NIMA-Related Kinase 6 as an Effective Target Inhibits the Hepatocarcinogenesis and Progression of Hepatocellular Carcinoma

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Research

Keywords: Hepatocellular carcinoma, NEK6, clinical characteristics, prognosis

Posted Date: September 2nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-836610/v1

Version of Record: A version of this preprint was published at Heliyon on June 1st, 2023. See the published version at https://doi.org/10.1016/j.heliyon.2023.e15971.

Abstract

Background

Never in Mitosis Gene A-Related Kinase (NEK), a member of the cell cycle-dependent protein kinase, encodes a serine/threonine protein kinase involved in mitosis of the G2-M transition period. Eleven NEKs have been identified, and NEK6 is one of them. It has been confirmed that NEK6 is over-expressed in some tumor cell lines. The following study aims to explore the expression of NEK6 in hepatocellular carcinoma (HCC) and the correlation between NEK6 expression and clinical features.

Methods

Liver tissues of 79 HCC patients and other patients were obtained during various liver surgeries. Total RNA from these tissue samples was extracted and QPCR was adopted to detect positive expression of NEK6. The correlation between NEK6 expression and the clinical characteristics of HCC was analyzed. The expression of NEK6 in different cell lines (LO-2, HepG2, Li-7, Huh-7, and BEL-7402) was quantified via QPCR. Scratch assay, Transwell assay, and tumor-formation assay in nude mice were used to evaluate the effects of NEK6 on the HCC progression in vitro and in vivo.

Result

The expression of NEK6 was up-regulated in HCC tissue compared with other tissues. The proportion of hepatitis B virus infection in the Nek6 Overexpression group was higher than the Control group (P=0.045). The multiple tumors and bigger tumors (>5 cm) seemed more common in the NEK6 overexpression group (P=0.018, and P=0.037, respectively). Further analysis showed that the overexpression of NEK6 was correlated with hepatitis B virus infection and tumor diameter (P=0.045; P=0.038). The 3-year disease-free survival (DFS) and overall survival (OS) of patients in the NEK6 overexpression group were 14.1% and 33.4%, which were worse than the control group. Tumor size over 5 cm and portal vein invasion were risk factors for both DFS and OS. Nek6 overexpression did not seem to affect the prognosis as a risk factor. Among the human cell lines, the expression of NEK6 was higher in Li-7 cells and HepG2 cells. The migration and invasion capabilities of Li-7 and HepG2 cells were suppressed when the NEK6 expression was down-regulated. The xenograft tumor weight was significantly lighter in the shNEK6 group ($P < 0.05$).

Conclusion

The results from the study demonstrated that the expression level of NEK6 was up-regulated in HCC and correlated with the HCC progression, suggesting it might be a clinically valuable biomarker and serve as a potential therapeutic target for treating HCC.

Background

Primary liver cancer (PLC) is one of the most common malignant tumors in human beings, accounting for 5.6% of the global cancer incidence in 2012, and 12.9% in China [1]. In 2015, PLC accounted for the fourth incidence of malignant tumors and the third mortality rate in China [2]. Hepatocellular carcinoma (HCC) is the most frequent type of PLC with higher incidence and earlier onset. Only 20% of HCC patients had the opportunity for radical resection [3], and 60% ~70% of them had tumor recurrence and metastasis within 5 years of surgical intervention. Therefore, it is of great clinical significance to deeply study the occurrence and invasive potential of HCC cells, to block the occurrence of HCC at an early stage, and to prevent recurrence and metastasis.

In 1987, Osmani et al. [4], when studying Aspergillus, discovered a gene related to the cell cycle, which was named NIMA (never-in-mitosis A). This gene, which encodes a Serine-threonine protein kinase, is involved in the regulation of the G2- M transition checkpoint in mitosis [5]. Later, Letwin et al. [6] and Bowers et al. [7] isolated some structural and functional genes

that were highly consistent with NIMA in mice and humans. These genetic families come together, collectively known as the Never in Mitosis Gene A-Related Kinase (NEK) gene family. NEK is actually a serine/threonine kinase, which has 11 members, including Nek1-11, in the human genome. NEK6 is one of them.

NEK6 is composed of 313 amino acids. Activated NEK6 can promote the non-adherent growth of tumor cells (suspended growth or cloned growth). The inhibition of endogenous NEK6 does not affect the function of normal fibroblasts but can induce apoptosis of cancer cell lines [8]. Overexpression of NEK6 can also inhibit apoptosis of p53 dependent cells and lead to cell cycle arrest [9]. The molecular mechanism of these effects is not clear, but to some extent, it reflects the non-mitotic role of NEK6. For example, NEK6 can activate tryptophan targets in the domain through phosphorylation Signal transduction and Activator of Transcription (STAT) C-terminal, thus increasing the transcriptional activity of cancer cells [10]. Above all, NEK6 is involved in the progress of mitotic phase, [11, 12]. Inhibition of NEK6 expression will lead to cell division stagnation and even apoptosis [11].

It has been confirmed that NEK6 is highly expressed in many tumors, such as gastric cancer [13], colon tumor [8, 14], lung cancer [8], esophageal cancer [15], breast cancer [8], cervical cancer [8], ovarian cancer [16] and some cancer cell lines [8]. However, the expression of NEK6 in HCC cell line has only been reported sporadically. Therefore, further research is needed to disclose the expression of NEK6 in human HCC, liver cirrhosis, and normal liver tissue, to reveal the potential links between over-expression of NEK6 and the clinical biological characteristics and prognosis of HCC.

