NEK2 regulates stem-like properties and predicts poor prognosis in hepatocellular carcinoma

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Abstract. NEK2 has been estimated to play an important role in cancer progression. However, its relevance in hepatocellular carcinoma (HCC) has not yet been explored. Immunohistochemistry revealed NEK2 expression was upregulated in HCC. NEK2-positive hepatocellular carcinoma patients were associated with poor prognosis after surgery compared with NEK2-negative patients based on Kaplan-Meier curves. Deletion of NEK2 reduced self-renewal properties and chemotherapeutic resistance, and decreased the stemness associated genes in cell lines. NEK2 was associated with unfavorable outcomes in HCC patients, and was revealed to regulate self-renewal property by means of Wnt/β-catenin signaling, and chemotherapeutic resistance by preferential regulation of the expression of ABCG2 and ALDH1A1 in HCC cells.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and the third leading cause of cancer-related deaths in the world (1). Recently data showed that 782,500 new cases and 745,500 deaths occurred worldwide during 2012, with China alone accounting for approximately 50% of the total number of cases and deaths (2). The majority of HCC occurs in the setting of chronic liver disease from viral hepatitis, alcohol abuse, heavy exposure to aflatoxin or algal hepatotoxins in contaminated water, betel nut chewing and diabetes mellitus (3). The process of HCC involves a series of sequential and complex steps. Over the past decades a large number of studies mostly focused on the cancer stem cells (CSCs). CSCs are a subset of tumor cells that are capable of self-renewal, chemo/radio-therapeutic resistance, tumorigenicity and differentiation, similar to normal stem cells (4,5), and these characteristics could further result in aggressive phenotype of cancer and poor prognosis. Therefore, CSCs may serve as an effective therapeutic target in the treatment of HCC and may improve the current poor prognosis of this disease.

NIMA-related kinase 2 (NEK2), a member of the Nek family of serine/threonine kinases, is structurally related to the essential mitotic regulator NIMA and is highly enriched at the centrosome (6). Recent data indicate that NEK2 has emerged as an important player in cancer progression. Overexpression of NEK2 in myeloma (7), colorectal carcinomas (8,9), breast carcinoma (10,11), and lung cancer (12) has been associated with aggressive disease, poor differentiation, development of metastases and poor clinical prognosis. Previous studies also revealed expression of Nek2 and β-catenin were correlated with each other in clinical specimens of colorectal cancer and breast carcinoma (8,10). Furthermore, β-catenin was proved as a Nek2 substrate involved in centrosome separation (13), and Nek2 could bind to β-catenin to prevent its ubiquitination and degradation (14). β-catenin was well-known as a key components of canonical wnt/β-catenin signal pathway which regulate the CSC features.

However, up to now, no data are available regarding the role of NEK2 in HCC and CSCs. In the present study, we evaluated that NEK2 is highly expressed in HCC, and associated with tumor recurrence and poor prognosis. Furthermore, this study also revealed the role of NEK2 in CSCs, including maintaining self-renewal property by means of Wnt/β-catenin signaling,
and influencing chemotherapeutic resistance by preferential regulating the expression of ABCG2 and ALDH1A1 in HCC cells.

Materials and methods

Patients and specimens. The tumor tissues with adjacent non-tumor tissues were from 40 patients, and tumor specimens with clinicopathological features and follow-up were obtained in 104 patients. All patients underwent curative hepatectomy for HCC at Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, from January 2006 to December 2010. All the cases conformed to the following criteria: diagnosed by postoperative histopathology, no perioperative extrahepatic metastasis, no other malignant diseases, did not die from perioperative complications and no preoperative anti-cancer therapy. Curative resection was defined as removal of all recognizable tumors with a clear microscopic margin. Follow-up data were recorded from the patient's medical records and completed by a telephone survey. All subjects selected were required to provide written informed consent on the use of clinical specimens for medical research. The study was approved by the Ethics Committee of our hospital.

