

# Clinical and Biological Significance of Never in Mitosis Gene A-Related Kinase 6 (NEK6) Expression in Hepatic Cell Cancer

Xiaolei Cao · Yunfei Xia · Junling Yang · Jinxia Jiang ·  
Li Chen · Runzhou Ni · Liren Li · Zhifeng Gu

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**Abstract** Nek6 is a cell cycle regulatory gene, which can control cell proliferation and survival. Recent studies suggested that desregulation of Nek6 expression plays a key role in oncogenesis. This study was aimed to investigate the potential roles of Nek6 in hepatocellular carcinoma (HCC) development. Immunohistochemistry and Western blot analysis was performed for Nek6 in 80 hepatocellular carcinoma samples. The data were correlated with clinicopathological features. The univariate and multivariate survival analyses were performed to determine the prognostic significance of Nek6 in HCC. In addition, Nek6 expression vector was used to detect its role in cell cycle control. Nek6 was overexpressed in hepatocellular carcinoma as compared with the adjacent normal tissue. High expression of Nek6 was associated with histological grade and the level of alpha fetal protein, and Nek6 was positively correlated with proliferation marker Ki-67. Univariate analysis showed that Nek6 expression was associated with poor prognosis. Multivariate analysis indicated that Nek6 and Ki-67 protein expression was an independent prognostic marker for HCC. While in vitro, following release from serum starvation of HuH7 HCC cell, the expression of Nek6 was upregulated. Overexpression

Nek6 in Huh7 cell could promote the cell cycle. In conclusion, Nek6 is involved in the pathogenesis of hepatocellular carcinoma. It may be a favorable independent poor prognostic parameter for hepatocellular carcinoma.

**Keywords** Human hepatocellular carcinoma (HCC) · Nek6 · Ki-67 · Prognosis

## Introduction

Hepatocellular carcinoma (HCC), which accounts for 80% to 90% of primary liver cancer, is the fifth most common cancer in the world, and the third most common cause of cancer-related death [1–3]. In worldwide, there are 500,000 to 1,000,000 new cases annually [4, 5]. Without specific treatment, the prognosis is still very poor, and the median survivals for patients with early and advanced tumors are 6–9 months and 1–2 months, respectively [5, 6]. Much effort has been spent to reveal the molecular pathogenesis of HCC, but the mechanisms underlying the pathogenesis are still far from clear. In these studies, cell cycle dysfunction plays an essential role in the process [7, 8], and the structure and expression of several tumor suppressor genes and oncogenes have been reported [9, 10].

Never in mitosis A (NIMA) was originally identified in *Aspergillus nidulans* as a serine/threonine kinase critical for cell cycle progression into mitosis. Dominant-negative version of NIMA can adversely affect the progression of human cells into mitosis [11]. Human NIMA-related kinases (NEKs) are a group of protein kinases that are homologous to NIMA, which have high homology to NIMA in their N-terminal catalytic domain sequences, but each diverges substantially from NIMA at its non-catalytic C-terminal [11, 12]. Eleven Neks have been identified in

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Xiaolei Cao and Yunfei Xia contributed equally in the work.

X. Cao · L. Chen  
Department of Pathology, Medical College, Nantong University,  
Nantong, Jiangsu 226001, People's Republic of China

Y. Xia · J. Yang · J. Jiang · R. Ni · L. Li (✉) · Z. Gu (✉)  
Department of Internal Medicine, Affiliated Hospital of Nantong  
University, Medical College, Nantong University,  
20 Xisi Road,  
Nantong, Jiangsu 226001, People's Republic of China  
e-mail: larry017@163.com  
e-mail: guzhifeng@126.com

human genome. Evidence suggests that NEKs perform functions similar to those of NIMA [13]. Nek6 is a recently identified serine/threonine kinase that belongs to the Neks (NIMA-related kinases) family, which has been implicated in mitosis control [14]. Previous studies revealed that Nek6 plays an important role in mitotic cell cycle progression. It has been shown that the protein level and the activity of Nek6 are increased during mitosis [14, 15]. Moreover, the overexpression of a dominant negative mutant form of Nek6 or of an RNAi designed to knock down Nek6 induced spindle defects, abnormal chromosome segregation, mitotic arrest and eventually apoptosis, indicating that Nek6 is an essential mitotic kinase [15, 16]. Several lines of evidence suggested that the function of Nek6 is related to tumorigenesis. For example, it was recently shown that Nek6 mRNA levels were upregulated in several tumor cell lines [17]. And overexpression of inactive Nek6 decreases the growth rate and induces apoptosis in human breast cancer cells [17, 18].

