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Original Article

Abnormal expression of Nek2 in pancreatic ductal adenocarcinoma: a novel marker for prognosis

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Abstract: Nek2 is a serine/threonine kinase that has a critical role in mitosis during the cell division process. Despite its importance in centrosome regulation and spindle formation, no direct binders are reported between human pancreatic cancer and Nek2 protein. Our aim in studying Nek2 expression and survival in PDA patients is to determine whether Nek2 is a valuable prognostic factor in PDA tumorigenesis. We found that Nek2 mRNA was elevated in PDA tissues. A high level of expression of Nek2 was significantly correlated with histological differentiation (P=0.042), lymph node metastasis (P=0.003) and tumor stage (P=0.001). Patients with a high Nek2 expression had a significantly worse overall survival (OS) than those patients with low Nek2 expression (P=0.002). Univariate and multivariate analysis revealed that high expression of Nek2 could serve as an independent predictor of poor prognosis. These results indicate that Nek2 could be a promising prognostic molecular marker and an attractive therapeutic target for PDA.

Keywords: Nek2, pancreatic ductal adenocarcinoma, prognosis

Introduction

Pancreatic cancer is one of the most fatal cancers and is the fourth leading cause of cancer-related deaths in the United States, with less than 5% overall 5-year survival [1]. According to the American Cancer Society, there were an estimated 45,220 new cases and 38,460 deaths from pancreatic cancer in the United States in 2013 [2]. Pancreatic ductal adenocarcinoma (PDA) that originates in the glandular epithelium accounts for more than 90% of all malignant pancreatic tumors and exhibits a high degree of malignancy. Distant metastasis occurs in more than 50% of PDA patients at the time of diagnosis, and most patients die within 1 year after diagnosis [3]. Even for the PDA patients who undergo pancreatectomy, the disease commonly recurs and the prognosis is poor, with a 5-year survival rate of 10-20% [4]. These grim statistics are due to late detection, aggressive nature, and resistance to traditional therapy.

Recent studies have identified some important molecular changes involving mutations or deletions affecting oncogenes and tumor suppressor genes in PDA, such as the Kirsten rat sarcoma viral oncogene homolog (KRAS), epidermal growth factor receptor (EGFR), tumor protein 53 (TP53), Sma- and Mad-related family member 4/deleted in pancreatic cancer locus 4 (SMAD4/DPC4) and P16/cyclin-dependent kinase inhibitor 2A (P16/CDKN2A) [5]. These changes affect key pathways that regulate proliferation, apoptosis, invasion and migration. However, the mechanisms of PDA tumorigenicity and progression remain unclear. Therefore, there is an urgent need to explore novel cancer-related markers to serve as diagnostic markers and molecular targets for PDA.

NIMA-related kinase 2 (Nek2) is an evolutionary conserved serine/threonine kinase involved in regulating cell cycle and mitosis by centrosome splitting during the cell division process [6]. Uncontrolled Nek2 activity can lead to chromo-

Table 1. Correlation between Nek2 protein expression and clinicopathologic features in PDA Patients

Variables	Nek2		χ^2	*P*-value
	Low	High		
Gender				
Male	28 (38.9)	44 (61.1)	2.768	0.096
Female	34 (53.1)	30 (46.9)		
Age (years)				
<60	25 (49.0)	26 (51.0)	0.387	0.534
≥60	37 (43.5)	48 (56.5)		
Tumor location				
Head	28 (41.8)	39 (58.2)	0.768	0.381
Body/tail	34 (49.3)	35 (50.7)		
Histological differentiation				
Well/moderately	41 (55.4)	33 (44.6)	6.307	**0.012**
Poorly	21 (33.9)	41 (66.1)		
Lymph node metastasis				
Present	30 (36.6)	52 (63.4)	6.748	**0.009**
Absent	32 (59.3)	22 (40.7)		
Clinical stage (pTNM)				
Early stage (I-II)	37 (54.4)	31 (45.6)	4.269	**0.039**
Advanced stage (III-IV)	25 (36.8)	43 (63.2)		
Respectability				
RR	54 (49.5)	55 (50.5)	3.459	0.063
PR	8 (29.6)	19 (70.4)		

