High *NEK2* Expression Is a Predictor of Tumor Recurrence in Hepatocellular Carcinoma Patients After Hepatectomy

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Abstract. *Background/Aim: Better prognosis of cancer including hepatocellular carcinoma (HCC) remains unsatisfactory due to recurrence and chemoresistance. In this respect it is important to identify molecular targets specific to the disease in order to design effective therapeutic strategies. In the present study, we investigated the prognostic role of Never-in-mitosis-A-related kinase 2 (NEK2) in HCC. Materials and Methods: Fifty HCC patients who underwent hepatectomy were enrolled in the study. NEK2 gene and protein expression was examined by quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry, respectively. Results: Higher expression of NEK2 was detected in HCC tumoral compared to adjacent non-tumor tissues (p<0.001), and protein expression was also relatively high in tumor than corresponding non-tumor tissues. Furthermore, high NEK2 expression was positively correlated with hepatic venous invasion (p=0.047), des-gammacarboxy prothrombin (p=0.003), and alpha-fetoprotein (AFP) (p=0.024). Patients with high NEK2 expression had significantly poor recurrence-free survival (p=0.042) and early recurrence. Conclusion: Overall, these results suggest that NEK2 could be a promising biomarker for HCC recurrence.*

Hepatocellular carcinoma (HCC) is the most common cancer and the third leading cause of cancer-related death worldwide (1). Long-term survival of hepatocellular carcinoma patients remains unsatisfactory due to repeated recurrence after hepatectomy and 5-year recurrence rate of liver cancer, to date, exceeds 70% (2).

Unfortunately, the chemoresistant nature of hepatocellular carcinoma makes it difficult to eradicate cancer cells by means of either conventional chemotherapy or transarterial chemoembolization. Moreover, most HCC patients are diagnosed at advanced stages and are not eligible for surgical treatment. Therefore, there is still a need to identify new therapeutic targets so that novel strategies of treatment can be developed.

Mitosis is a complicated course that depends on the coordination of microtubules and various kinases are involved in a proper spindle formation and correct chromosome separation in proliferating cells and thus, holding promise as anticancer therapeutic targets (3, 4). Centrosomal kinases are vital regulators of cell division and uncontroled expression of mitotic kinases enhances tumor progression by encouraging chromosomal instability (CIN) (5-7). Never-in-mitosis-A-related kinase 2 (Nek2) is an evolutionary serine/threonine kinase that has a critical role in mitosis during cell division (8). Nek2 is often overexpressed in cancers and the mitosis-specific roles of Nek2 and its worse clinical outcome in human cancers make Nek2 as an alternative target for cancer therapy (7-9).

Increased Nek2 activity leads to abnormal chromosome content often more than twice the content of a normal diploid cell (10) and an over four-fold expression of Nek2 has been observed in various types of cancer cells (11). In xenograft studies, knockout of Nek2 from the tumor significantly reduced tumor size, suggesting that Nek2 inhibition could counter further tumor progression (12).

Recently it has been demonstrated that elevated detection of Nek2 induced therapy resistance through efflux pumps activation in myeloma (7) that could implicate the involvement of Nek2 in cancer chemoresistance. Nek2 collaborated with receptor tyrosine kinase (RTK), to boost local invasion, distant migration of tumor cells as well as enhance the levels of the activated Akt protein (13).

Altogether, the reported findings propose that Nek2 governs tumor development and its blockage could be a new approach for cancer treatment. So far, no study has reported any significant prognostic importance of *NEK2* in HCC. Thus, investigation of *NEK2* in HCC could strengthen what

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Key Words: Hepatocellular carcinoma, never-in-mitosis-A-related kinase 2, NEK2, tumor recurrence.

has been previously reported and supplement alternative therapeutic strategies for the treatment of HCC. The current study present the clinical and prognostic significance of *NEK2* in human HCC patients.