Cell lines and cell culture. Normal human liver cell line (Chang liver) and HCC cell lines Hep-G2, Hep-3B, HuH-7, SMMC7721, HCCLM3, Snu387 and Snu475 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) or the Cell Bank of Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences (Shanghai, China). Chang liver, HuH-7, SMMC7721 and HCCLM3 were routinely maintained in Dulbecco's modified Eagle's medium (DMEM). Hep-G2 and Hep-3B were cultured in Minimum Essential Medium (MEM), Snu387 and Snu475 were in RPMI-1640 medium. All cell lines were supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen, Carlsbad, CA, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified incubator under 5% CO2.

Immunohistochemistry (IHC) and IHC evaluation. Immunohistochemical stainings were performed following standard procedure. Briefly, formalin-fixed and paraffin embedded human samples were first cut into 5-μm-thick sections. Then the antigen retrieval was accomplished by deparaffinization, rehydration, and boiling in a microwave oven with citrated buffer. Hydrogen peroxide (3%) in PBS was used to block endogenous peroxidase activity and BSA was used to block the antigen. Endogenous peroxidase activity was eliminated by incubation in 3% H2O2 in PBS for 10 minutes. All sections were washed in PBS and incubated with rabbit anti-β-catenin as ‘NEK2/β-catenin positive’ and a high (++++) score of NEK2/β-catenin as ‘NEK2/β-catenin negative’ and a high (++++) score of
Briefly, 6x10^3 cells in 100 µl medium was dispensed into a 96-well plate. After overnight incubation, cells were exposed to 5-fluorouracil (5-Fu) and cisplatin for 72 h, respectively. Then CCK-8 was added to the wells and incubated for 1 h. Finally, the absorbance of the sample taken from each well was detected at 450 nm.

**Table I. Sequences of gene-specific primers used for real-time RT-PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence forward (5'-3')</th>
<th>Primer sequence reverse (5'-3')</th>
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<tr>
<td>NEK2</td>
<td>CATTGGCACAGGCTCCTAC</td>
<td>GAGCCCATGTAAGTCTTTTCCCA</td>
</tr>
<tr>
<td>ABCG2</td>
<td>CACCTATTGGCCTCAGGAA</td>
<td>CCTGCTTGGAAAGGCTCTATG</td>
</tr>
<tr>
<td>ALDHIA1</td>
<td>TGGAATGTGGGAGGGCCCGT</td>
<td>CAACAAAGGGGCACACTGGGCA</td>
</tr>
<tr>
<td>β-catenin</td>
<td>GTCTTACCTGGACTCTGGAATCC</td>
<td>GGTATCCACATCCCTTGTCCTCAG</td>
</tr>
<tr>
<td>c-Myc</td>
<td>GGCTCTGGCAGAACAGGTA</td>
<td>CTGCCTAGGTCTGCTGTATG</td>
</tr>
<tr>
<td>EpCAM</td>
<td>TAAACGTCATGCGAGTGTACCTC</td>
<td>CTTCCTCAAGTGGTTGAGCCAT</td>
</tr>
<tr>
<td>CD133</td>
<td>TGGATGCAAAACTTGACACGT</td>
<td>ATACCTGCTACGACAGTGTGCTG</td>
</tr>
<tr>
<td>K19</td>
<td>TTTGAGACACGAAAAGCAGCTT</td>
<td>ATGAAATCCCTGGACCTGCT</td>
</tr>
<tr>
<td>LIN28</td>
<td>TGTAAGTGTCACTCACCTGCG</td>
<td>CTTACCCTCCCTCCAAGTCE</td>
</tr>
<tr>
<td>NOTCH1</td>
<td>GAGCGTGCCAGAGCTATGC</td>
<td>CTGTAATCCCTCCCTGAAGTGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGTTCCTCTCTGACTTCAACA</td>
<td>GGTAGGTCTCCTCTCTTCCCT</td>
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Figure 1. NEK2 expression is upregulated in HCC. (A and B) Analysis from the Oncomine database shows that mRNA expression levels of NEK2 is significantly higher in HCC compared to normal liver tissues. Data were pooled together from two published HCC gene expression studies Wurmbach Liver Statistics (A), Hepatology 2007/04/01 and Roessler Liver Statistics (B), Cancer Res 2010/12/15 (all P<0.001). (C) The mRNA level of NEK2 in Chang liver and 7 HCC cell lines with different differentiation status. Data were normalized against the NEK2 expression level in Chang liver cells. (D) Immunohistochemical staining of NEK2 expression in human HCC specimens and their matched normal tissues. Negative (-) (a) or weak (+) (b) staining were defined as negative expression; moderate (++) (c) or strong (+++) (d) staining were defined as positive expression. Original magnification, x200.

The statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS version 19.0 (SPSS, Inc., Chicago, IL, USA). A statistical analysis for group differences was performed by using the Chi-square test or the Student’s t-test. The mean SD of three independent
experiments is reported. Recurrence free survival (RFS) and overall survival (OS) after the operation were calculated using the Kaplan-Meier method. Multivariate analysis of prognostic factors for survival was performed by a Cox stepwise regression model. A P-value of <0.05 was considered to be statistically significant.

**Results**

**NEK2 expression is upregulated in HCC.** To determine the significance of NEK2 in HCC, we first analyzed multiple microarray data sets in the Oncomine database (www.oncomine.com). As showed in Fig. 1A and B, we found NEK2 mRNA levels were significantly increased in HCC samples as compared to normal liver tissue from two published HCC gene expression studies Wurmbach Liver Statistics (18) and Roessler Liver Statistics (19) (all P<0.001). We further assessed the mRNA expression of NEK2 in multiple HCC cell lines by real-time RT-PCR test. As shown in Fig. 1C, the mRNA levels of NEK2 were higher in the seven HCC cell lines including Hep-G2, Hep-3B, HuH-7, SMMC7721, HCCLM3, Snu387 and Snu475 than that in Chang liver.

To verify the microarray analysis results, we performed IHC experiments on 40 pairs human HCC specimens and their matched normal tissues. Immunohistochemical analysis showed NEK2 positive staining was detected in the nuclear and cytoplasmic regions. The expression of NEK2 was classified into negative (-) or weak positive (+) and moderate positive (++) or strong positive (+++) staining in Fig. 1D. Positive staining of NEK2 could be observed in 23 of 40 (57.5%) cases of HCCs, whereas NEK2 showed positive staining in only 3 of 40 (7.5%) cases of adjacent non-tumor tissues.

**Correlation between NEK2 expression and clinical outcome.** To better understand the clinical significance of NEK2 expression in HCC, we investigated the clinicopathological features of NEK2 in 104 HCC samples. As shown in Table II, a positive NEK2 protein level was significantly associated with β-catenin expression (P<0.001). In contrast, NEK2 expression was not correlated with age, gender, HBsAg, differentiation, tumor number, tumor size, TNM stage, BCLC stage and portal vein tumor thrombus (PVTT (all P>0.05).
To assess the prognostic significance of NEK2 expression, Kaplan-Meier curves for OS and RFS were analyzed. The OS and RFS rates at 5 years were 51.7 and 31.7% for NEK2 positive patients (n=60) compared with 72.7 and 54.5% for NEK2 negative patients (n=44), respectively (OS P<0.05, Fig. 2A; RFS P<0.01, Fig. 2B).

Univariate Cox regression analysis was conducted to identify important prognostic factors of OS. NEK2 expression (P=0.014), PVTT (P<0.001), TNM stage (P<0.001) and tumor size (P=0.034) were identified as important risk factors for OS (Table III). However, in multivariate Cox analysis, NEK2 expression (P=0.033) and tumor size (P=0.032) were found to be independent negative prognostic factors for OS (Table III).

These results indicated that the positive expression of NEK2 was associated with unfavorable outcomes in HCC patients.