Bold values indicate P<0.05. RR indicates radical resection; PR indicates palliative resection.

some instability (CIN) as well as abnormal chromosome content [7, 8]. The elevation of Nek2 contributes to the formation of centrosomal abnormalities, monopolar spindles, and promotes aneuploidy by disrupting the control of the mitotic checkpoint [9-11]. Nek2 overexpression has been observed in several human cancer cell lines, including breast cancer, cervical cancer, prostate cancer and lymphomas [12], and its downregulation inhibits cell proliferation [13]. Increased expression of Nek2 has been reported to be involved in tumor progression and cell-cycle progression, and associated with poor prognosis in breast cancer [14-18]. Meanwhile, experimental evidence has suggested that Nek2 could predict treatment resistance in ovarian and breast cancers [19-21].

So far, numerous efforts have focused on the functional investigation of Nek2 in centrosome regulation and spindle formation. However, no direct binders are reported between Nek2 protein and human pancreatic cancer. In the present work, we demonstrated that Nek2 was frequently overexpressed in PDA tissues. Associations between Nek2 expression, clinicopathological features and clinical outcome were investigated in PDA tissue specimens obtained from patients with resected pancreatic cancer. Our results provide evidence that Nek2 may be an important prognostic biomarker and a promising target for molecular therapy in PDA patients.

Materials and methods

Patients and tissue specimens

A total of 136 patients with PDA who underwent surgical resection from January 2002 to January 2013 at the Department of General Surgery at the First Affiliated Hospital of Dalian Medical University were investigated in this study. Information on patient demographics (gender and age) and clinicopathologic features (tumor location, histological differentiation, lymph node metastasis and TNM stage) were available for all patients (**Table 1**). Histological PDA grading was based upon the World Health Organization grading system. Disease stage was classified in accordance with the criteria proposed by UICC/AJCC. In the present study, stage I and II cancers were defined as early-stage, whereas stage III and IV were defined as advanced-stage. None of the patients underwent palliative resection, preoperative or postoperative chemotherapy, or radiotherapy. All patients were followed up postoperatively until May 2013 or death. Overall survival (OS) was defined as the interval between the dates of surgery and death. Ethical approval for the project was obtained from the First Affiliated Hospital of Dalian Medical University Research Ethics Committee. All fresh samples were confirmed by hematoxylin-eosin staining in frozen sections with histopathologic analysis.

Immunohistochemistry

Formalin-fixed and paraffin-embedded surgical samples were sectioned at a thickness of 4 μm and then mounted on silane-coated glass sec-

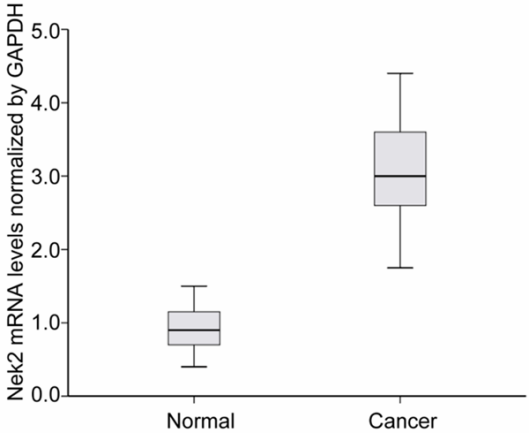

Figure 1. Nek2 mRNA expression in the fresh-frozen PDA tissues compared with the normal carcinoma-adjacent tissues. Nek2 mRNA expression was significantly higher in all 26 PDA tissues (T) than in the paired normal carcinoma-adjacent tissues (N) from the same patients (P<0.05).

tions. Sections of each sample were stained with hematoxylin-eosin to confirm the diagnosis and the presence of cancer cells. Sections were deparaffinized in xylene and rehydrated in a series of graded alcohol. To enhance the immunoreactivity, high-temperature antigen retrieval was carried out in ethylene diamine tetraacetic acid (pH=8.0) for 10 min with a stream oven. Then, sections were immersed in 3.0% hydrogen peroxide for 10 min at room temperature to block endogenous peroxidase and incubated in blocking serum for 15 min to reduce non-specific binding. The sections were incubated in the primary antibody against Nek2 (SC-33167, 1:200, Santa Cruz Biotech, CA, USA) at dilution of 1:50 in phosphate-buffered solution (PBS) overnight at 4°C. Sections were then incubated with secondary biotinylated antibody followed by streptavidin-biotinylated horseradish peroxidase complex. 3,30-Diaminobenzidine solution was added to visualize the reaction; then the sections were counterstained with hematoxylin, dehydrated in graded alcohol and xylene and then mounted with neutral balsam. For negative controls, sections were incubated with PBS instead of the primary antibody.