Materials and Methods

Patients. A total of 50 HCC patients who underwent a curative hepatectomy were included in the study. The study was authorized by the Institutional Review Board of the University of Tokushima Graduate School, and informed consent was obtained from each patient. The study participants were followed-up regularly in the outpatient clinic and checked prospectively for recurrence by following previously established guidelines. In brief, serum alpha-fetoprotein (AFP), des-gammacarboxy prothrombin (DCP) levels and ultrasound or contrast computed tomography (CT) were checked. Patients were followed-up every 2 months during the first postoperative year and at least every 3-4 months thereafter. AFP examination and liver ultrasound were performed during each visit. Bone scanning or magnetic resonance imaging (MRI) was performed when localized bone pain was reported. A diagnosis of recurrence was based on typical imaging appearance in CT and/or MRI and elevated AFP and/or DCP levels.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted from HCC tumor and its adjacent non-tumor tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 2.5 μg total RNA by reverse transcription using the SuperScript RT kit (Promega, Madison, WI, USA) following the manufacturer's instructions. qRT-PCR was performed using the Applied Biosystems 7500 real-time PCR system, TaqMan Gene Expression Assays on demand, and TaqMan Universal Master Mix (gene-specific TaqMan probes on a StepOne Plus; Applied Biosystems, Foster City, California, USA). Human NEK2 (Hs00601227_mH) TaqMan primer was used. GAPDH was used as an internal control for normalization. Expression levels of a gene was calculated as a ratio to GAPDH. Amplification data were analyzed with an Applied Biosystems Prism 7500 Sequence Detection System version 1.3.1 (Applied Biosystems).

Immunohistochemistry. Sections were de-paraffinized in xylene and hydrated through a series of graded ethanol, then rehydrated and quenched with 3% hydrogen peroxide for 10 min at room temperature. Antigen retrieval of the sections was achieved in a multifunctional microwave histoprocessor at 100˚C by microwave heating of slides in 0.01 mol/L of pH 6.0 citrate buffer for about 24 min. The sections were incubated with primary antibody against Nek2 (aa287-299, LS-B129-LSBio) at a dilution of 1:100 in phosphate-buffered solution (PBS) for one hour at room temperature. Sections were then incubated with secondary biotinylated antibody followed by streptavidin-biotinylated horseradish peroxidase complex. Finally, positive staining was visualized with diaminobenzidine and cell nuclei were counterstained with Mayer's hematoxylin. Paraffin-embedded sections of human testis was used as positive controls. Nek2 protein expression was evaluated by staining intensity (intensity score: 0=negative, 1=weak, 2=moderate, and 3=strong) (14).

Statistical analysis. Data are expressed as the mean±standard error of the mean (SEM) for continuous variables. The differences between means in two groups were analyzed by the Student's *t*-test.

Table I. *Correlation between NEK2 expression with clinicopathological factors in hepatocellular carcinoma patients*.

Variables	*NEK2* expression in HCC tumor tissue (n=50)		
	Low n=25	High n=25	*p*-Value
Average age (years)	71±6.3	64±0.1	0.156
Gender (female/male)	8/17	8/17	0.992
HBV (no/yes)	22/3	19/6	0.269
HCV (no/yes)	10/15	12/13	0.569
ALT (IU/l)	50.1±0.2	57±0.4	0.483
AST (IU/l)	48.3±0.3	46.5±0.3	0.483
AFP (</>200ng/ml)	21/4	13/11	0.024
DCP (</>40mAU/ml)	10/15	1/23	0.003
PT(s)	11.8±1.5	11.4±1.0	0.773
TNM stage (I-II/III-IV)	12/13	10/13	0.753
Tumor number (1/>1)	14/11	16/9	0.564
Average tumor size (cm)	4.2±1.8	4.2±3.2	0.196
vp (no/yes)	22/3	16/9	0.047
vv (no/yes)	23/2	19/6	0.123
im (no/yes)	17/8	18/7	0.758
Tumor differentiation (well/moderate or poor)	4/21	3/22	0.684

NEK2, Never in mitosis A-related kinase 2; *HBV*, hepatitis B virus; *HCV*, hepatitis C virus; *ALT*, alanine aminotransferase; *AST*, aspartate aminotransferase; *AFP*, alpha fetoprotein; *DCP*, desgammacarboxy prothrombin; *PT*, prothrombin time; *vp*, portal venous invasion; *vv*, hepatic venous invasion; im, intrahepatic metastasis.

Pearson's χ^2 test was used to compare qualitative variables. Univariate analysis was performed using the Kaplan-Meier method, and disease-free survival was compared using the log-rank test. Multivariate analysis was conducted using the Cox proportional hazards regression model. Statistical analyzes were performed using SPSS for Windows version 21.0 (SPSS Inc, Chicago, IL, USA). Statistical significance was determined at $p<0.05$.

