First Strand cDNA Synthesized Kit (Fermentas, Burlington, Canada). First Strand cDNA was subsequently subjected to Corbett RG-6000 PCR system (QIAGEN, Dusseldorf, German) using Fast Start Universal SYBR Green Master Mix (Roche, Basel, Switzerland). The sense and antisense primers were synthesized as follows: GAPDH 5′-GCAAGTTCAAC GGCACAG-3′, 5′-GCCAGTAGACTCCACGACAT-3′; NEK3 5′-GGGGTACCGAGCCACCATGGATGACTACATGGTC-3′, 5′-AATTTGCGGCCGCCATCTGTCGCACAGGCCTTG-3′. Quantitative real-time PCR were carried out on the Corbett RG-6000 PCR system under the following condition: after an initial denaturation at 95°C for 5 minutes, 40 cycles of denaturation (94°C for 15 seconds), annealing (60°C for 20 seconds), and extension (72°C for 20 seconds) for the target gene. The fold change in gene expression was evaluated by the $2^{-\Delta\Delta}$Ct method.

Table 1

The correlation between clinicopathological factors and NEK3 expression.

		NEK3 expression		
	Patients, n	Low, n (%)	High, n (%)	*P* value
All patients	168	43 (25.60)	125 (74.40)	
Age, years				
≤60	62	19 (30.65)	43 (69.35)	.251
>60	106	24 (22.64)	82 (77.36)	
Gender				
Male	108	30 (27.78)	78 (72.22)	.384
Female	60	13 (21.67)	47 (78.33)	
Tumor size, cm				
≤5	90	22 (24.44)	68 (75.56)	.714
>5	78	21 (26.92)	57 (73.08)	
Tumor site				
Upper	66	15 (22.73)	51 (77.27)	.493
Middle/Lower	102	28 (27.45)	74 (72.55)	
Degree of differentiation				
Well/ Moderate	61	17 (27.87)	44 (72.13)	.610
Poor/Not	107	26 (24.30)	81 (75.70)	
pT stage				
T1/T2	70	26 (37.14)	44 (62.86)	**.004**
T3/T4	98	17 (17.35)	81 (82.65)	
pTNM stage				
I/II	68	27 (39.71)	41 (60.29)	**.001**
III/IV	100	16 (16.00)	84 (84.00)	
Lymph node metastasis				
No	57	22 (38.60)	35 (61.40)	**.006**
Yes	111	21 (18.92)	90 (81.08)	

NEK3 = never in mitosis gene A-related kinase 3; pTNM = pathologic TNM.

2.3. Western blot analysis

Western blot was performed in accordance with a previous study.[20] In brief, the tissue samples were immediately homogenized in a lysis buffer and complete protease inhibitor cocktail (Roche Diagnostics), and then centrifuged at 12,000*g*, 4°C for 15 minutes to collect the supernatant. The protein samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation and then transferred to polyvinylidene difluoride filter (PVDF) membranes (Millipore, Bedford, MA), then incubated with rabbit polyclonal anti-NEK3 antibody (1:200, Abgent) and mouse monoclonal anti-β-actin antibody (1:500, Santa Cruz) overnight at 4°C, then incubated with horseradish peroxidase-linked goat anti-rabbit or mouse IgG (Pierce Biotechnology, Rockford, IL) at a dilution of 1:5000. The detection of chemiluminescent signals was performed by the electrochemiluminescent method (ZhongShan Biotech Company, China).

2.4. Immunohistochemistry

Immunohistochemistry was performed in accordance with previous studies.[20,21] In brief, sample sections were incubated overnight at 4°C with rabbit polyclonal anti-NEK3 antibody (1:200, Abgent), followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:500, Santa Cruz, Bolivia). The sections were counterstained with hematoxylin, and then mounted for observation under the DM IL LED microscope (Leica Microsystems GmbH). The immunostaining results were independently assessed by 2 pathologists who blinded to the clinical data of the patients. The intensity of immunostaining was graded as 0 (no or weak staining), 1 (moderate staining), and 2 (strong staining). At least 5 areas of each section were viewed and the percentage of $NEK3^+$ cells was scored according to the following criteria: 1 (<50% $NEK3^+$ cells), 2 (50–75% $NEK3^+$ cells), and 3 (>75% $NEK3^+$ cells). Then, A semiquantitative histopathology score was obtained by multiplying the staining intensity score with the percentage score. The average of histopathology score was applied as the cut-off to differentiate between low and high expression of NEK3.