In the present study, we compared the expression of Nek6 protein in normal human hepatic and HCC tissues using immunohistochemical and immunoblotting methods. And we also investigated its associations with clinical and pathologic factors, as well as the prognostic implications. At the same time, we have detected the function of Nek6 in cell cycle control by siRNA and overexpression in the HepG2 cell lines.

## Materials and Methods

### Patients and Tissue Specimens

HCC tissues were obtained from 80 patients. All underwent hepatic surgical resection without postoperative systemic chemotherapy at the Surgery Department, the Affiliated Hospital of Nantong University between January 2003 and December 2006. The diagnosis was confirmed histologically in all cases, based mainly on examination of sections stained with hematoxylin and eosin. After obtaining informed consent, patients were interviewed to obtain information on demographic characteristics, and clinical data were collected. The study population consisted of 58 males and 22 females, and their ages ranged from 32 to 75 years, with an average age of 54.8 years. Histological grades were classified according to the International Union Against Cancer TNM classification system, and assemble to well (grade I), moderately (grade II), and poorly differentiated (grade III). The main clinicopathological variables of the patients are shown in Table 1.

### Immunohistochemistry

For histological examination, tissue specimens were immediately processed after surgical removal. All tumorous and

surrounding nontumorous tissue portions were processed into 10% buffered formalin-fixed, paraffin-embedded blocks. The sections were deparaffinized using a graded ethanol series, and endogenous peroxidase activity was blocked by soaking in 0.3% hydrogen peroxide. Thereafter, the sections were processed in 10 mmol/L citrate buffer (pH 6.0) to retrieve the antigen. After rinsing in PBS (pH 7.2), sections were blocked for 1 h at room temperature to remove any nonspecific reactions. The sections were then incubated overnight at 4°C with anti-Nek6 rabbit polyclonal antibody (diluted 1:100; Santa Cruze), and anti-Ki-67 mouse monoclonal antibody (diluted 1:100, Santa Cruze). Negative control slides were also processed in parallel using a nonspecific immunoglobulin IgG (rabbit and mouse, Santa Cruze) at the same concentration as the primary antibody. Immunostaining were performed using the avidin biotin peroxidase complex method and antigen-antibody reactions were visualized with diaminobenzidine tetrahydrochloride method. Two observers (X.C. and L.C.) independently evaluated the immunostaining results, similar results were obtained in these samples. For assessment of Nek6 and Ki-67, five highpower fields in each specimen were selected randomly, and nuclear (cytoplasm) staining was examined under high power magnification. More than 500 cells were counted to determine the labeling index, which represented the percentage of immunostained cells relative to the total number of cells.

### Cell Culture and Cell Cycle Analysis

The human hepatocarcinoma cell line Huh7 was obtained from the Institute of Cell Biology, Academic Sinica and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in 5% CO<sub>2</sub> at 37°C. The cell proliferation assay was performed with Brdu assay kit according to the kit's protocol. Generally, cells were incubated with 100 µM Brdu labeling solution for 4 h at 37°C. After removing the culture media, the cells were fixed and the DNA was denatured by FixDenat solution. The anti-Brdu-POD working solution and substrate solution were then added, and the absorbances of the samples were measured by an ELISA plate reader at 370 nm wavelength.

### Expression Plasmid and Transient Transfection

Full-length Nek6 cDNA was generated by polymerase chain reaction from a cDNA library of HCC. The polymerase chain reaction fragment was cloned into the pcDNA3.1-myc expression vector at the EcoRI/XhoI. The proper construction of the plasmid was confirmed by DNA sequencing and the plasmid for transfection was prepared using a Plasmid Extra Kit (Sigma). Transfections were

**Table 1** Nek6 and Ki67 expression and clinicopathological parameters in 80 HCC specimens

	Total	Nek6		<i>P</i>	Ki67		<i>P</i>
		Low expression	High expression		Low expression	High expression	
Year							
≤45	32	16	16	0.348	18	14	0.448
>45	48	23	25		26	22	
Gender							
Male	58	24	24	0.510	23	25	0.210
Female	22	12	10		9	13	
Histological grade							
Well	27	18	9	0.014	20	7	0.007
Mod	30	16	14		15	15	
Poor	23	6	17		4	19	
Metastasis							
Negative	57	24	33	0.136	25	22	0.336
Positive	23	10	13		11	12	
Tumor size (cm)							
≤5	46	22	24	0.953	27	19	0.453
>5	34	16	18		17	17	
HBsAg							
Negative	22	11	11	0.543	10	12	0.543
Positive	58	28	30		23	25	
Cirrhosis							
Negative	23	12	11	0.678	17	5	0.002
Positive	57	30	27		20	37	
AFP (ng/mL)							
≤50	34	24	10	0.034	25	9	0.048
>50	46	10	36		8	38	