Results

Expression of NEK2 in HCC paired hepatic tissues. We examined *NEK2* mRNA expression levels in 50 pairs of human HCC and their corresponding non-tumor liver tissues using qRT-PCR. The patients were divided into two groups based on the median value: high *NEK2* expression group (n=25) and low *NEK2* expression group (n=25). *NEK2* was found to be significantly ($p<0.001$) highly expressed in HCC tumor tissue compared to corresponding non-tumor tissues, with an average mRNA expression level of 0.62±0.4 and 0.18±0.3, respectively (Figure 1A). In IHC staining of tumor and non-tumor tissues, protein expression of Nek2 was more intense in tumor tissue than its non-tumor counterparts (Figure 1B).

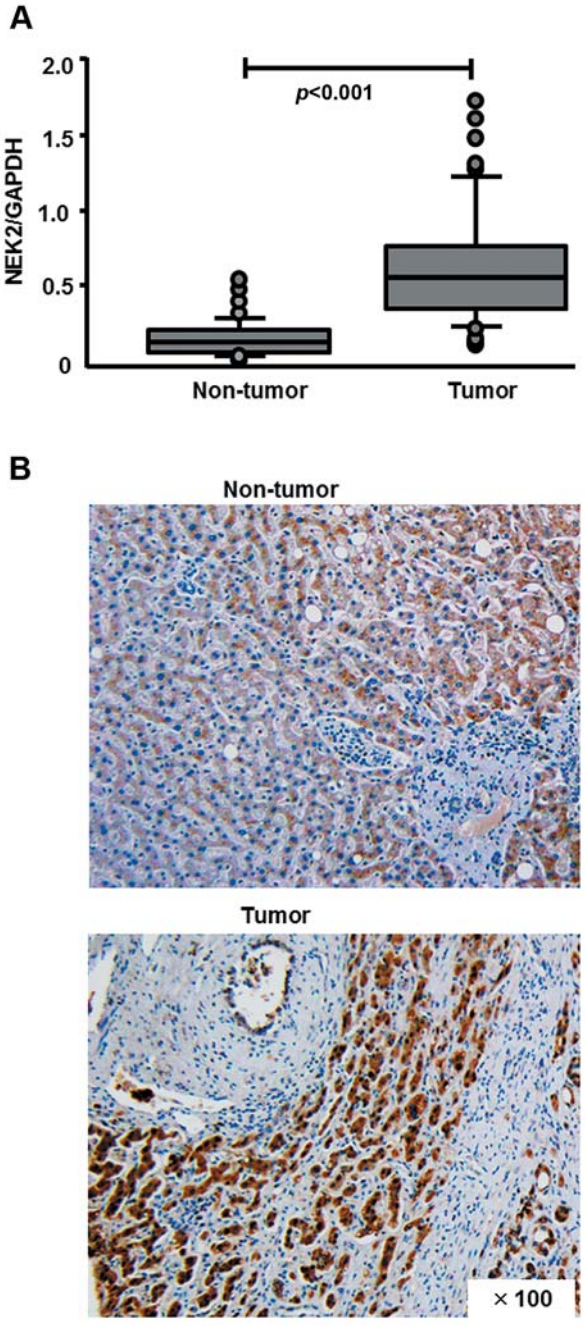

Figure 1. *NEK2 expression in tumor and non-tumor tissues of hepatocellular carcinoma. (A) Increased expression of NEK2 in tumor tissue (n=50) compared to its non-tumor counterparts (n=50) (p<0.001). (B) Representative image of Nek2 protein expression in a randomly selected HCC tumor and non-tumor tissue (×100) (n=15).*