Evaluation of the immunohistochemical staining

Nek2 expression in the paraffin-embedded specimens was examined according to standard immunohistochemical method [22]. For scoring Nek2 expression, the intensity (intensity score: 0=negative, 1=weak, 2=moderate, and 3=strong) and percentage of the total cell population that expressed YWHAZ (proportion score: 0<10%, 10%≤1≤33%, 34%≤2≤66%, 67%≤3≤100%) were evaluated for each case. The expression of YWHAZ was regarded as high (intensity plus proportion scores≥4) or low (intensity plus proportion scores≤3) using high-powered (×200) microscopy.

Quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA extraction from tumor cells was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was prepared with a a First Strand cDNA Synthesis Kit (Promega, Wisconsin, CA, USA). Real-time reverse transcription-polymerase chain reaction (PCR) analysis of expression of the Nek2 gene was performed using 2 μL of cDNA and SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) as recommended by the manufacturer for with the following primers: Nek2, 5'-CCAGCCCTGTATTGAGTG-3' (forward) and 5'-ACTTCCGTTCCTTTAGCA-3' (reverse); GAPDH, 5'-CACCATTGGCAATGAGCGGTTC-3' (sense strand) and 5'-AGGTCTTTGCGGATGTCCACGT-3' (antisense strand). GAPDH was used as a normalization control. The PCR conditions were as follows: UDG pre-treatment at 50°C for 2 min, 1 cycle; initial denaturation at 95°C for 10 min; denaturation at 95°C for 15 s, annealing at 53°C for 30 s and extension at 72°C for 30 s, 40 cycles. All experiments were performed in triplicate.

Statistical analysis

Statistical analyses were performed using SPSS software package version 17.0. Pearson's χ^2 test or Fisher's exact test was used to compare qualitative variables. Univariate survival analysis was performed according to the Kaplan-Meier method, and OS was compared using the log-rank test. Multivariate analysis was conducted using the Cox proportional hazards regression model. The hazard ratios (HRs) between prognostic groups and their 95% confidence intervals were analyzed. The quantitative data derived from three independent experiments are expressed as means (±S.D.). Unpaired Student's t-tests were used to analyze between group differences that is repeated. P<0.05 was considered statistically significant.