2.5. Statistical analysis

Statistical analysis was performed by statistics package for social science 21.0 (SPSS 21.0). The expression of NEK3 mRNA and protein of samples was analyzed using a *t*-test. The relationship between NEK3 expression and clinicopathological features was analyzed using the Pearson χ^2 test. Multivariate analysis was constructed using the Cox regression model. The overall survival (OS) and disease-free survival (DFS) of patients were performed using the Kaplan–Meier curves and differences were analyzed using the log-rank test. A *P*-value <.05 were considered statistically significant.

3. Results

3.1. NEK3 expression was upregulated in gastric cancer tissues

The expression pattern of NEK3 in 6 paired cancer and adjacent normal tissues was detected by reverse transcription polymerase

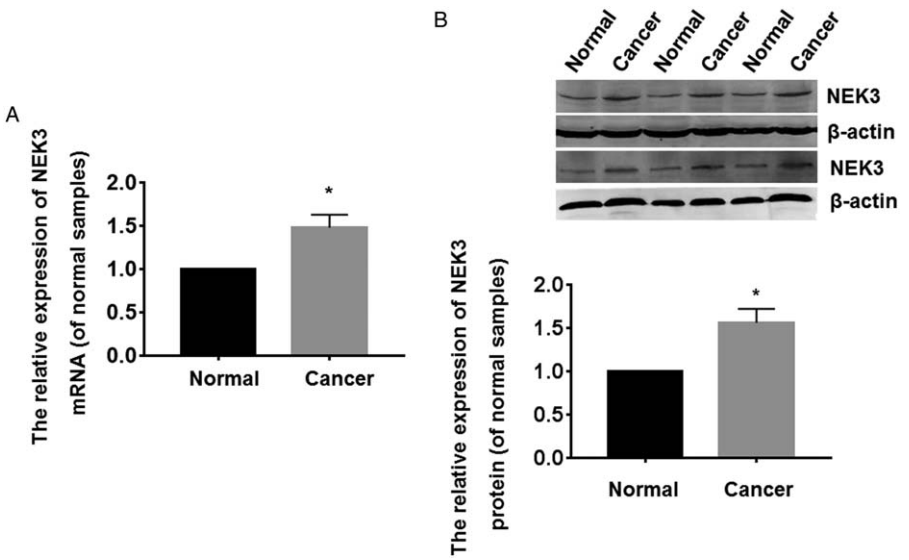

Figure 1. (A) The expression of NEK3 mRNA in cancers and adjacent normal tissues was detected by RT-PCR. The NEK3 mRNA expression was remarkably upregulated in cancer tissues, compared with adjacent normal ones. (B) The expression of NEK3 protein in cancers and adjacent normal tissues was detected by western blot. The NEK3 protein expression was remarkably upregulated in cancer tissues, compared with adjacent normal ones. $^{*}P<.05$ vs normal. NEK3 = never in mitosis gene A-related kinase 3, RT-PCR = reverse transcription polymerase chain reaction.

chain reaction (RT-PCR) and western blot analysis. Compared with adjacent normal tissues, the NEK3 mRNA and protein expression in cancer tissues was significantly upregulated (Fig. 1A and B), and the difference between the cancer and normal samples was statistically significant (Fig. 1A and B). Then, the NEK3 expression in 168 specimens was further investigated using immunohistochemical assay. In this way, high NEK3 expression was found in most cancer tissues, whereas low or no expression of NEK3 was observed in adjacent normal tissues (Fig. 2A). The high expression of NEK3 was more frequent in 125 of