Materials And Methods

Tissue samples preparation

The study was approved by the Medical Ethics Committee of Sichuan Provincial People's Hospital, School of Medicine, University of Electronic Science and Technology of China. A total of 30 pairs of HCC tumor tissues and corresponding paracancerous tissues were enrolled in this study. Inclusion criteria: (1) the patients had a history of hepatitis B virus infection; (2) None of the patients had received pre-operative therapy; (3) Hepatectomy was performed and the diagnosis was confirmed by pathology and/or histochemical staining as HCC; (4) about 1cm HCC and 1cm Paracancerous tissues (at least 1cm away from the tumor) could be obtained; (5) no perioperative death; (6) the expected survival time was more than 3 months. Exclusion criteria: (1) patients who could not undergo radical resection, (2) patients with other types of tumors. We defined HCC tissue as HCC group (Group I) and paracancerous tissues as Paracancerous group (Group II). At the same time, 15 cases of normal liver tissues (Normal group, Group III) were obtained from specimens of hepatic hemangioma patients undergoing liver resection; meanwhile, 10 cases of liver cirrhotic tissues (Cirrhotic group, Group IV) were taken from the patients who underwent portal azygous devascularization for posthepatitic liver cirrhosis; at last, 49 cases paraffin specimens (Paraffin group, Group V) diagnosed as HCC with hepatitis B virus infection were collected, which meets HCC Group's in- and exclusion criteria. Also, all the tissues were collected from September 2016 to March 2017, paraffin sections were collected from January to August 2015. The clinical data of HCC patients were collected. Written consent for all patients conformed to the ethical guidelines of the Helsinki Declaration.

Also, among these patients and specimens, 20 cases of HCC and its precancerous tissues, 7 cases of posthepatitic liver cirrhosis tissues were collected in the West China Hospital of Sichuan University. 10 cases of HCC and paracancerous tissues, 3 cases of posthepatitic liver cirrhosis tissues, 15 cases of normal liver tissues, and all paraffin sections were all from Sichuan Academy of Medical Sciences·Sichuan Provincial people's Hospital.

Cell culture

Human Hepatic cell line: LO-2, and Human Hepatocellular carcinoma cell lines: HepG2, Li-7, Huh-7, BEL-7402 were purchased from the Cell Resource Center of Shanghai Institute of Life Sciences, Chinese Academy of Sciences. All the cells

were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA), supplemented with 10%% fetal bovine serum (Hyclone, USA) in 5% CO2 at 37°C.

Plasmid construction and Real-time Quantitative PCR Detecting System (qPCR)

Total RNA from tissue samples and cells were extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The optical absorbance ratio at 260/280nm was measured using Scandrop 100 (Analytic Jena, Germany) to determine the concentration and quality of the RNA. Complementary DNA (cDNA) was synthesized was generated using a TUREscript First-Strand cDNA Synthesis Kit (Aidlab, China). Real-time qPCR was performed to validate gene expression using 2×SYBR® Green Supermix on an analytikjena-qTOWER2.2 PCR System (Analytik Jena, Germany) with the following thermal cycling conditions: 95°C for 3min, followed by 39 cycles at 95°C for 10s and 60°C for 30s. Nek6 primer sequences were: forward 5'- GGACAGGAAGACAGTGGC-3', reverse 5'- GATATTTGGGTGGTTCAGTT-3'. GAPDH primer sequences were: forward 5'-CGGAGTCAACGGATTTGGTC-3', reverse 5'-CGGTGCCATGGAATTTGCCA-3'. Data were analyzed using the ΔΔCt method.

Transfection of HCC cell lines

A lentivirus-based NEK6-homo-657 RNA plasmid (shNEK6) and an NC-shRNA plasmid were constructed (GenePharma, Shanghai, China). The NEK6 gene was cloned in LV3 (H1/GFP&Puro) plasmids. LV3-NEK6-homo-657 and LV3-shNC plasmids were transfected into 293T cells using RNAi-mate (GenePharma) according to the manufacture's protocol. The cells were harvested 72h after transfection and stable cells were selected and detected by Fluorescence microscope (Motic, China).

Migration and invasion assays

Scratch Assay $5X10^5$ cells/well HCC cells, HCC cells transfected with LV3-NEK6-homo-657, and cells transfected with LV3-shNC were seeded in a 24-well plate respectively and incubated to reach confluence. Then, the monolayer was scratched with a germ-free toothpick and washed with serum-free medium to remove detached cells. The cells were incubated for 72h, and the width of five randomly chosen areas was measured and photographed (Olympus IX73) at 0 h, 24 h, 48 h, and 72 h.

Transwell Assay the suspension cells of HCC cells, HCC cells transfected with LV3-NEK6-homo-657 and cells transfected with LV3-shNC (1 × 105 cells/well) were added to the upper chamber of transwell 24-well plates with 8 μm pore filters (costar 3422, USA), 10 % fetal bovine serum was added to the lower chambers and the cells were cultured for 24 hours. Then, the cells attached to the lower surface of the membranes were stained with 0.1% crystal violet for 20 minutes. The level of migration was observed under an optical microscope (Olympus IX73) and the number of cells in 3 randomly selected views was calculated and recorded.

