## ORIGINAL ARTICLE \_\_\_

## Nek2B accelerates the progression of hepatocellular carcinoma through regulating SFRP1 to activate the Wnt/β-catenin pathway

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## Summary

**Purpose:** The purpose of this study was to clarify the expression pattern of Nek2B in hepatocellular carcinoma (HCC) and its influence on malignant phenotypes of HCC through regulating SFRP1 and the Wnt/ $\beta$ -catenin pathway.

Methods: Nek2B levels in 64 paired HCC tissues and adjacent normal ones were detected by quantitative real-time polymerase chain reaction (qRT-PCR). The correlation between Nek2B level and clinical parameters of HCC patients was analyzed. Regulatory effects of Nek2B and SFRP1 on clonality, proliferation and apoptosis of MHCC97H and Hep3B cells were determined through functional experiments. Western blot was conducted to detect protein levels of SFRP1,  $\beta$ -catenin, c-myc, cyclinD1 and MMP7 in HCC cells with overexpression or knockdown of Nek2B. At last, rescue experiments were performed to clarify the role of Nek2B/SFRP1 regulatory loop in aggravating the progression of HCC.

**Results:** Nek2B was upregulated in HCC tissues and cells.

HCC patients expressing a high level of Nek2B were in more advanced tumor stage and had worse prognosis. Overexpression of Nek2B in MHCC97H cells enhanced clonality, 5-Ethynyl-2'- deoxyuridine (EdU)-positive ratio and suppressed apoptosis. Besides, knockdown of Nek2B in Hep3B cells yielded the opposite results. SFRP1 was downregulated in HCC, and low level of SFRP1 predicted worse prognosis of HCC. Overexpression of Nek2B downregulated SFRP1, but upregulated  $\beta$ -catenin, c-myc, cyclinD1 and MMP7 in HCC cells. Importantly, Nek2B/SFRP1 regulatory loop was identified to aggravate the progression of HCC.

**Conclusions:** Nek2B is upregulated in HCC, and closely linked to tumor stage and poor prognosis in HCC patients. *Through interaction with SFRP1, Nek2B aggravates the pro*gression of HCC by activating the Wnt/ $\beta$ -catenin pathway.

*Key words:* Nek2B, SFRP1, Wnt/β-catenin, hepatocellular carcinoma (HCC), malignant progression

## Introduction

and hepatocellular carcinoma (HCC) is the most veloped countries, the 5-year survival of HCC is important pathological subtype [1-3]. In 2018, lower than 15% [6, 7]. In recent years, the inci-WHO reported that HCC is the fifth prevalent ma- dence of HCC is on the rise because of the populignancy in males and the ninth in females [4-6]. larization of HCV infection, steatohepatitis and It is estimated that there are 8.82 million newly some other underlying diseases [8]. Complex eti-

Liver cancer is a common clinical malignancy, onsets of HCC, and over 50% are in China. In de-

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ology, high malignancy and strong heterogeneity of HCC result in the poor prognosis of affected patients [8,9]. So far, tumor stage-guided radical surgery and palliative treatment are preferred for HCC treatment. Nevertheless, clinical outcomes of HCC are unsatisfactory because of high recurrence, ineffective drug therapy and lack of individualized treatment [9-11].

Nek2B is a serine threonine kinase that localizes in the centrosome, and it is upregulated in many human cancers [12,13]. Nek2B can initiate and promote the replication and maturation of the centrosome, maintain the stability of the centrosome structure, and promote centrosome separation during the progress of replication, thus forming a bipolar spindle [14,15]. Hence, Nek2B is of significance in the process of cell mitosis. It not only directly participates in cell division by acting on chromatin, but also indirectly influences cell division by regulating centrosomes [16,17]. Downregulation of Nek2B has little effect on intracellular chromatin condensation, but it is required for mitotic cessation. Deficiency of Nek2B leads to delayed cell division, cell cytokinesis failure and abnormal division at the late phase [18,19]. Additionally, Nek2B is reported to influence tumor cell behaviors [13,17,18].

The activation of Wnt/ $\beta$ -catenin pathway triggers the occurrence and progression of HCC via targeting its downstream gene SFRP1 [20,21]. In this paper, we mainly investigated the involvement of Nek2B, Wnt/ $\beta$ -catenin pathway and SFRP1 in aggravating the progression of HCC.