Statistical analyses were performed by the Pearson  $\chi^2$  test.  $P < 0.05$  was considered significant

performed using lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacture's protocol. The cells were harvested 48 h after transfection and used for the experiment. The experiments were repeated at least 3 times.

#### Immunoblot Analysis

For immunoblot examination, all tumor and surrounding nontumor tissue specimens were immediately processed after surgical removal, and stored at  $-80^{\circ}\text{C}$ . Tissue and cell protein were promptly homogenized in SDS loading buffer, centrifuged at 10,000 g for 30 min to collect the supernatant. Protein concentrations were determined with a Bio-Rad protein assay (Bio-Rad). Proteins were separated with SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride filter (PVDF) membranes (Millipore). The membranes were blocked with 5% dried skim milk in TBST for 2 h at room temperature. The membranes were then incubated overnight with polyclonal antibody against using the primary antibodies. And then, horseradish peroxidase-linked IgG was used as the secondary antibody. Immunoreactive bands were visu-

alized by chemiluminescence (Cellsignal). After the chemiluminescence was exposed to X-ray films, scanned using a Molecular Dynamics densitometer (Imaging Technology, Ontario, Canada). Values are responsible for at least three independent reactions.

#### Statistical Analysis

Statistical analysis was performed using the Stat View 5.0 software package. The association between Ki-67 and Nek6 expression and clinico-pathological features was analyzed using  $\chi^2$  test. Ki-67 and Nek6 expression in human hepatocellular carcinoma (HCC) was studied using the Spearman rank correlation test because the data were not normally distributed. Survival curves were calculated using the Kaplan–Meier method, and the log-rank test was used for analysis. Multivariate analysis was performed using Cox's proportional hazards model. One-way-ANOVA followed by the Tukey's post-hoc multiple comparison tests was used for statistical analysis of the data of immunoblot. The results of the HCC cells are expressed as the mean  $\pm$  SE.  $P < 0.05$  was considered statistically significant.

## Results

### The Expression of Nek6 Protein in HCC and Adjacent Normal Tissue

Of the 80 HCC, Nek6 was predominantly found in the nucleus, although weak cytoplasmic immunoreaction was also observed (Fig. 1). Representative examples of reactivity for Nek6 and Ki-67 are shown in Fig. 1. There was no or low Nek6 and Ki-67 expression in adjacent normal tissue (Fig. 1a,c). The percentage of Nek6 positive tumor cells ranged from 5% to 80% with a mean of  $53 \pm 23.23\%$ . Ki-67 expression in HCC was scored as positive when strong nuclei was stained (Fig. 1b,d). Its LI ranged from 0.85% to 84.38%. with a mean percentage of  $23 \pm 12.53\%$  (Fig. 2).