Tumor-formation assay in nude mice

All animal experiments were approved by the Medical Ethics Committee of Sichuan Provincial People's Hospital and were performed by the guidelines. Fifteen nude mice (BALB/c-nu, 5 weeks old, female, purchased from Laboratory Animal Service Center, Kunming Medical University, Qualification Certificate No: SCXK (Dian) K2015-0002) were subcutaneously injected with HCC cells (0.5 x 10^5/mouse) into the right axilla. After the tumor volume reached 0.6 cm3 (calculated according to the following formula: Volume = width2 × length/2), the nude mice were randomly divided into 3 groups (Blank group, shNEK6 group, and NC-control group). Then the same volume of saline, LV3-NEK6-homo-657, and LV3-shNC (both 50ul, titer: 10^8 pfu/ml) were injected into the tumors of the corresponding groups of mice. Ten days later, the mice were weighed and sacrificed. The tumor tissues were harvested, the tumor weight and volume were recorded and the HE and TUNEL staining were performed regularly.

Statistical analysis

Statistical analysis was performed by SPSS 22.0 Statistical Package (IBM SPSS STATISTICS). Continuous variables conformed to normal distribution were shown as the mean ± standard deviation and analyzed by independent T-test, otherwise, they were recorded as median (P25, P75) and analyzed by Mann-Whitney test. The measured variables were calculated by χ^2 test or fisher's exact test. The correlation between variables was compared by Spearman's correlation analysis. The clinical and pathological variables that affected the postoperative survival were analyzed by Kaplan–Meier method firstly, and then the variables with a p-value ≤ 0.1 were included in the multivariate COX regression mode. A p-value of less than 0.05 was accepted as statistically significant.

Result

Expression of NEK6 in different cell lines

The expression of NEK6 in cell lines was varied. The lowest expression of Nek6 was in Human Hepatocyte cell line: LO-2, in which the measured value was 0.965 ± 0.116; followed by two Hepatocellular carcinoma cell lines: huh-7 and BEL-7402, with the measured values 1.066 ± 0.093 and 1.015 ± 0.094 respectively. The highest ones were Li-7 and HepG-2, both were Hepatocellular carcinoma cell lines, and the corresponding measurements were 1.497 ± 0.303 and 1.633 ± 0.582, respectively. (Fig. 1 and Table 1) Further statistical analysis showed the expression of NEK6 both in Li-7 and HepG-2 were significantly higher than that in LO-2 (P = 0.006 and 0.036, respectively). So, these two HCC cell lines were selected for further study. (Table 1)

Effect of down-regulating the Nek6 expression on Hepatocellular Carcinoma in vitro and in vivo

To evaluate NEK6 function in vitro, the effect of down-regulation of NEK6 expression on the migration and invasion of the Li-7 and HepG2 cells was studied. In the Wound scratch assay,5 randomly chosen area distances in each cell line in each group were measured and statistical analysis was performed. We observed that the migration capability of Li-7 and HepG2 cells (Experimental group) at the edge of the scratch was significantly decreased (both P < 0.05) following down-regulating the Nek6 expression compared with the control (Control group). (Fig. 2, Fig. 3) Further study was performed by transwell assay to compare the migration and invasion capability between Li-7 and HepG2 cells and those transfected with shNEK6. The number of cells attached to the lower surface of the membranes in 3 randomly selected views were calculated and recorded. When silenced the expression of NEK6, the number of cells passing through the transwell chamber in the Experimental group showed a significant decline (both P < 0.01) (Fig. 4). The scratch assay and transwell assay revealed that the migration and invasion capability of Li-7 and HepG2 cells was decreased when they were transfected with the LV3-NEK6-homo-657 virus.

We further carried out an animal model to test the functional activity of NEK6 in vivo. We measured the sizes of the subcutaneous tumors in nude mice formed by the Li-7 HCC cells before and after being injected with saline (Blank group), shNEK6 (shNEK6 group), and shNC (NC-control group) respectively. (Fig. 5) The real volume and increased volume of tumors in the shNEK6 group seemed smaller than those in the NC-control group and the Blank group but without statistical significance (both P > 0.05). Furthermore, the weight of the tumors removed from the nude mice in the three groups was recorded and compared, which were 1.14 ± 0.39 g, 0.60 ± 0.20 g, and 1.08 ± 0.30 g, respectively. The tumor weight was significantly lighter in the shNEK6 group than the other two groups (P < 0.05). (Table 2)

Table 2
The tumor-formation assay in nude mice.

	Blank Group	shNEK6 group	NC-control group	F_1	P_1	T_2	P_2	T_3	P_3	T_4	P_4
Real volume (mm^3) *	1282.08 ± 284.95	1202.15 ± 368.56	2247.61 ± 835.65	1.370	0.291						
Increased volume (mm^3) #	1044.54 ± 264.26	851.19 ± 358.50	1949.60 ± 1794.24	1.509	0.260						
Tumor weight (g)	1.14 ± 0.39	0.60 ± 0.20	1.08 ± 0.30	4.611	0.033	2.749	0.025	2.954	0.018	0.271	0.793
*: The real volume of tumors when harvested. #: The increased volume when the real volume minus the basic volume.											
F_1, P_1: Comparing the results among the Blank Group, shNEK6 group, and NC-control group by one-way ANOVA analysis.											
T_2, P_2: Comparing the results between Blank Group and shNEK6. T_3, P_3: Comparing the results between NC-control Group and shNEK6 group. T_4, P_4: Comparing the results between Blank Group and NC-control group.											