## Methods

#### Patients and HCC samples

HCC tissues and paracancerous tissues were surgically resected from 64 patients, and they were pathologically diagnosed. Samples were preserved at -80°C. None of HCC patients were treated with preoperative anti-tumor therapy. Clinical indexes and follow-up data of enrolled patients were collected. Patients and their families in this study have been fully informed. This study was approved by Ethics Committee of Beijing University of Chinese Medicine Affiliated Shenzhen Hospital.

#### Cell culture

HCC cells (MHCC97H, Huh7 and Hep3B) and normal hepatocytes (HL-7702) were provided by American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Life Technologies, Gaithersburg, MD, USA) and maintained in a 37°C, 5% CO<sub>2</sub> incubator. Cell passage was conducted at 80-90% confluence using  $1 \times typsin+EDTA$  (ethylenediaminetetraacetic acid).

#### Transfection

Transfection vectors were constructed by GenePharma (Shanghai, China). Cells inoculated in a 6-well plate were transfected at 70% confluence using Lipofactamine 3000 (Invitrogen, Carlsbad, CA, USA). At 48 h, cells were harvested for subsequent experiments.

#### Cell proliferation assay

A total of 2.0×10<sup>3</sup> cells per well were inoculated in a 96-well plate. Viability was determined at the appointed time points using cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan). Absorbance at 490 nm was recorded for plotting the viability curve.

#### Colony formation assay

Cells were seeded in a 6-well plate with 200 cells per well and incubated for 14 days. Medium was replaced once in the first week and twice in the second week. Subsequently, cells were fixed in 100% methanol and dyed with 0.5% crystal violet for 20 min. Colonies were captured and calculated in a good light environment.

#### 5-Ethynyl-2'-deoxyuridine (EdU) proliferation assay

Cells were labeled with 50  $\mu$ M EdU (R&D Systems, Minneapolis, MN, USA) for 2 h, dyed with AdoLo and 4',6-diamidino-2-phenylindole (DAPI) in dark for 30 min. EdU-positive rate was calculated as the ratio of EdU-positive cells (red) and DAPI-stained cells (blue) under a fluorescent microscope.

#### Flow cytometry analysis

Cells were collected, washed with phosphate buffered saline (PBS) twice, centrifuged and resuspended in binding buffer. After incubation with 5  $\mu$ L of AnnexinV-FITC (fluorescein isothiocyanate) and 5  $\mu$ L of Propidium Iodide (PI) in dark for 15 min, cells were subjected to flow cytometry (Partec AG, Arlesheim, Switzerland) for determining the apoptotic rate.

#### Quantitative real-time PCR (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for extracting cellular RNA, followed by purification by DNase I treatment. Extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Japan). The cDNA was amplified by real-time quantitative PCR using SYBR®Premix Ex Taq<sup>TM</sup> (TaKaRa, Otsu, Japan).  $\beta$ -actin was used as an internal reference.

#### Western blot

Cells were lysed for extracting total protein using radioimmunoprecipitation assay (RIPA), quantified by bicinchoninic acid (BCA) method and loaded for electrophoresis (Beyotime, Shanghai, China). After transferring on polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland), the proteins were blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by electrochemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

#### Statistics

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean  $\pm$ standard deviation. The student t-test was applied for analyzing differences between the two groups. Kaplan-Meier was introduced for survival analysis, followed by Log-rank test for comparing two curves. P<0.05 was considered statistically significant.

### Results

#### Nek2B was highly expressed in HCC

Nek2B levels in 64 paired HCC tissues and adjacent normal ones were determined. It is shown that Nek2B was upregulated in HCC tissues (Figure 1A). Identically, Nek2B was highly expressed in HCC cells relative to normal hepatocytes (Figure 1B). Notably, among the six tested HCC cell lines, MHCC97H cells expressed the lowest level of Nek2B and Hep3B cells expressed the highest level. These two cell lines were selected for establishing *in vitro* overexpression and knockdown models of Nek2B, respectively.