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NEK2 knockdown decreases the self-renewal property of CSCs. β-catenin is a key downstream molecule in the Wnt/β-catenin signaling pathway, which is one of the well-known pathways to play a critical role in CSC formation and maintenance (20,21). The results from the clinical samples indicated a correlation between NEK2 levels and β-catenin, so we next investigated whether NEK2 knockdown influenced the stemness characteristics of CSCs in HCC. Given that self-renewal is a hallmark of CSCs, we performed sphere forming assay and colony formation assay to investigate the role of NEK2 in maintaining self-renewal property in HCC cells.

siRNA technology was used to knockdown NEK2, and its levels were effectively downregulated in HCCLM3 and Hep-3B, verified by qRT-PCR (all P<0.001; Fig. 3A) and western blotting (Fig. 3B). In the sphere formation assay, we found that NEK2 knockdown group and control group grew...
in the form of suspended individual cells on the first day. As 7 days passed, HCCLM3 and Hep-3B cells in which NEK2 expression was knocked down exhibited fewer and smaller spheres (all P<0.01; Fig. 3C and D). Colony formation assay showed that NEK2 deletion formed less and smaller colonies than their control groups. NEK2 knocked-down also resulted in generation of a decreased ability to form colonies in HCCLM3 and Hep-3B cells (all P<0.01; Fig. 3E and F).

Deletion of NEK2 reduces the expression of stemness associated genes of CSCs. NEK2 had been proved to influence self-renewal of HCC CSCs, then we investigate whether the knockdown of NEK2 suppresses stemness associated genes by means of western blot analysis and qRT-PCR. We investigated whether NEK2 influences the expression of transcriptional factors including Bmi-1, Sox2 and Nanog, which are essential for maintaining stem cell phenotypes. Compared with the control group, western blot analysis showed NEK2 knock-down decreased the levels of stem cell genes Bmi-1, Sox2 and Nanog in HCCLM3 (Fig. 4A) and Hep-3B (Fig. 4B) cells. Additionally, the expression levels of the hepatic CSC markers EpCAM, CD133, K19, LIN28 and NOTCH1 were analyzed

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>Age (years) (≤50 vs. &gt;50)</td>
<td>0.981 (0.559-1.720)</td>
<td>0.946</td>
</tr>
<tr>
<td>Gender (Male vs. female)</td>
<td>1.213 (0.619-2.376)</td>
<td>0.573</td>
</tr>
<tr>
<td>Cirrhosis (Absent vs. present)</td>
<td>1.627 (0.911-2.908)</td>
<td>0.100</td>
</tr>
<tr>
<td>Tumor number (Single vs. multiple)</td>
<td>1.384 (0.588-3.255)</td>
<td>0.457</td>
</tr>
<tr>
<td>Tumor size (≤5 vs. &gt;5 cm)</td>
<td>1.827 (1.046-3.191)</td>
<td>0.034</td>
</tr>
<tr>
<td>TNM stage (I vs. II/III)</td>
<td>3.129 (1.707-5.735)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BCLC stage (A vs. B)</td>
<td>1.566 (0.801-3.063)</td>
<td>0.190</td>
</tr>
<tr>
<td>Differentiation (Well/medium vs. poorly/undifferentiated)</td>
<td>1.285 (0.738-2.240)</td>
<td>0.376</td>
</tr>
<tr>
<td>PVTT (Absent vs. present)</td>
<td>5.765 (2.925-11.364)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NEK2 expression (Positive vs. negative)</td>
<td>2.122 (1.162-3.875)</td>
<td>0.014</td>
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</table>