NEK6 Expression in different liver tissues

As we mentioned before, NEK6 had been confirmed with overexpression in many tumors [8, 13–15]. We studied the expression of NEK6 in different liver tissues. The positive detection rate of NEK6 among different tissues varied greatly, which were 90% (27/30, HCC group), 100% (30/ 30, Paracancerous group), 86.67% (13/15, Normal group), 80.00% (8/10, Cirrhotic group), and 69. 39% (34/49, Paraffin group), respectively. The value of NEK6 expression in each group was recorded by median (P25, P75) and compared by Mann-Whitney analysis. (Fig. 6, Table 3)

The expression of NEK6 in HCC was significantly higher than in other groups (all $P < 0.05$). Also, the Paraffin group showed a higher level of NEK6 among the remaining groups (all $P < 0.05$). There was no significant difference among the Paracancerous group, the Normal group, and the Cirrhotic group (all $P > 0.05$). (Table 3)

Analysis of clinical data of HCC cases

Definition of NEK6 overexpression

Of all the 5 liver tissue groups, the specimen of the HCC group and Paraffin group were taken from HCC patients. We set double the median value of Nek6 expression of the Normal group as the cut-off value, and the expression level which was higher than that was defined as overexpression. In this way, of the total of 79 cases in the HCC group and Paraffin group, 43 cases (54.43%) with higher expression level were classified as the Nek6 Overexpression group, the remaining 36 cases (45.57%) were classified as the Control group.

Baseline characteristics of patients

Among the 79 cases, 63 males and 16 females were enrolled in the study, and each case was followed up and the liver function, tumor markers, and CT/MRI outcome were recorded every 3 months. Tumor recurrence time was confirmed based on the results of enhanced CT or MRI. The median follow-up period was 26 months, and the deadline for follow-up was January 2020. The clinicopathologic characters of the patients, including age, gender, Child-Pugh, tumor number and size, tumor differentiation, BCLC staging, and AJCC staging, were summarized in Table 4.

Table 4
Baseline characteristics of patients in Nek6 Overexpression group and Control group.

Characteristics		Control group N(%)	Nek6 Overexpression group N (%)	t/χ^2 value	P value
Age	Mean ± SD	52.56 ± 11.111	55.53 ± 10.338	-1.233	0.221
Gender	Male	28(44.4)	35(55.6)	0.159	0.690
	Female	8(50.0)	8(50.0)		
HBV infection[1]	Positive	23(39.7)	35(60.3)	4.034	0.045
	Negative	12(66.7)	6(33.3)		
Liver cirrhosis	Yes	16(39.0)	25(61.0)	0.040	0.842
	No	14(36.8)	24(63.2)		
Child-Pugh	A	32(44.4)	40(55.6)	0.061	0.805
	B + C	4(57.1)	3(42.9)		
AFP[1]	≥ 400μg/L	14(48.3)	15(51.7)	0.197	0.657
	< 400μg/L	22(53.7)	19(46.3)		
Tumor number	Single	30(61.2)	19(38.8)	5.549	0.018
	Multiple	6(30.0)	14(70.0)		
Tumor size	≤ 5 cm	21(58.3)	15(41.7)	4.344	0.037
	5 cm	15(34.9)	28(65.1)		
Satellite lesions	Positive	6(50.0)	6(50.0)	0.112	0.738
	Negative	30(44.8)	37(55.2)		
Lymph node metastasis	Positive	0(0.0)	3(100)	—[2]	0.2462
	Negative	36(47.4)	40(52.6)		
Margin	Positive	2(33.3)	4(66.7)	—[2]	0.683
	Negative	34(46.6)	39(53.4)		
Portal vein invasion	Positive	0(0.0)	4(100.0)	—[2]	0.1212
	Negative	36(48.0)	39(52.0)		
Metastasis	Positive	1(16.7)	5(83.3)	1.108	0.293
	Negative	35(47.9)	38(52.1)		
Major hepatectomy	Yes	13(39.4)	20(60.6)	0.871	0.351
	No	23(50.0)	23(50.0)		
Tumor differentiation	Well	3(37.5)	5(62.5)	0.645	0.725
	Moderate	19(44.2)	24(55.8)		
	Poorly	12(52.2)	11(47.8)		

[1]: Recorded cases with results only; [2]: Fisher test; 3: Mann-Whitney U test.

Characteristics		Control group N(%)	Nek6 Overexpression group N (%)	t/χ^2 value	P value
Lymphovascular invasion	Positive	12(50.0)	12(50.0)	0.273	0.601
	Negative	24(43.6)	31(56.4)		
p53[1]	Negative	18(40.9)	26(59.1)	3.033	0.082
	Positive	14(63.6)	8(36.4)		
VEGF[1]	Negative	16(55.2)	13(44.8)	0.022	0.881
	Positive	16(57.1)	12(42.9)		
Ki-67[1]	Median (P25, P75)	30(15,40)	20(10.45)	-0.697[3]	0.486
BCLC staging	A + B(%)	34(50.0)	34(50.0)	3.865	0.049
	C + D(%)	2(18.2)	9(81.8)		
AJCC staging	I	20 (55.6)	16 (44.4)	4.758	0.190
	II	10 (45.5)	12 (54.5)		
		5 (35.7)	9 (64.3)		
		1(14.3)	6(85.7)		

[1]: Recorded cases with results only; [2]: Fisher test; 3: Mann-Whitney U test.