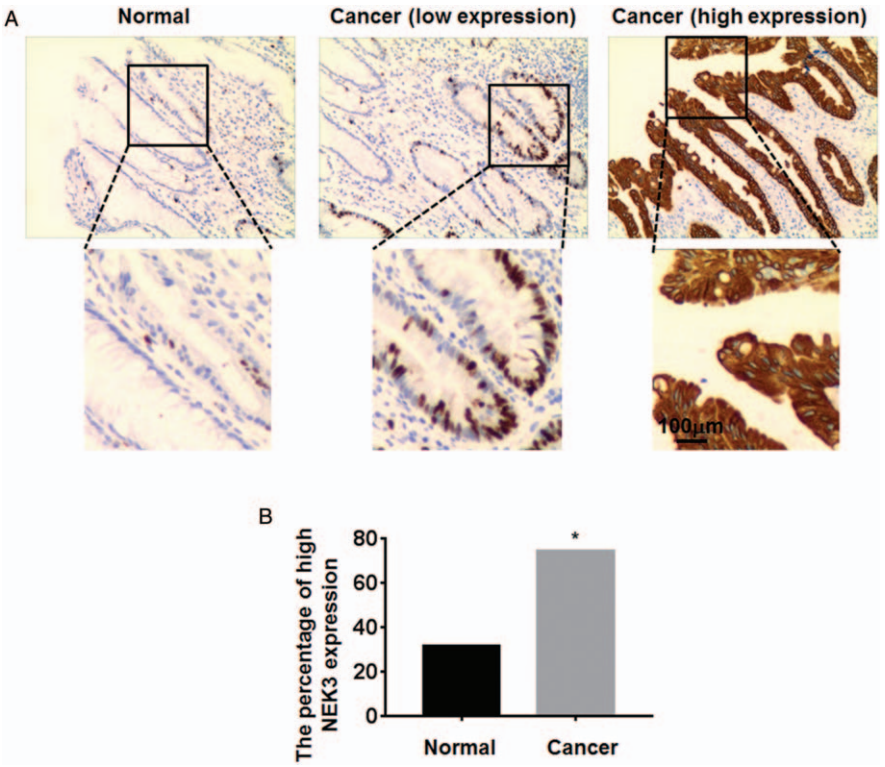

Figure 2. NEK3 expression in 168 specimens was detected using immunohistochemistry. (A) NEK3 was highly expressed in most tumor tissues, whereas low or no expression of NEK3 was observed in adjacent normal tissues. Bar=100 μm. (B) The high expression of NEK3 was more frequent in 125 of 168 (74.40%) cases than that in normal samples (53 of 168; 31.55%). $^{*}P<.05$, vs normal. NEK3 = never in mitosis gene A-related kinase 3.

168 (74.40%) cases than that in normal samples (53 of 168; 31.55%) (Fig. 2B).

3.2. Relationships between NEK3 expression and clinicopathological features in patients with gastric cancer

The association of NEK3 expression with clinicopathological features of 168 patients with gastric cancer was evaluated by Pearson χ^2 test. The results showed that NEK3 expression was significantly correlated with pT stage, pTNM stage, and lymph node metastasis (Table 1). The results indicated the over-expression of NEK3 may be indicative in the determination of clinical outcome of gastric cancer.

3.3. Prognostic significance of NEK3 expression in gastric cancer patients

When the relationship between all clinicopathological features and survival status was explored by the Pearson χ^2 test, it was found that degree of differentiation, pT stage, pTNM stage, lymph node metastasis, and NEK3 expression significantly influenced the patients' survival status (Table 2). The multivariate Cox regression analysis model showed that degree of differentiation, pTNM stage, lymph node metastasis, and NEK3 expression were independent prognostic factors in patients with gastric cancer (Table 3). Moreover, at the end of clinical follow-up, the correlation between NEK3 expression and OS or DFS was analyzed by Kaplan–Meier analysis. The Kaplan–Meier survival curves revealed that the OS and DFS of gastric cancer patients with high NEK3 expression were worse than those of patients with low NEK3 expression (Fig. 3). Take these results together, high expression of NEK3 may serve as a predictor of poor prognosis in gastric cancer.

Table 2

The correlation between clinicopathological factors and survival status in 168 gastric cancer patients.

	Patients, n	Survival		*P* value
		Yes, n (%)	No, n (%)	
All patients	168	47 (27.98)	121 (72.02)	
Age, years				
≤60	62	20 (32.26)	42 (67.74)	.344
>60	106	27 (25.47)	79 (74.53)	
Gender				
Male	108	33 (30.56)	75 (69.44)	.318
Female	60	14 (23.33)	46 (76.67)	
Tumor size, cm				
≤5	90	24 (26.67)	66 (73.33)	.685
>5	78	23 (29.49)	55 (70.51)	
Tumor site				
Upper	66	18 (27.27)	48 (72.73)	.870
Middle/Lower	102	29 (28.43)	73 (71.57)	
Degree of differentiation				
Well/ Moderate	61	25 (40.98)	36 (59.02)	**.005**
Poor/not	107	22 (20.56)	85 (79.44)	
pT stage				
T1/T2	70	28 (40.00)	42 (60.00)	**.003**
T3/T4	98	19 (19.39)	79 (80.61)	
pTNM stage				
I/II	68	30 (44.12)	38 (55.88)	**.000**
III/IV	100	17 (17.00)	83 (83.00)	
Lymph node metastasis				
No	57	26 (45.61)	31 (54.39)	**.000**
Yes	111	21 (18.92)	90 (81.08)	
NEK3 expression				
Low	43	20 (46.51)	23 (53.49)	**.002**
High	125	27 (21.60)	98 (78.40)	

NEK3 = never in mitosis gene A-related kinase 3; pTNM = pathologic TNM.

Table 3

The Cox multivariate analysis for OS.

Factors	Hazard ratio	95% CI	*P* value
Age	0.643	0.269–1.537	.320
Gender	0.473	0.146–1.364	.166
Tumor size	0.795	0.296–2.136	.648
Tumor site	1.005	0.641–1.573	.984
Degree of differentiation	2.206	1.164–4.180	**.015**
pT stage	1.932	0.864–4.321	.109
pTNM stage	1.520	1.005–2.300	**.047**
Lymph node metastasis	2.300	1.225–4.318	**.009**
NEK3 expression	0.156	0.060–0.402	**<.001**

NEK3 = never in mitosis gene A-related kinase 3, OS = overall survival.