Nek2B expression was correlated with tumor staging and overall survival in HCC patients

Chi-square analysis was conducted to analyze the correlation between Nek2B level and clinical parameters of HCC patients. The data uncovered that Nek2B level was positively correlated with the tumor stage of HCC patients (Table 1). Kaplan-Meier curves illustrated that HCC patients expressing a high level of Nek2B suffered worse survival than those expressing a low level.

#### Nek2B regulated proliferation and apoptosis in HCC

Through transfection of pcDNA-Nek2B in MH-CC97H cells and sh-Nek2B in Hep3B cells, *in vitro* 



**Figure 1.** Nek2B was highly expressed in HCC. **A:** Nek2B level in HCC tissues and adjacent normal ones. **B:** Nek2B level in HCC cells (MHCC97H, SMMC-7221, Huh7 and Hep3B) and normal hepatocytes (HL-7702). (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).

Item	Number of cases	Nek2B expression		р
	-	Low (n=38)	<i>High (n=26)</i>	
Age (years old)				0.418
<60	26	17	9	
≥60	38	21	17	
Gender				0.067
Male	31	22	9	
Female	33	16	17	
T stage				0.010
T1-T2	37	27	10	
T3-T4	27	11	16	
Lymph node metastasis				0.935
No	39	23	16	
Yes	25	15	10	
Distance metastasis				0.568
No	52	30	22	
Yes	12	8	4	

Table 1. Clinicopathologic characteristics of the included HCC patients and grouping based on Nek2B expression

overexpression and knockdown models of Nek2B were successfully established, respectively (Figure 2A). Overexpression of Nek2B in MHCC97H cells markedly elevated viability, clonality and EdUpositive ratio, indicating the accelerated prolifera-

tive ability (Figure 2B-2D). Conversely, silence of Nek2B in Hep3B cells yielded the opposite trends. Flow cytometry uncovered attenuated apoptosis after overexpression of Nek2B, and it was enhanced by silence of Nek2B (Figure 2E).



**Figure 2.** Nek2B regulated proliferation and apoptosis in HCC. MHCC97H cells were transfected with NC or pcDNA-Nek2B. Hep3B cells were transfected with sh-NC or sh-Nek2B. **A:** Nek2B level detected by RT-PCR; **B:** Viability at day 1, 2, 3 and 4 via CCK-8; **C:** Relative colony number; **D:** EdU-positive ratio; **E:** Apoptotic rate: overexpression of Nek2B attenuated cell apoptosis, while sh-Nek2B promoted the apoptosis (\*p<0.05).



**Figure 3.** Nek2B mediated the Wnt/ $\beta$ -catenin pathway in HCC. **A:** Western blot analyses of SFRP1,  $\beta$ -catenin, c-myc, cyclinD1 and MMP7 in MHCC97H cells transfected with NC or pcDNA-Nek2B, and Hep3B cells transfected with sh-NC or sh-Nek2B. **B:** SFRP1 level in HCC tissues and adjacent normal ones. **C:** SFRP1 level in HCC cells (MHCC97H, HepG2, MHCC97H, SMMC-7221, Huh7 and Hep3B) and normal hepatocytes (LO2). (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).



**Figure 4.** SFRP1 was responsible for Nek2B-mediated HCC progression. MHCC97H cells were transfected with NC+NC, pcDNA-Nek2B+NC or pcDNA-Nek2B+pcDNA-SFRP1. Hep3B cells were transfected with sh-NC+si-NC, sh-Nek2B+si-NC or sh-Nek2B+si-SFRP1. **A:** Nek2B level; **B-C:** Viability at day 1, 2, 3 and 4 detected by CCK-8; and cell proliferation explored by EdU-positive ratio: Viability and EdU-positive ratio were enhanced by overexpression of Nek2B (\*p<0.05).

#### *Nek2B mediated the Wnt/β-catenin pathway in HCC*

In MHCC97H cells transfected with pcDNA-Nek2B, protein level of SFRP1 was downregulated, while  $\beta$ -catenin, c-myc, cyclinD1 and MMP-7 were markedly upregulated. The opposite trends were observed in Hep3B cells with Nek2B knockdown (Figure 3A).