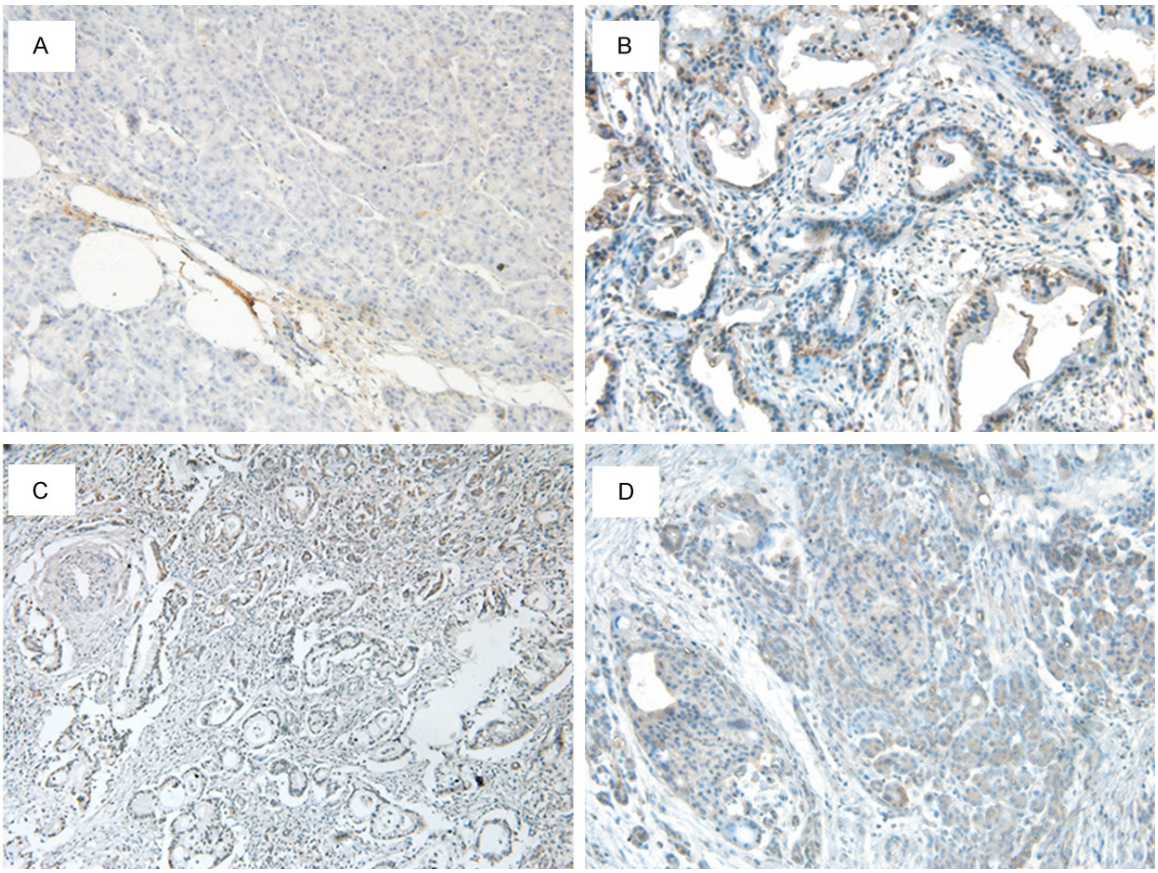

Figure 2. Expression of Nek2 is elevated in pancreatic cancer tissue sections with different differentiation types compared with the normal carcinoma-adjacent tissues. A. Normal carcinoma-adjacent tissues showed no or weak immunoreactions for Nek2 (×200). B. A low level expression of Nek2 in tissue sections of a well-differentiated PDA (×200). C. A low level expression of Nek2 in tissue sections of a moderately-differentiated PDA (×200). D. High level expression of Nek2 in tumor tissue sections of a poorly differentiated PDA (×200).

Results

Nek2 expression in PDA tissues

The expression of Nek2 was assessed by immunohistochemistry in 136 pancreatic cancer samples and 26 adjacent noncancerous tissues. Immunolocalization of Nek2 was observed in the nuclei and cytoplasm of cancer cells, including well-differentiated and moderately/poorly differentiated tumor tissue (**Figure 2**). In general, Nek2 expression was absent or very weak in the normal carcinoma-adjacent tissues (**Figure 2A**). This finding is consistent with those of earlier reports. In 136 PDA patients, 72 (52.9%) had strong staining in lesion tissues, which were classified as the high expression group. The other 64 (47.1%) patients had weak staining in lesion tissues, which were classified as the low expression group.

qPCR analysis was carried out to investigate the levels of Nek2 protein expression in freshly isolated PDA tissues. As shown in **Figure 1**, Nek2 mRNA was significantly higher in all 26 PDA tissues (T) than in the paired normal carcinoma-adjacent tissues (N) from the same patients ($P<0.05$).

Correlation between Nek2 expression and clinicopathologic features

Clinicopathologic features of all the cases included in this study are shown in **Table 1**. The histological grades and tumor stages (pTNM) were classified into 2 groups as follows: 74 cases of well-differentiated (54.4%) and 62 cases of moderately and poorly differentiated tumors (45.6%); 68 patients (50.0%) with PDA were in early stages (I-II), whereas 68 patients (50.0%) were in advanced stages (III-IV). Lymph