4. Discussion

More recent studies have indicated that human NEK3 have a similar preference, which was involved in cell migration, cell proliferation, cell viability, and neuronal development.[12,14–16] But its role in cancer development is still unclear. To date the relative researches are still very little. Loss of heterozygosity (LOH) studies showed chromosome region 13q14 frequently is lost in several cancers such as prostate,[22,23] hepatocellular,[24,25] lung,[26,27] and oral cancer.[28,29] Interestingly, NEK3 is located on this region. Hernandez and Almeida[17] found an A insertion deletion polymorphism in exon 9 and an alternative transcript variant that skip exon 10 by analyzing the coding region of NEK3 in a set of prostate tumors, xenografts, and cell lines. An association between NEK3 A insertion/deletion polymorphism and cancers with alterations at 13q14 was observed. The result indicated NEK3 may play a role in cancer development. Miller et al[30] reported that a significant upregulation of NEK3 expression in malignant breast cancer tissue versus normal specimens. They documented that NEK3 contributed to prolactin-mediated breast cancer motility through mechanisms involving Rac1 activation and paxillin phosphorylation. Harrington and Clevenger[14] found threonine residue 165 (Thr-165) as a major site that regulated the activity and function of NEK3, the phosphorylation of NEK3 Thr-165 contributed to its regulation of breast cancer cell migration, focal adhesion remodeling, and actin cytoskeletal rearrangement, which promoted an invasive breast cancer phenotype.

Although the role of NEK3 has been documented in breast cancer, whether NEK3 was involved in gastric cancer has yet not been defined. In this report, we explored the potential role of NEK3 in gastric cancer. Western blot analyses showed that NEK3 protein was significantly upregulated in 6 paired fresh gastric cancer tissues, compared with the adjacent normal tissues. To confirm this result, NEK3 level in gastric cancer and its adjacent normal samples of 168 cases were detected by immunohistochemistry. Immunohistochemistry staining assay showed NEK3 expression in gastric cancer samples was significantly higher than that in adjacent normal samples. Moreover, we evaluated the associations of NEK3 expression and clinicopathological characteristics and prognosis in gastric cancer. Notably, NEK3 overexpression was correlated with pT stage, pTNM stage, and lymph node metastasis. In addition,

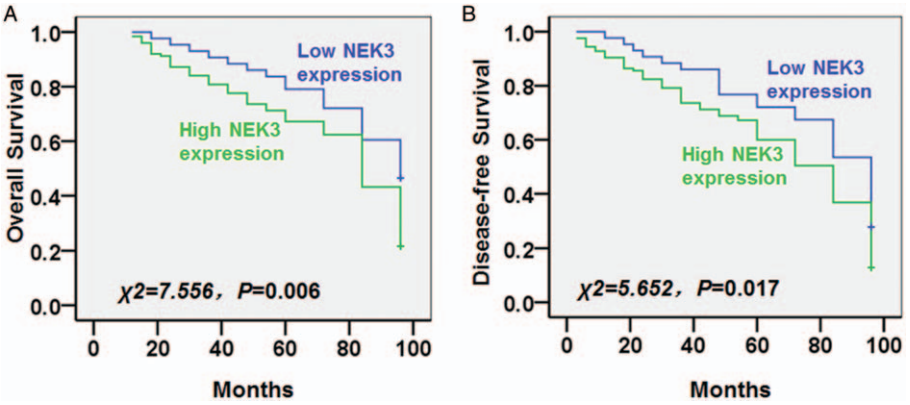

Figure 3. The correlation between NEK3 expression and patients' survival was explored by Kaplan–Meier analysis. The Kaplan–Meier survival curves revealed that gastric cancer patients with high NEK3 expression had significantly worsened OS and DFS, compared with those with low NEK3 expression. DFS = disease-free survival, NEK3 = never in mitosis gene A-related kinase 3, OS = overall survival.

gastric cancer patients with high NEK3 expression possessed a significantly shorter OS and DFS, compared with patients with low NEK3 expression. Furthermore, multivariate analyses demonstrated that NEK3 served as an independent prognostic factor for survival of patients with gastric cancer.

5. Conclusion

Taken together, our data demonstrate that NEK3 is overexpressed in gastric cancer, which promotes the malignancy of gastric cancer. NEK3 may be as a prognostic biomarker and a potential therapeutic target for gastric cancer. However, further investigations are certainly needed in order to deeply understand its role in gastric cancer.

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