#### SFRP1 was lowly expressed in HCC

SFRP1 levels in HCC tissues and cells were determined as well. Conversely to that of Nek2B, SFRP1 was downregulated in HCC (Figure 3B, 3C). The prognostic potential of SFRP1 was identified through Kaplan-Meier curves, that was, low level of SFRP1 indicated worse survival in HCC patients. Moreover, Nek2B level was found to be negatively correlated with that of SFRP1 in HCC tissue.

# SFRP1 was responsible for Nek2B-mediated HCC progression

As proved a negative interaction between SFRP1 and Nek2B, we next analyzed the biological function of SFRP1 in HCC cells. Nek2B level was lower in MHCC97H cells co-overexpressed with Nek2B and SFRP1 than those overexpressing Nek2B. In addition, Nek2B level was higher in Hep3B cells cotransfected with sh-Nek2B and si-SFRP1 than those transfected with sh-Nek2B (Figure 4A). Viability and EdU-positive ratio were enhanced by overexpression of Nek2B, and they were partially reduced after co-overexpression of Nek2B and SFRP1. Similarly, decreased viability and EdU-positive ratio in Hep3B cells with Nek2B knockdown were partially reversed by co-silence of Nek2B and SFRP1 (Figure 4B, 4C). Therefore, SFRP1 was proved to be necessary during Nek2B-mediated HCC progression.

## Discussion

HCC is one of the epithelial malignancies that severely threaten human lives [1-3]. Atypical symp-

toms in the early stage, rapid progression and high rate of metastasis are the major reason for the severity of HCC [4,5]. It is necessary to uncover regulatory networks involving in the progression of HCC [6-9]. Growth factors in the tumor microenvironment participate in complex signaling networks formed in precancerous or cancerous cells, thus affecting multiple events in HCC [10, 11]. Nek2B is an important molecule involving in the inflammation-cancer transformation, which exerts an oncogenic role in HCC [15].

Multiple regulatory factors, including p53, BRCAl, Aurora-A, Plk1, and Cyclin B1, are associated with tumorigenesis, and some of them have been shown to be closely related to centrosomes [11,13,14]. Nek2B is a newly discovered protein kinase responsible for the assembly of centrosomes and microtubes [12-14]. Injection of Nek2B antibody or inactivated Nek2B could result in the fragmentation of centrosomes. Deficiency of Nek2B leads to microtubule assembly disorders and centrosome maturation disorders [15]. Besides, Nek2B can also bind to the spindle detection point proteins. Its deficiency may cause damage to the mitotic detection point and abnormal cell chromosome separation [16].

So far, most studies reporting the role of Nek2B in mitosis are limited to the early embryos [13-16]. The biological function of Nek2B in tumor diseases are rarely reported. In this paper, Nek2B was upregulated in HCC tissues and cells. HCC patients expressing a high level of Nek2B were in more advanced tumor stages and had worse prognosis. Overexpression of Nek2B in MHCC97H cells enhanced clonality, EdU-positive ratio and suppressed apoptosis. Knockdown of Nek2B in Hep3B cells yielded the opposite results. It is suggested that Nek2B served as an oncogene in the malignant progression of HCC.

The Wnt/ $\beta$ -catenin pathway is closely linked to the malignant progression of HCC [22-24]. SFRP1 is a key regulator in the Wnt/ $\beta$ -catenin pathway. Once the cytoplasmic concentration of SFRP1 reaches a certain level, it can affect proliferative ability of tumor cells [22-24]. Here, protein level of SFRP1 was downregulated, while  $\beta$ -catenin, c-myc, cyclinD1 and MMP-7 were markedly upregulated by overexpression of Nek2B. It is suggested that Nek2B activated the Wnt/ $\beta$ -catenin pathway in HCC. Moreover, tumors expressing a low rate of positive-SFRP1 are characterized by poor differentiation, high rate of lymphatic metastasis and advanced tumor staging [25,26]. Our rescue experiments identified the interaction between Nek2B and SFRP1. During the progression of HCC, SFRP1 was necessary in Nek2Bmediated cellular phenotype changes in tumor cells.

## Conclusions

Nek2B is upregulated in HCC, and closely linked to tumor staging and poor prognosis in HCC

patients. Through interaction with SFRP1, Nek2B aggravates the progression of HCC by activating the Wnt/ $\beta$ -catenin pathway.

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## **Conflict of interests**

The authors declare no conflict of interests.

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