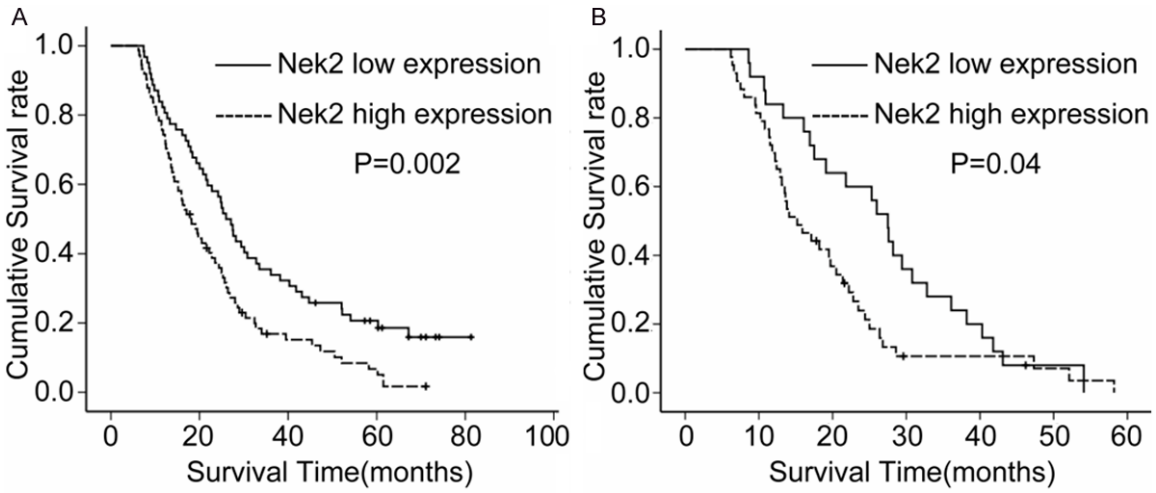

Figure 3. Kaplan-Meier survival curves of OS in patients with PDA according to Nek2. A. High expression of Nek2 was significantly associated with poor OS (P=0.002). B. High expression of Nek2 have shorter survival time in the advanced stage subgroup, especially.

Table 2. Univariate and multivariate analyses for Survival in PDA Patients

Variables	Univariate Analysis				Multivariate Analysis			
	RR	95% CI		P	RR	95% CI		P
		Lower	Upper			Lower	Upper	
Gender	1.149	0.802	1.646	0.448				
Age	0.926	0.640	1.339	0.683				
Nek2 level	0.922	0.875	0.972	**0.002**	0.940	0.890	0.993	**0.028**
Tumor location	0.550	0.626	1.283	0.550				
Histological differentiation	1.455	1.013	2.089	**0.042**	1.068	0.724	1.575	0.741
Lymph node metastasis	0.920	0.870	0.972	**0.003**	0.941	0.889	0.996	**0.037**
TNM stage	1.928	1.320	2.817	**0.001**	1.739	1.172	2.579	**0.006**
Respectability	1.511	0.980	2.331	0.062				

Bold values indicate P<0.05.

node metastasis appeared in 82 cases of PDA (60.3%).

We evaluated the correlation between Nek2 expression and clinicopathologic features, including age, gender, tumor stage, differentiation, and metastasis. As shown in **Table 1**, Nek2 expression was significantly associated with histological differentiation (P=0.042), lymph node metastasis (P=0.003) and tumor stage (P=0.001). No significant difference was observed regarding age, gender or tumor location.

Correlation between Nek2 expression and patient survival

We assessed the prognostic value of Nek2 expression for survival in PDA patients. The median survival time for all patients was 21.5 months (n=136). The 5-year survival rate was 10.3%. Kaplan-Meier analysis demonstrated that PDA patients with high Nek2 expression had a significantly poorer OS compared to the patients with low Nek2 expression (**Figure 3A**) (log-rank P=0.002).