### Correlation of Nek6 Expression with Clinicopathological Parameters in HCC

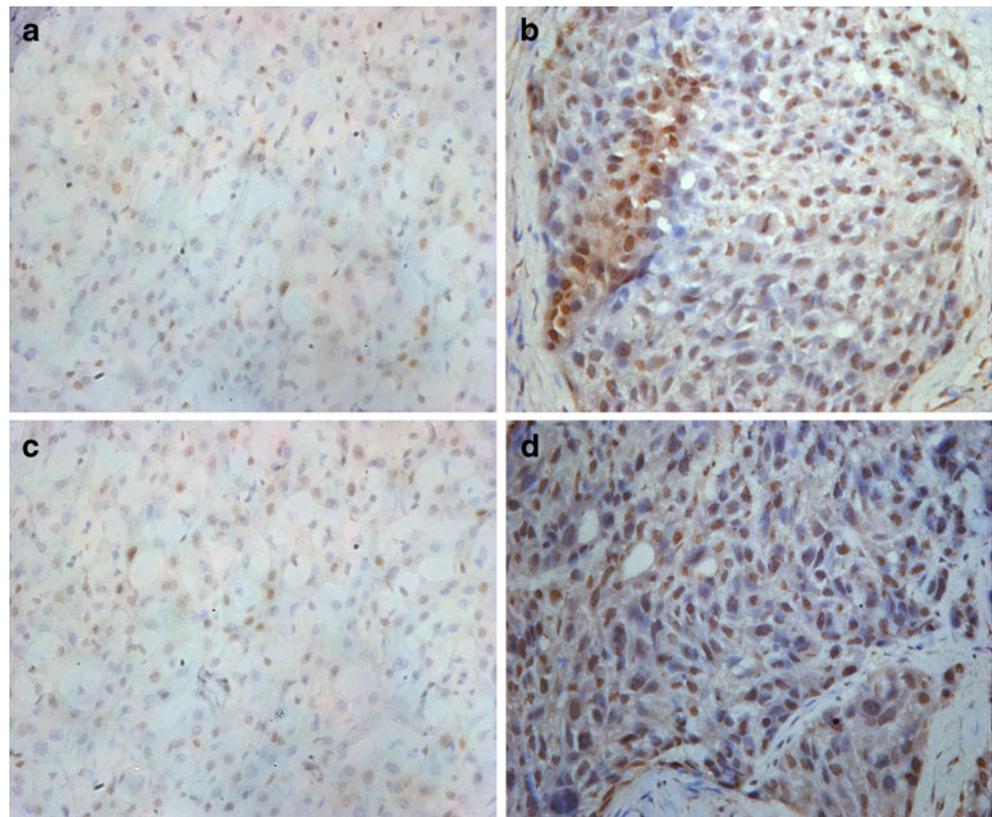
The clinico-pathological data of the patients are summarized in Table 1. As shown in Table 1, we evaluated the association of Ki-67 and Nek6 expression with clinical variables. For statistical analysis of the expression of Nek6 and Ki-67, the carcinoma specimens were divided into two groups: high expression and low expression, according to

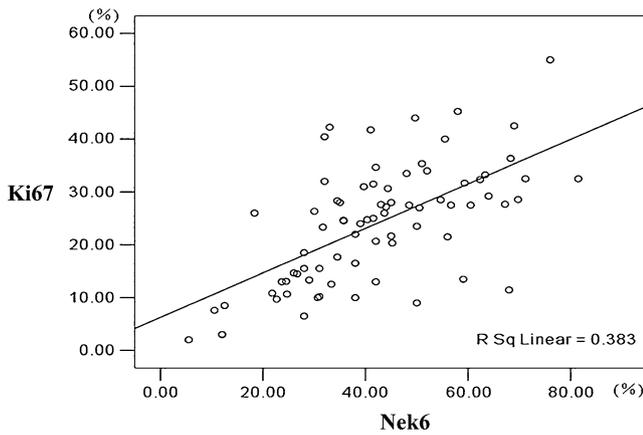
the percentage of Nek6 and Ki-67 positive cells, using a cut off level of 53% and 23% representing the mean value of Nek6 and Ki-67 expression. Nek6 expression did not correlate significantly with gender, age, metastasis, tumor size, HBsAg, cirrhosis, but its expression was significantly correlated with histological grade ( $P=0.009$ ) and the level of AFP ( $P=0.003$ ) (Table 1). Furthermore, in most specimens, the proportion of Nek6 positive tumor cells was similar to the proportion of Ki-67 positive tumor cells. A positive correlation between Nek6 expression and Ki-67 based proliferate activity was found ( $P<0.01$ ; Fig. 3).

### Survival Analysis

At the end of clinical follow-up, survival information was available in 80 cases of 80 patients (100%). Of these 80 patients, only 16 of 47 (33%) patients in the Nek6 high-expression group were alive versus 24 of 33 (72%) in the Nek6 low-expresser group (Table 2). When all variables were compared separately to survival status, only Nek6 ( $P=0.004$ ), Ki-67 ( $P=0.000$ ), and histological grade ( $P=0.006$ ) significantly influenced survival (Table 2). In univariate analysis, the Kaplan–Meier survival curves showed that high Nek6 expression related to a poor survival with statistical significance (Fig. 3). The Cox's proportional

**Fig. 1** Immunohistochemical staining of Nek6 and Ki-67 in adjacent normal tissues and HCC tissues. Paraffin-embedded tissue sections were stained with antibodies for Nek6 and Ki-67 and counterstained with hematoxylin. **a** Nek6 negative staining was shown in adjacent normal tissues. **b** Nek6 immunoreactivity was detected in HCC, with staining predominant in the nucleus. **c** Ki-67 negative staining was shown in normal human hepatic tissue. **d** Ki-67 positive nuclear staining was shown in HCC ( $\times 400$ )