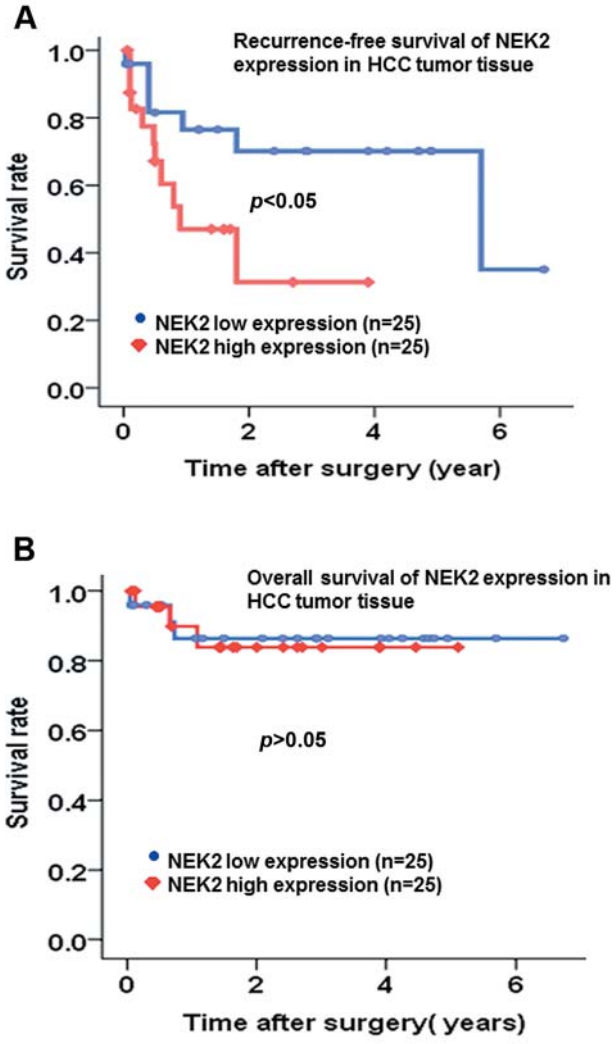

Figure 2. *Prognostic roles of NEK2 expression in HCC. Survival curve of HCC patients with high and low NEK2 expression was plotted using Kaplan-Meier analysis and their difference was evaluated by the log-rank test. High expression was correlated with poor recurrence-free survival (p<0.05). (A) Recurrence-free survival, (B) overall survival.*

Correlation between NEK2 expression and clinicopathological parameters. Correlation between *NEK2* expression and clinicopathological features were investigated in HCC patients (Table I). High *NEK2* expression significantly correlated with, presence of portal venous invasion (vp), high des-gamma carboxy prothrombin (DCP), and high alpha-fetoprotein (AFP) levels ($p<0.05$) and tended to correlate with hepatic venous invasion (vv). However, we found no statistically significant correlation between *NEK2* expression with viral infection and other socio-demographic variables.

Prognostic significance of NEK2 expression. We evaluated the prognostic role of *NEK2* expression for disease-free and overall survival in HCC patients. The 1- and 3-year recurrence-free survival rates in the low-expression group was 77% and 70% compared to 45% and 31% in the high-expression group respectively ($p<0.05$) (Figure 2A). In

Table II. *Risk factor analysis of tumor recurrence in tumor tissue.*

Prognostic factors	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*p*-Value	HR (95%CI)	*p*-Value
Age (<69/≥69)	1.893 (0.731-4.899)	0.288		
Gender (female/male)	0.621 (0.270-1.433)	0.807		
HBV (no/yes)	1.091 (0.313-3.802)	0.891		
HCV (no/yes)	1.114 (0.429-2.895)	0.825		
ALT (<47/≥47 IU/l)	1.013 (0.384-2.675)	0.979		
AST (<37/≥37 IU/l)	0.762 (0.280-2.073)	0.594		
AFP (<200/>200 ng/ml)	0.807 (0.260-2.509)	0.807		
PT (<11/>11s)	0.677 (0.251-1.820)	0.439		
Tumor size (<3.5/≥3.5 cm)	1.636 (0.604-4.430)	0.333		
Tumor number (1/>1)	1.029 (0.391-2.704)	0.954		
vv (no/yes)	5.704 (2.063-15.772)	0.001	4.036(1.153-14.131)	0.029
vp (no/yes)	2.589 (0.953-7.034)	0.052	1.149(0.348-3.790)	0.820
im (no/yes)	2.118 (0.817-5.493)	0.114	1.581(0.555-4.501)	0.391
Differentiation (well/moderate or poor)	1.228 (0.294-5.637)	0.737		
NEK2 expression (low/high)	2.891 (1.037-8.058)	0.042	2.836 (0.959-8.389)	0.060

Vv, Hepatic venous invasion; vp, portal venous invasion; im, intrahepatic metastasis.

addition, *NEK2* high-expression group showed tumor recurrence within two years. However no statistically significant association was observed between *NEK2* expression and overall survival (Figure 2B). Univariate analysis revealed that vp, vv, and *NEK2* expression were a significant prognostic factors for recurrence-free survival (Table II). Multivariate analysis using the Cox proportional hazards model showed vv was an independent prognostic factor, whereas high *NEK2* expression tended to be an independent prognostic factor in HCC patients (Table II).