The age of the patients enrolled in this study was 52.56 ± 11.111 years and 55.53 ± 10.338 years, the sex ratio of male to female was 28:8 and 35:8, respectively (both P>0.05). The proportion of hepatitis B virus infection in the Nek6 Overexpression group was higher than the Control group (P = 0.045), while 3 other patients with Hepatitis C Virus (HCV) infection, one in the control group and the remaining in the overexpression group, were not analyzed in the discussion. There was no significant difference between the two groups in the number of cirrhotic patients (P = 0.842), Child-Pugh (P = 0.805), and AFP (≤ 400 ug /L) (P = 0.197). (Table 4)

The multiple tumors and bigger tumors (>5 cm) seemed more common in the NEK6 overexpression group (P = 0.018, and P = 0.037, respectively). There was no significant difference between the 2 groups in the Characteristics of satellite lesions (P = 0.738), lymph node metastasis (P = 0.246), margin positive rate (P = 0.683), portal vein invasion (P = 0.121), metastasis (P = 0.293), and Major hepatectomy (P = 0.351).

Also in the two groups, the immunohistochemical analysis showed the tumor differentiation, the positive rate of Lymphovascular invasion, p53, VEGF, and the level of Ki-67 were the same (all P>0.5). While in the control group, there were fewer numbers (2:34, 5.56%) in stage C + D of BCLC stage than that (9:34, 20.93%) in the NEK6 overexpression group (P = 0.049). There was no significant difference in AJCC staging between the two groups (P = 0.190), but the incidence rate of AJCC in the NEK6 overexpression group was 13.95% (6:37), which seemed higher than that in the control group (2.78%, 1:35). (Table 4)

Correlation analysis between NEK6 overexpression and other clinical factors

In Table 5, hepatitis B virus infection, tumor number, tumor size, p53 and BCLC stage (A + B: C + D) in the Control group and NEK6 overexpression group were analyzed by Spearman rank correlation coefficient test. The results showed that the

overexpression of NEK6 was correlated with hepatitis B virus infection and tumor diameter (ρ = 0.230, P = 0.045; ρ = 0.234, P = 0.038). (Table 5)

Table 5
Correlation analysis by Spearman's correlation

Characteristics		HBV infection	Tumor number	Tumor size	p53	BCLC(C + D)
NEK6 overexpression	ρ value	0.230*	0.124	0.234*	0.214	0.221
	P value	0.045	0.278	0.038	0.084	0.050
	N	76#	79	79	66#	79

*: P < 0.05 was regarded as having statistical significance. #: Analyzed cases with results only.

Prognosis and survival analysis

Among all 79 patients, only 14 were survival after the follow-up, 55 died because of tumor-related diseases, and 10 were lost. Sixty-nine patients (87.34%) developed tumor recurrence during the follow-up period: 63 patients were diagnosed with intrahepatic recurrences firstly, and 6 patients had distant recurrences firstly. Reoperation, TACE, microwave ablation, targeted therapy, immunotherapy, and chemotherapy were used alone or in combination to control these recurrent tumors. The median (P25, P75) follow-up time of all the patients was 26.00 (10.00, 39.00) months.

The 3-year disease-free survival (DFS) of the Control group and the Nek6 overexpression group were 27.1% and 14.1%, respectively. The patients in the Control group had a lower tumor recurrent rate (P = 0.038). Also, the 3-year overall survival (OS) rate of the Control group was higher than the Nek6 overexpression group (46.4% VS 33.4%, P = 0.026). (Fig. 7)

According to univariate analysis for DFS and OS, tumor size > 5 cm (both P = 0.000), portal vein invasion (both P = 0.000), Nek6 overexpression (both P < 0.05) were the risk factors for prognosis. Meanwhile, lymph node metastasis, positive margin, and metastasis were the risk factors for OS (all P < 0.05). Furthermore, the P-value of lymph node metastasis, positive margin, and metastasis for DFS and that of satellite Lesions for both DFS and OS were less than 0.1. As we mentioned before, all these factors were enrolled in Multivariate Cox regression analysis. (Table 6)

Table 6
Survival analysis by Kaplan-Merier analysis.

Characteristics	n	three-year DFS			three-year OS		
		Survival rate (%)	χ2 value	P value	Survival rate (%)	χ2 value	P value
Age							
< 60	47	11.3	2.035	0.154	30.5	1.450	0.228
≥ 60	32	29.6			53.8		
Gender							
Male	63	19.3	1.472	0.225	34.7	1.030	0.310
Female	16	25.0			56.3		
HBV + HCV infection							
Yes	61	18.8	0.484	0.486	37.8	0.586	0.444
No	18	24.4			44.9		
AFP (μg/L)[1]							
≥ 400	29	25.8	0.981	0.322	34.1	1.911	0.167
< 400	41	10.9/-			23.2		
Child-Pugh[1]							
A	72	20.9	1.928	0.381	40.7	1.196	0.550
B	6	16.7/-			33.3		
Tumor number							
Single	59	22.2	1.047	0.306	40.4	0.413	0.520
Multiple	20	14.2			36.9		
Tumor size (cm)							
≤ 5	36	37.4	14.677	0.000	65.4	17.935	0.000
> 5	43	5.9			18.4		
Satellite Lesions							
Yes	12	8.3	3.770	0.052	16.7	3.728	0.054
No	67	22.6			44.2		
Lymph node metastasis							
Yes	3	0.00	3.430	0.064	0.00	8.152	0.000
No	76	20.7			40.6		
Margin							
Positive	6	0.00	2.852	0.091	16.7	3.878	0.049

[1]: Recorded cases with results only.