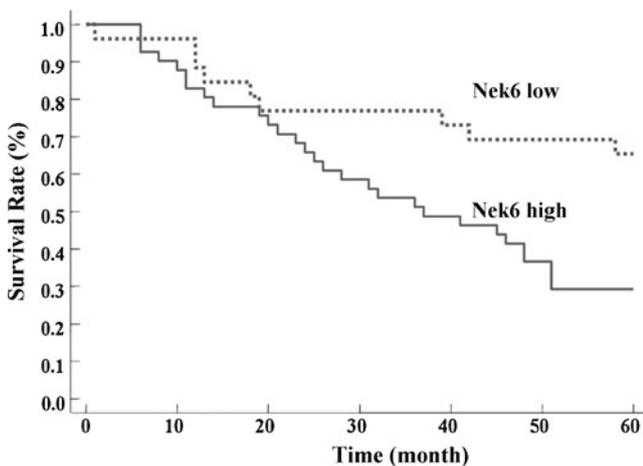


**Fig. 2** Relationship between Ki-67 proliferation index and Nek6 expression in HCC. Scatterplot of Ki-67 versus Nek6 with regression line showing a correlation of them using the Spearman’s correlation coefficient

hazards regression model proved that Nek6, Ki67 expression and histological grade were independent prognostic factors in patients with HCC (Table 3).

**Nek6 Promotes Cell Proliferation in HCC cells**

Based on our current study, we further detected the role of Nek6 in the proliferation of HCC cells. As previous reported, HuH7 cells were arrested in G1 phase by serum deprivation for 72 h. Upon serum addition, the cells were reentered S phase. Western blot displayed that the expression of Nek6 was increased as early as 12 h after serum stimulation (Fig. 4a). To further detect the role of Nek6 in cell proliferation, myc-peptide target Nek6 expression vector were transfected to Huh7 cell line (Fig. 4b). Cell



**Fig. 3** Kaplan–Meier survival curves for Nek6 expression. Kaplan–Meier survival curves for low Nek6 expression versus high Nek6 expression in 63 patients of HCC showed a highly significant separation

**Table 2** Survival status and clinicopathological parameters in 43 HCC specimens

	Total	Survival status		P
		Survival	Dead	
Year				
≤45	32	18	14	0.648
>45	48	21	27	
Gender				
Male	58	25	29	0.210
Female	22	14	8	
Histological grade				
Well	27	21	6	0.023
Mod	30	18	12	
Poor	23	9	14	
Metastasis				
Negative	57	28	29	0.436
Positive	23	11	12	
Tumor size (cm)				
≤5	46	21	25	0.753
>5	34	17	17	
HBsAg				
Negative	22	10	12	0.543
Positive	58	31	27	
Cirrhosis				
Negative	23	13	10	0.467
Positive	57	26	31	
AFP (ng/mL)				
≤50	34	15	16	0.044
>50	46	14	32	
Nek6				
Low expression	33	24	9	0.004
High expression	47	16	31	
Ki67				
Low expression	27	14	13	0.000
High expression	53	15	38	

Statistical analyses were performed by the Pearson  $\chi^2$  test.  $P < 0.05$  was considered significant

proliferation were enhanced in the Nek6 overexpression cells (Table 4).

**Discussion**

HCC is one of the most common cancers worldwide and is a major cause of death in many countries, especially in Asia [2, 4]. Although morbidity and mortality rates have decreased in recent years in patients with surgically treated HCC, the prognosis of HCC remains unsatisfactory and the 5-year survival rate is limited to 25–49% after surgery [19]. A deeper understanding of the molecular events associated with the HCC is necessary [20, 21].

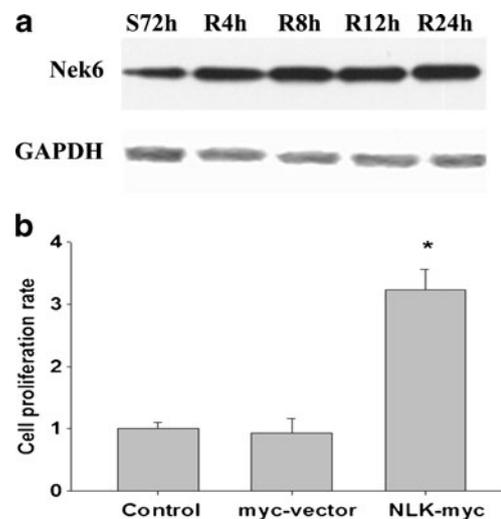
**Table 3** Contribution of various potential prognostic factors to survival by Cox regression analysis in 80 HCC specimens