Discussion

It is has been previously shown that chromosomal instability is involved in initiation of mutations and is one of the possible causes of genetic variation enhancing tumor progression. Recent experimental evidence have shown that, Nek2 encourages tumorigenesis by up-regulating numerous mitotic abnormalities (15). In the process of cell division, Nek2 promotes the centrosome splitting at the start of mitosis by phosphorylation of multiple linker components (8, 16), and also regulates microtubule organization of the centrosome (17).

Many former studies have demonstrated that unusual centrosome was a significant feature of most cancer cells and the majority of breast tumors displays an abnormal centrosome (18). Additionally, high expression of Nek2 induces tumor proliferation and drug resistance in many cancers (7, 19, 21). Furthermore, up-regulation of Nek2 has been demonstrated to be associated with reduced prognosis in colorectal carcinoma (22), breast carcinoma (23) and myeloma (7). However, it remains unclear whether NEK2 expression is associated with HCC prognosis.

In compliance with the results of studies of other tumor types, the current study also revealed that *NEK2* expression was significantly up-regulated in HCC tumor tissues than adjacent non-tumor tissues. Moreover our study showed that uncontrolled expression of NEK2 was associated with unfavorable clinicopathological factors of HCC, including vp, AFP, and DCP. Hence, these results confirm the intriguing possibility that an oncogenic role of *NEK2* activation may contribute to progression of HCC.

Previous reports have indicated that Nek2 overexpression promotes tumor invasion and metastasis through combinatorial activation of the Akt pathway, stabilization of β-catenin, up-regulation of Wnt/Wg, inhibition of apoptosis and deregulation of the Rho1-Rac1 balance (7, 8). Remarkably, uncontrolled Nek2 expression in A549 cells resulted in over-reduction of β-catenin signaling at the cell–cell adherence junction complexes and activation of β-catenin signal in and around the nucleus, indicating that reduced β-catenin in the adherent junction and its translocation to the nucleus is indicative of invasive state of cells, consequently supporting our finding that Nek2 could have regulatory role in tumor invasion.

The present results on *NEK2* have a notable similarity to those of another centrosomal kinase Aurora-A. Earlier evidence suggests that Aurora-A high expression also induces tumor metastasis (24). Taken together, these results powerfully suggest that centrosomal kinases, like Nek2 and Aurora-A, can also activate the Akt signaling pathway to enhance tumor invasion and metastasis.

To confirm the possible clinical impact of NEK2, we evaluated the prognostic role of NEK2 in HCC. Based on the time of relapse, recurrence can occur early (within 2 years) characteristically, resulting from dissemination of metastatic HCC cells, while late recurrence (more than 2 years) is the result of a *de novo* developed tumor in the liver (25). Our findings verified that many patients with high expression of *NEK2* have recurrence within two years, indicating that *NEK2* may be a potential factor for early relapse in HCC patients. Multivariate survival analysis further confirmed that uncontrolled *NEK2* expression still remained nearly an independent prognostic factor for the unfavorable survival which could be significant if the sample size is increased.

In summary, the current study revealed that high expression of *NEK2* was observed in HCC tumor tissue and was significantly associated with unfavorable prognosis. The present findings lay the basis for future studies that can further investigate the role of *NEK2* in cancer development as well as develop more reasonable plans to inhibit the cancer-promoting *NEK2* signaling pathway. Furthermore, we suggest that a novel potent non-toxic *NEK2*-targeting anticancer agent should be developed for efficient cancer treatment.

Competing Interests

All Authors declare that no competing interests exist.

Acknowledgements

This study was supported by the Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan.

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Received December 12, 2015
Revised January 14, 2016
Accepted January 15, 2016