Characteristics	n	three-year DFS			three-year OS		
		Survival rate (%)	χ2 value	P value	Survival rate (%)	χ2 value	P value
Negative	73	22.3			61.1		
Portal vein invasion							
Yes	4	0.000	12.922	0.000	0.00	15.876	0.000
No	75	21.3			41.9		
Metastasis							
Yes	6	0.00	2.940	0.086	0.00	7.318	0.007
No	73	21.7			42.6		
Tumor differentiation[1]							
Well	8	43.8	1.827	0.401	57.1	2.982	0.225
Moderate	44	23.6			44.5		
Poor	22	12.3			29.0		
Lymphovascular invasion							
Yes	24	17.0	1.719	0.190	20.5	2.116	0.146
No	55	21.1			47.4		
Liver cirrhosis							
Yes	41	17.2	0.485	0.486	41.9	0.493	0.483
No	38	21.9			33.9		
p53[1]							
Positive	44	17.7	0.084	0.772	44.4	1.077	0.299
Negative	22	14.9			35.0		
VEGF[1]							
Positive	29	14.4	0.170	0.680	36.2	0.198	0.656
Negative	28	19.3			49.2		
Ki-67[1]							
Positive	46	13.2	0.693	0.405	39.8	1.723	0.189
Negative	20	30.0			47.7		
NEK6 expression							

[1]: Recorded cases with results only.

Characteristics	n	three-year DFS			three-year OS		
		Survival rate (%)	χ2 value	P value	Survival rate (%)	χ2 value	P value
Control group	36	27.1	4.304	0.038	46.4	4.934	0.026
overexpression group	43	14.1			33.4		
[1]: Recorded cases with results only.							

The multivariate Cox regression analysis showed Tumor size over 5 cm increased the HR by 2.3-fold (95% CI: 1.254–4.379, P = 0.008) in DFS and by 2.8-fold (95% CI: 1.492–5.347, P = 0.001) in OS. Whilst the portal vein invasion boosted the HR by 3.7-fold (95% CI: 1.064–12.545, P = 0.040) in DFS and by 4.27-fold (95% CI: 1.143–15.915, P = 0.031) in OS respectively. Satellite lesions, lymph node metastasis, metastasis, positive margin, and Nek6 overexpression did not influence the HR, according to multivariate analysis. (Table 7)

Table 7
Disease-free survival and Overall survival analysis by Multivariate Cox regression.

Characteristics	DFS					OS				
	Regression coefficients	P value	HR	HR95% CI		Regression coefficients	P value	HR	HR95% CI	
Tumor size (>5cm)	0.852	0.008	2.343	1.254	4.379	1.038	0.001	2.824	1.492	5.347
Satellite lesions	0.089	0.839	1.093	0.464	2.573	-0.131	0.768	0.877	0.367	2.094
Lymph node metastasis	0.836	0.286	2.307	0.497	10.714	1.344	0.096	3.835	0.788	18.673
Portal vein invasion	1.295	0.040	3.653	1.064	12.545	1.451	0.031	4.265	1.143	15.915
Metastasis	0.252	0.667	1.287	0.407	40.64	0.902	0.130	2.465	0.767	7.922
Margin	-0.050	0.927	0.951	0.328	2.758	0.161	0.780	1.174	0.380	3.624
NEK6 overexpression	0.308	0.307	1.361	0.754	2.459	0.402	0.188	1.495	0.822	2.721

Discussion

NEK6 is a member of the NIMA-related serine/threonine kinase family and serves as a novel target of the DNA damage checkpoint for cell self-repair to monitor the process of mitosis [20]. Whether in solid tumors [8, 13–15] or non-solid tumors [21], compared with normal tissues and fibroblasts, the transcription, protein expression, and kinase activation levels of NEK6 were higher in malignant tumor tissues and human tumor cell lines, which indicated that NEK6 played an important role in tumorigenesis [8]. In our study, we compared the expression levels of NEK6 among normal human liver cell (LO-2) and human liver cancer cells (HepG2, Li-7, Huh-7, BEL-7402). We proved NEK6 had the highest expression in Li-7 and HepG-2 cell lines. At the same time, whether in fresh or paraffin HCC tissues, the expression level of NEK6 was much higher than in paracancerous, cirrhosis, and normal liver tissues.

The mechanism by which NEK6 promoted tumorigenesis was still unclear. Some studies speculated that this might be related to the following three aspects: NEK6 overexpression could inhibit the expression of wild-type p53 gene [22], activated the Signal Transducers and Activators of Transcription 3 (STAT3) signaling pathway [10], and blocked the TGF-β/Smad signaling pathway [23]. Meanwhile, Jee et al. found that NEK6 overexpression could induce early resistance of tumor cells

to the anticancer drugs: camptothecin and doxorubicin [24]. While decreasing the NEK6 expression could improve the sensitivity of human tumor cells to anticancer drug therapy, inhibit tumor growth in xenograft mouse models, and promote tumor cell apoptosis [25].