	Hazard ratio	95% Confidence interval	P
Year	2.5890	0.8345–8.0123	0.067
Gender	0.6213	0.2341–2.7654	0.532
Histological grade	2.4567	1.3546–4.8765	0.002
Metastasis	0.6753	0.2345–1.6754	0.345
Tumor size	1.1456	0.4567–2.8796	0.762
HBsAg	1.5467	0.6223–3.4565	0.346
Cirrhosis	0.6234	0.2876–1.7232	0.324
AFP (ng/mL)	1.4567	0.5674–3.4262	0.234
Nek6	1.0507	1.0223–1.0875	0.002
Ki67	1.0234	1.0032–1.0567	0.009

Statistical analyses were performed by the log-rank test.  $P < 0.05$  was considered significant. The RR is given as low versus high expression

Recently studies have demonstrated that the Aurora kinase family, polo-like kinases family and NIMA kinase family could control cell cycle in prokaryotic and eucaryotic cells [22, 23]. NIMA is a serine/threonine kinase and could control cell cycle progression into mitosis. NEKs are a group of protein kinases that homologous to NIMA, and have high homology sequence in their N-terminal catalytic domain sequences. Neks have been identified in 11 isotype according its non-catalytic C-terminal [11, 22]. Nek6 is a recently identified serine/threonine kinase belongs to the Neks family, which has been implicated in mitosis control. Previous studies revealed that Nek6 plays an important role in mitotic cell cycle progression, and could arrest cell in G2/M phase if the expression was blocked. Over expression of a dominant negative mutant form of Nek6 or of an RNAi was designed to knock down Nek6 induced spindle defects, abnormal chromosome segregation, mitotic arrest and eventually apoptosis, indicating that Nek6 is an essential mitotic kinase [14–16]. Several lines of evidence suggest that the function of Nek6 is related to tumorigenesis, and it was recently shown that Nek6 mRNA levels were upregulated in several tumor cell lines [22, 23]. Overexpression of inactive Nek6 decreases the growth rate and induces apoptosis in human breast cancer cells [17, 18]. In HCC, previous studies have indicated its expression was up-regulated in more HCC cell lines [24]. In our studies, we have also found the expression of Nek6 was up-regulated in the HCC tissues compared with the benign normal tissue, which showed no or low Nek6 expression, and that Nek6 was mainly located in the nuclei of tumor cells. In further, we have also found the expression of Nek6 was increased accompanying with the increasing of the HCC tissue grade. Our study suggested that increased Nek6 levels may be closely associated with the pathogenesis of HCC. We also investigated the correlation between Nek6 expression and Ki-67 immunoreactivity, which has been reported to be a useful marker of tumor proliferative activity [25–27]. A positive correlation between Nek6 and Ki-67 was observed in HCC.

The malignant conversion of a tumor is a complex process. Overexpression of Nek6 could promote cell proliferation in Huh7 cell line. So Nek6 may represent an important mechanism of development of HCC. In addition, we evaluated the correlation between Nek6 and clinicopathological parameters, as well as the prognosis of patients. We found that Nek6 expression was significantly associated with histological grade and the level of AFP. Results of the survival analysis showed that Nek6 high expression was significantly associated with poor prognosis. These findings suggested that Nek6 might be a reliable indicator of prognosis in HCC patients. Previous studies has reported Nek6 could regulated Pin 1 expression and sate-1 activity in HCC, in which the two proteins played an essential role of HCC development [24, 28].



**Fig. 4** The function of Nek6 in HCC cells proliferating regulation. **a** HuH7 cells were serum starved for 72 h and upon serum releasing, cell lysates were prepared and analyzed by Western blot using antibodies directed against Nek6.  $\beta$ -actin was used as a control for protein load and integrity. **b** HuH7 cells were transfected with myc-NLK vector and then serum starved for 72 h and upon serum releasing, MTT assay shown cell proliferation rate. The data are means  $\pm$  SEM ( $n=3$ ,  $*P < 0.01$ , compared with controls)

In conclusion, we have showed that Nek6 expression was increased in HCC, and positively correlated with HCC cell proliferation. These results indicate that Nek6 may play an important role in HCC and its evaluation provides important prognostic information on HCC. Further studies are necessary to elucidate the molecular mechanisms of Nek6 in HCC pathogenesis.

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