PVTT, portal vein tumor thrombus.
Figure 5. NEK2 regulates the self-renewal property of CSCs through the canonical Wnt/β-catenin signal pathway. (A) Western blot analysis was performed for β-catenin in HCCLM3 and Hep-3B cells transfected with siControl or siNEK2. (B) QRT-PCR was performed using primers for β-catenin in HCC cells transfected with siControl or siNEK2; (all P<0.01). (C) QRT-PCR was performed using primers for c-Myc and EpCAM in HCCLM3 and Hep-3B cells transfected with siControl or siNEK2; (all P<0.01). (D and E) Wnt3a (20 ng/ml) was added in cultures after cells were transfected with siRNA for 24 h. Wnt3a addition increased the ability of tumorsphere formation to partially compensate NEK2 inhibition in HCCLM3 (P<0.01; D) and Hep-3B (P<0.01; E).
NEK2 regulates the self-renewal property of CSCs through the canonical Wnt/β-catenin signal pathway. Next, we explored the molecular mechanisms by which NEK2 affects the self-renewal traits of CSCs. Recent studies indicate that Wnt/β-catenin pathway play a key role in hepatic CSCs (22) and its deregulation has been extensively reported in HCC (23,24). We examined both expression of the NEK2 and β-catenin from previous HCC specimens by IHC. Nek2 positive expression was found to associate with β-catenin positive expression (r=0.358, P<0.001; Table III). Western blot analysis also showed NEK2 knockdown reduced β-catenin protein (Fig. 5A), but the mRNA level of β-catenin was not altered (P<0.01; Fig. 5B) in HCC cells, which meant NEK2 regulated β-catenin through post-translational modification. This result was in accordance with that previously shown, i.e. NEK2 binds to β-catenin to prevent its ubiquitination and degradation (14). Subsequently, we used qRT-PCR to assessed the expression of downstream target genes of Wnt/β-catenin signal pathway including EpCAM and c-Myc. The expression of EpCAM and c-Myc in NEK2 knockdown group was significantly decreased compared with controls (all P<0.01; Fig. 5C).

NEK2 deletion reduces chemotherapeutic resistance of CSCs. Chemotherapeutic resistance is an important characteristic of CSCs, which are known to show strong resistance to chemotherapy, thus, we examined 5-Fu and cisplatin resistance of HCC cells for NEK2 inhibition. Compared with control cells, NEK2 knockdown cells displayed significantly higher sensitivity to 5-Fu and cisplatin chemotherapeutic agents in HCCLM3 (P<0.01; Fig. 6A) and Hep-3B cells (P<0.01; Fig. 6B). Finally, to investigate the mechanism responsible for 5-Fu and cisplatin resistance, we analyzed the mRNA expressions of multidrug resistant genes (ABCG2) and aldehyde

![Graphs showing cell viability and mRNA expression](image-url)
activation of Wnt/β-catenin signaling emerged as a critical regulator of stem cells. In many tissues, Wnt/β-catenin signaling is associated with cancer. This has raised the possibility that the tightly regulated self-renewal property of stem cells is converted in cancer cells to allow malignant proliferation (35). Further investigation revealed that NEK2 influenced the self-renewal properties in HCC cells. 

CSC was first reported in acute myeloid leukemia (AML) (27), and subsequently found in some solid tumors, including HCC. NEK2 was associated with cancer cells in proliferation, drug resistance, apoptosis, tumorigenicity, invasion and migration (11,28,29). To better elucidate the role of NEK2 in CSCs, we investigated the effect of NEK2 depletion on the self-renewal properties. As expected, the ability to form spheres and colonies was inhibited. Nanog, Sox2 and Bmi-1 are three core transcription factors regulating cellular pluripotency and are known to suppress differentiation in ES cells (4,30). CD133, EpCAM, K19, LIN28 and NOTCH1 which is frequently expressed in HCC, has been predicted to be a CSC marker (31-34). Silencing NEK2 expression in HCC cells also downregulated the expression of stemness associated genes of CSCs, including Nanog, Sox2, Bmi-1, EpCAM, CD133, K19, LIN28 and NOTCH1.

The canonical Wnt/β-catenin signaling pathway has emerged as a critical regulator of stem cells. In many tissues, activation of Wnt/β-catenin signaling has also been associated with cancer. This has raised the possibility that the tightly regulated self-renewal mediated by Wnt/β-catenin in stem and progenitor cells is converted in cancer cells to allow malignant proliferation (35). Further investigation revealed that NEK2 influenced the self-renewal properties in HCC cells. ABCG2 and ALDH1A1 are ABC transporter of ABCG2 and ALDH1A1. The present study provides a foundation for future studies that hold great promise for the development of a novel clinical biomarker for prediction of the malignant potential of HCC, and therapeutic strategies for HCC patients.

Acknowledgements

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References