We conducted experiments in vitro, which showed that inhibiting the expression of NEK6 could reduce the migration, repair, and invasion abilities of Li-7 and HepG-2 cell lines. In addition, when we performed the tumor-formation assay in nude mice, the result showed that the weight of the tumors of tumor-bearing nude mice with the injection of shNC was significantly lighter than the others. At the same time, the overexpression of NEK6 in human cancer cells further produces a large number of NEK6, to promote the production and proliferation of their own tumor cells [8]. Therefore, NEK6 may be an important tumor promoter and its overexpression accelerated the cell cycle process, speeded up the self-reproduction of tumor cells, enhanced their transmural invasion ability, and promoted the infiltration and metastasis of HCC cells [27].

In addition, in a xenograft nude mice experiment, Nassirpour et al. [8] found that the overexpression of exogenous wild-type NEK6 promoted the non-anchorage-dependent growth of a variety of human cancer cell lines, leading to distant metastasis of cancer cells. They inhibited the proliferation of HeLa cells (a type of malignant tumor cell) and reduced the tumor diameter by reducing the expression of NEK6. Zou et al. [23] also discovered that NEK6 expression level was significantly up-regulated both in HCC patients with portal vein tumor thrombi and in HCC cell lines with strong metastasis ability.

It indicated that NEK6 overexpression was related to the metastatic ability of HCC cells and easily promoted the metastasis of HCC by portal vein invasion, lymph node, and surrounding tissue infiltration, which was also consistent with our findings. In our study, the weight of the tumors of tumor-bearing nude mice in the NEK6 group was significantly heavier than in other groups. And the NEK6 expression level in HCC patients was positively correlated with tumor diameter. Furthermore, HCC patients with NEK6 overexpression had more tumor numbers, larger tumor diameters, and more cases classified as BCLC C/D stages, which indicating these patients could have a higher possibility of portal vein invasion, lymph node metastasis, and distant metastasis.

When we analyzed the data of different clinical characteristics, we also found that there were quite a few cases of hepatitis virus infection in the NEK6 overexpression group. Further correlation analysis suggested that the expression level of NEK6 was positively correlated with hepatitis B virus infection. Although there was no solid evidence to suggest that NEK6 expression was directly related to hepatitis virus infection, existing studies still showed some potential indirect association between them. For example, Chen et al. [28] detected the levels of Peptidyl-prolyl Isomerase (PIN1) and NEK6 mRNA in 40 pairs of HCC and adjacent tissues. The results indicated that the expression of NEK6 was positively correlated with PIN1 and was involved in the carcinogenesis of HCC. PIN1 overexpression promoted the occurrence of HCC [29–31]. It could both enhance the stability of hepatitis virus X protein (HBx), encoded by HBV and play as a main carcinogenic component in HBV-induced HCC [32] and increase the replication and proliferation of HCV [31]. Therefore, to some extent, NEK6 overexpression might be associated with HBV or HCV infection through increasing the expression of PIN1. This correlation was partially verified in this study, but the specific mechanism was still unclear.

The previous discussion mentioned that inhibiting the expression of NEK6 will not affect p53-induced senescence, but may promote wild-type p53-induced apoptosis [22]. At the same time, inhibition of NEK6 expression was sufficient to eliminate the cell transformation activity in a variety of aggressive cancer cell lines without causing normal cell death [8]. Therefore, many researchers took NEK6 as a potential cancer drug treatment target and conducted related experimental studies [8, 10, 22–25].

Conclusion

This study confirmed that NEK6 expression was up-regulated in HCC and this overexpression promoted the infiltration and metastasis of HCC and deteriorated the prognosis of patients, suggesting it might be a clinically valuable biomarker and a potential therapeutic target. However, due to the limited sample size and the inconsistent definition of NEK6 overexpression,

some bias in the research results was inevitable. Further studies should enroll more cases, focus on the mechanism of NEK6 promoting tumorigenesis, and develop the corresponding therapeutic drugs.

Abbreviations

AFP	alpha-fetoprotein
AJCC	American Joint Commission for Cancer
BCLC	Barcelona Clinic Liver Cancer
CDKs	cyclin-dependent kinases
DFS	disease-free survival
HBV	Hepatitis B Virus
HBx	hepatitis B virus Xprotein
HCC	hepatocellular carcinoma
HCV	Hepatitis C Virus
HR	hazard ratio
NEK	Never-in-mitosis A-related kinase
OS	overall survival
PCR	Polymerase Chain Reaction
PIN1	Peptidyl—prolyl Isomerase
PLC	Primary liver cancer
PLK1	Polo-like kinase1
qPCR	Real-time Quantitative PCR Detecting System
STAT	Signal transduction and Activator of Transcription
TACE	transcatheter arterial chemoembolization

Declarations

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of Sichuan Provincial People's Hospital, School of Medicine, University of Electronic Science and Technology of China.

Consent for publication:

Not applicable.

Availability of data and materials:

All data generated or analysed during this study are included in this published article.

Competing interests:

The authors declare that they have no competing interests.

Funding:

Not applicable.

Authors' contributions:

Hao Zhang and Bo Li contributed to the conception of the study;

Hao Zhang contributed significantly to analysis and manuscript preparation;

Hao Zhang and Bo Li performed the data analyses and wrote the manuscript.