Table 2 shows the results of univariate and multivariate analyses of factors related to patient prognosis. Univariate analysis showed that Nek2 level (HR: 0.922; 95% CI: 0.875-0.972; P=0.002, **Table 2**), histological differentiation (HR: 1.455; 95% CI: 1.013-2.089; P=0.042, **Table 2**), lymph node metastasis (HR: 0.920; 95% CI: 0.870-0.972; P=0.003, **Table 2**) and tumor stage (HR: 1.928; 95% CI: 1.320-2.817; P=0.001, **Table 2**) were significant prognostic factors of PDA. **Figure 3B** shows that

high levels of Nek2 have shorter survival time in the advanced stage subgroup, especially. Multivariate analysis showed that Nek2 level (adjusted HR: 0.940; 95% CI: 0.890-0.993; P=0.028), lymph node metastasis (adjusted HR: 0.941; 95% CI: 0.889-0.996; P=0.037), and TNM stage (adjusted HR: 1.739; 95% CI: 1.172-2.579; 0.006) were independent prognostic factors of survival.

Discussion

Nek2 is a serine-threonine kinase of the NIMA-related kinase family that localizes to the centrosomes which is the microtubule-organizing centers of a cell and regulates its separation [6]. NIMA-related kinase, or 'Nek', family consists of eleven different members (Neks 1-11) [24]. In humans, Nek2 exhibits the greatest sequence identity to NIMA [6]. In the process of cell division, Nek2 promotes centrosome splitting at begin of mitosis by phosphorylation of multiple linker components [25]. Besides centrosome separation, Nek2 also regulates the microtubule organization capacity of centrosome [26, 27]. Many former studies had demonstrated that abnormal centrosome was a significant feature of most cancer cells. The vast majority (80-100%) of breast tumors displays abnormal centrosome [28]. Additionally, recent studies have shown that high expression of NEK2 induced abnormal tumour proliferation and drug resistance in breast and ovarian cancer [14, 21, 29]. Furthermore, the significantly up-regulation of Nek2 has been demonstrated to be associated with the progression and poor prognosis in a series of malignant tumors originating in different organs and tissues, such as colorectal carcinoma [30], breast carcinoma [20] and myeloma [31].

However, it remains unclear whether Nek2 expression is associated with the clinicopathologic features and prognosis of PDA. In the present study, we detected Nek2 protein expression by immunohistochemistry in PDA tissues and found that the expression of Nek2 protein was mainly in the nuclei and cytoplasm of cancer cells, which was in accordance with the findings of studies of other cancers. Our study showed that Nek2 was highly expressed in the PDA tissues, and no or very weak staining of Nek2 was detected in the adjacent noncancerous tissues. This is the first study to demonstrate that high expression of Nek2 protein was significantly associated with several unfavorable clinicopathologic factors of PDA, including differentiation grading, lymph node metastases, and tumor stage. Hence, these results strongly confirm the intriguing possibility that an oncogenic role of Nek2 activation may contribute to tumorigenesis and progression of PDA. Nek2 can be used as a marker to identify distinct types of tumors with aggressive clinical behaviors for PDA.

To validate the potential clinical utility of Nek2, we evaluated the prognostic power of Nek2 protein to predict OS for the first time in PDA. Univariate survival analysis demonstrated that patients with high expression of Nek2 protein had a significantly poorer OS, suggesting that Nek2 may be a potential prognostic factor for PDA patients. To eliminate the impact of mixed factors correlated with prognosis on survival analysis, only those factors with significant values in univariate survival analysis were further enrolled in multivariate survival analysis, and it was found that high expression of Nek2 protein still remained an independent prognostic factor for unfavorable survival.

Despite its importance in centrosome regulation and spindle formation, the mechanism of the abnormal expression and regulation of Nek2 remains unclear. Further studies are needed to clarify the mechanism that underlies the role of Nek2. Current studies have shown that Nek2 regulates central pathways critical for in cell shape, tissue morphogenesis and tumorigenesis, such as wnt, PI3K and MAPK pathways [32]. Additionally TRF1 is required for overexpressed Nek2 to trigger abnormal mitosis and chromosomal instability [33, 34].

In summary, the current study demonstrated for the first time that high expression of Nek2 protein was common in PDA tissues and was significantly associated with unfavorable clinicopathologic and poor prognosis. Our data also suggest that Nek2 may not only be a useful molecular marker for predicting the survival but may also be an effective therapeutic target for pancreatic cancer. For Nek2 to be widely used in clinic, further clinical validation and standardization need to be accomplished. Further work is required to determine the downstream signal pathways regulated by Nek2, which may

help us to design an effective therapeutic modality to control pancreatic cancer.

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