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

Yongfeng Cao, MD\textsuperscript{a,b}, Jiaye Song, MD\textsuperscript{b}, Jia Chen, MD\textsuperscript{b}, Jinzhang Xiao, MD\textsuperscript{b}, Jingyi Ni, MD\textsuperscript{b}, Changping Wu, PhD\textsuperscript{a,*}

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.\textsuperscript{[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 \textless 25\%.\textsuperscript{[4]} Currently, therapeutic strategies for gastric cancer involving surgery, chemotherapy and radiotherapy remain unsatisfactory.\textsuperscript{[5]} Furthermore, due to late diagnosis, most patients are diagnosed at an advanced stage, which usually indicates a poor prognosis.\textsuperscript{[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.\textsuperscript{[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.\textsuperscript{[11]} Previous studies showed that NEKs were involved in cell cycle, checkpoint control, and cancer.\textsuperscript{[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.\textsuperscript{[13]} Previous studies indicated that human NEK3 has a similar preference, which was involved in cell migration, cell proliferation, cell viability, and neuronal development.\textsuperscript{[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.\textsuperscript{[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
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.\textsuperscript{[18]}

2.2. RT-PCR analysis

The total RNA was isolated from cancer and paracarcinoma specimens were analyzed using protocol described previously by Li.\textsuperscript{[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, Germany) using Fast Start Universal SYBR Green Master Mix (Roche, Basel, Switzerland). The sense and antisense primers were synthesized as follows: GAPDH 5'-GGCAAGTTCACAGGCACAG-3', 5'-GCCCAGTAGACTCCACGACAT-3'; NEK3 5'-GATTTGCGGCCGCCATCTGTC-3', 5'-AAATTTGCGGCCGCCATCTGTCGCAAGCGCCTTG-3'. Quantitative real-time PCR were carried out on the Corbett RG-6000 PCR system under the following conditions: 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\textsuperscript{-ΔΔCt} method.

2.3. Western blot analysis

Western blot was performed in accordance with a previous study.\textsuperscript{[20]} In brief, the tissue samples were immediately homogenized in a lysis buffer and complete protease inhibitor cocktail (Roche Diagnostics), and then centrifuged at 12,000g, 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.\textsuperscript{[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\textsuperscript{+} cells was scored according to the following criteria: 1 (<50% NEK3\textsuperscript{+} cells), 2 (50–75% NEK3\textsuperscript{+} cells), and 3 (>75% NEK3\textsuperscript{+} 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\textsuperscript{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

\begin{table}[h]
\centering
\caption{The correlation between clinicopathological factors and NEK3 expression.}
\begin{tabular}{|c|c|c|c|c|}
\hline
& Patients, n & Low, n (%) & High, n (%) & P value \\
\hline
All patients & 168 & 43 (25.60) & 125 (74.40) & \\
Age, years & & & & \\
\leq 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 & & & & \\
\leq 5 & 90 & 22 (24.44) & 68 (75.56) & .714 \\
>5 & 78 & 21 (26.92) & 57 (73.08) & \\
Tumor site & & & & \\
Upper & 66 & 15 (23.13) & 51 (77.27) & .493 \\
Middle/Lower & 102 & 28 (27.55) & 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 & 98 & 27 (27.55) & 71 (72.45) & .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) & \\
\hline
\end{tabular}
\end{table}
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 168 specimens (74.40%) than that in normal samples (53 of 168; 31.55%). $P<.05$, vs normal. NEK3 = never in mitosis gene A-related kinase 3.

**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.

**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.
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 χ² 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 overexpression of NEK3 may be indicative in the determination of clinical outcome of gastric cancer. 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, 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.

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 χ² 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

Table 2
The correlation between clinicopathological factors and survival status in 168 gastric cancer patients.

| Patients, n | Yes, n (%) | No, n (%) | P value |
|------------|------------|-----------|---------|
| All patients | 168 | 47 (27.98) | 121 (72.02) | .428 |
| 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) | .870 |
| 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/I | 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 | .220 |
| 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 | 0.884–4.321 | .109 |
| pTNM stage | 1.520 | 1.005–2.300 | .047 |
| Lymph node metastasis | 2.300 | 1.226–4.318 | .009 |
| NEK3 expression | 0.156 | 0.060–0.402 | <.001 |

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.

References

[1] Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010;127:2893–917.
[2] Siegel R, Ma J, Zou Z, et al. Cancer statistics, 2014. CA Cancer J Clin 2014;64:9–29.
[3] Malvezzi M, Bonifazi M, Bertuccio P, et al. The prognostic value of E-cadherin in gastrointestinal cancer. Exp Rev Anticancer Ther 2007;7:465–9.
[4] Catalano V, Labianca R, Beretta GD, et al. Gastric cancer. Crit Rev Oncol Hematol 2009;71:127–64.
[5] Mayer RJ, Venook AP, Schilsky RL. Progress against GI cancer during the American Society of Clinical Oncology’s first 50 years. J Clin Oncol 2014;32:1521–30.
[6] Chiba T, Marusawa H, Ushijima T. Inflammation-associated cancer development in digestive organs: mechanisms and roles for genetic and epigenetic modulation. Gastroenterology 2012;143:550–63.
[7] Luo D, Zhang B, Lv L, et al. Methylation of CpG islands of p16 associated with progression of primary gastric carcinomas. Oncogene 2006;25:1050–7.
[8] Lin YW, Sheu JC, Liu LY, et al. Loss of heterozygosity at chromosome 13q in hepatocellular carcinoma. J Pathol 2002;196:8–13.
[9] Lin YW, Sheu JC, Liu LY, et al. Loss of heterozygosity at chromosome 13q in hepatocellular carcinoma: identification of three independent regions. Eur J Cancer 1999;35:1730–4.
[10] Wong CM, Lee JM, Lau TC, et al. Clinicopathological significance of loss of heterozygosity on chromosome 13q in hepatocellular carcinoma. Clin Cancer Res 2002;8:2266–72.
[11] Goeze A, Schluns K, Wolf G, et al. Chromosomal imbalances of primary and metastatic lung adenocarcinomas. J Pathol 2002;196:8–16.
[12] Beder LB, Gunduz M, Ouchida M, et al. Genome-wide analyses on loss of heterozygosity in neuroblastoma cell lines. J Pediatr Hematol Oncol 2003;25:31–40.
[13] Huang XP, Wei F, Liu XY, et al. Allelic loss on 13q in esophageal squamous cell carcinomas from northern China. Cancer Res 2000;60:4894–906.
[14] Gozee A, Schwab K, Wolf G, et al. Chromosomal imbalances of primary and metastatic lung adenocarcinomas. J Pathol 2002;196:8–16.
[15] Chang J, Baloh RH, Milbrandt J. The NIMA-family kinase Nek3 regulates microtubule acetylation in neurons. J Cell Sci 2009;122:pt 13;2274–82.
[16] Kimura M, Okano Y. Molecular cloning and characterization of the human NIMA-related protein kinase 3 gene (NEK3). Cytogenet Cell Genet 2001;95:177–82.
[17] Hernandez M, Almeida TA. Is there any association between nek3 and cancers with frequent 13q14 deletion? Cancer Invest 2006;24:682–8.
[18] Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol 2010;17:1471–4.
[19] Lin H, Qin J, Jin G, et al. Overexpression of Lhx8 inhibits cell proliferation and induces cell cycle arrest in PC12 cell line. In Vitro Cell Dev Biol Anim 2015;51:329–35.
[20] Xu B, Jiang D, Chen Y, et al. High CHMP4B expression is associated with accelerated cell proliferation and resistance to doxorubicin in hepatocellular carcinoma. Tumour Biol 2015;36:2569–81.
[21] Lv L, Wan C, Chen B, et al. Nemo-like kinase (NLK) inhibits the progression of NSCLC via negatively modulating WNT signaling pathway. J Cell Biochem 2014;115:81–92.
[22] Girard L, Zochbauer-Muller S, Virmani AK, et al. Assessing the location of a putative human prostate cancer tumor suppressor gene at chromosome 13q14.3. Oncogene 1999;18:7576–83.
[23] Lin YW, Sheu JC, Liu LY, et al. Loss of heterozygosity at chromosome 13q in hepatocellular carcinoma: identification of three independent regions. Eur J Cancer 1999;35:1730–4.
[24] Hirata H, Meziade P, Kato S, et al. Genome-wide allelotyping of lung cancer identifies novel regions of allelic loss, differences between small cell lung cancer and non-small cell lung cancer, and loci clustering. Cancer Res 2000;60:4894–906.
[25] Goeze A, Schwab K, Wolf G, et al. Chromosomal imbalances of primary and metastatic lung adenocarcinomas. J Pathol 2002;196:8–16.
[26] Beder LB, Gunduz M, Ouchida M, et al. Genome-wide analyses on loss of heterozygosity in head and neck squamous cell carcinomas. J Pathol 2003;202:859–866.
[27] Huang XP, Wei F, Liu XY, et al. Allelic loss on 13q in esophageal squamous cell carcinomas from northern China. Cancer Lett 2002;181:155–64.
[28] Miller SR, Antico G, Raghunath PN, et al. Nek3 kinase regulates prolactin-mediated cytoskeletal reorganization and motility of breast cancer cells. Oncogene 2007;26:4668–78.