Acknowledgment: Not applicable.

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Figures

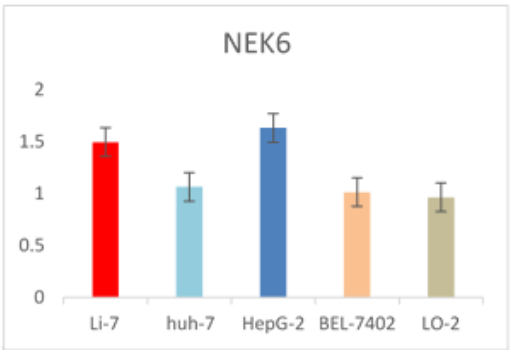

Figure 1

Expression of NEK6 in hepatic cell line and HCC cell lines.

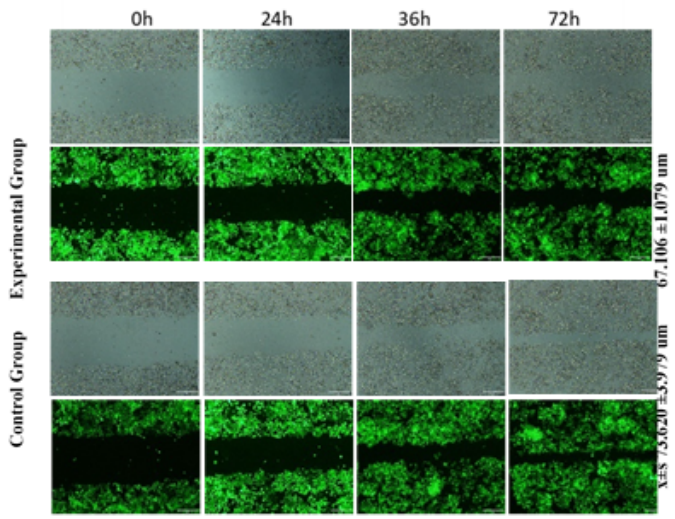

Figure 2

The wound scratch assay of Li-7 cell. (× 40 times) (Experimental group vs Control group, P = 0.019).

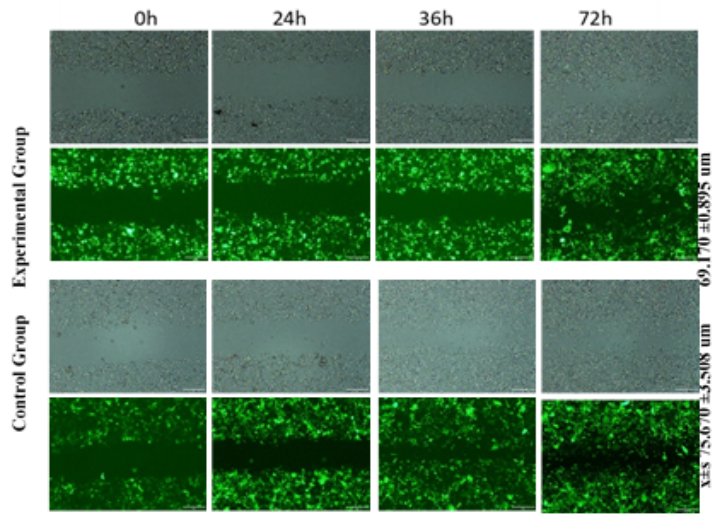

Figure 3

The wound scratch assay of HepG2. (× 40 times) (Experimental group vs Control group, P = 0.004).

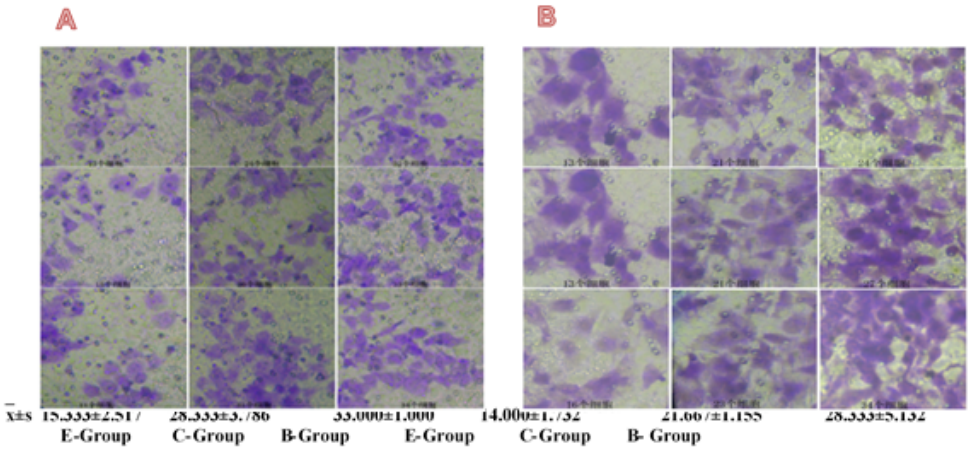

Figure 4

The transwell assay of Li-7(A) and HepG2(B). (Crystal purple staining × 100). E-group Experimental group C-group Control group B-group Blank group A: E-group vs C-group P=0.008; E-group vs B-group P < 0.001. B: E-group vs C-group P=0.003; E-group vs B-group P < 0.010.

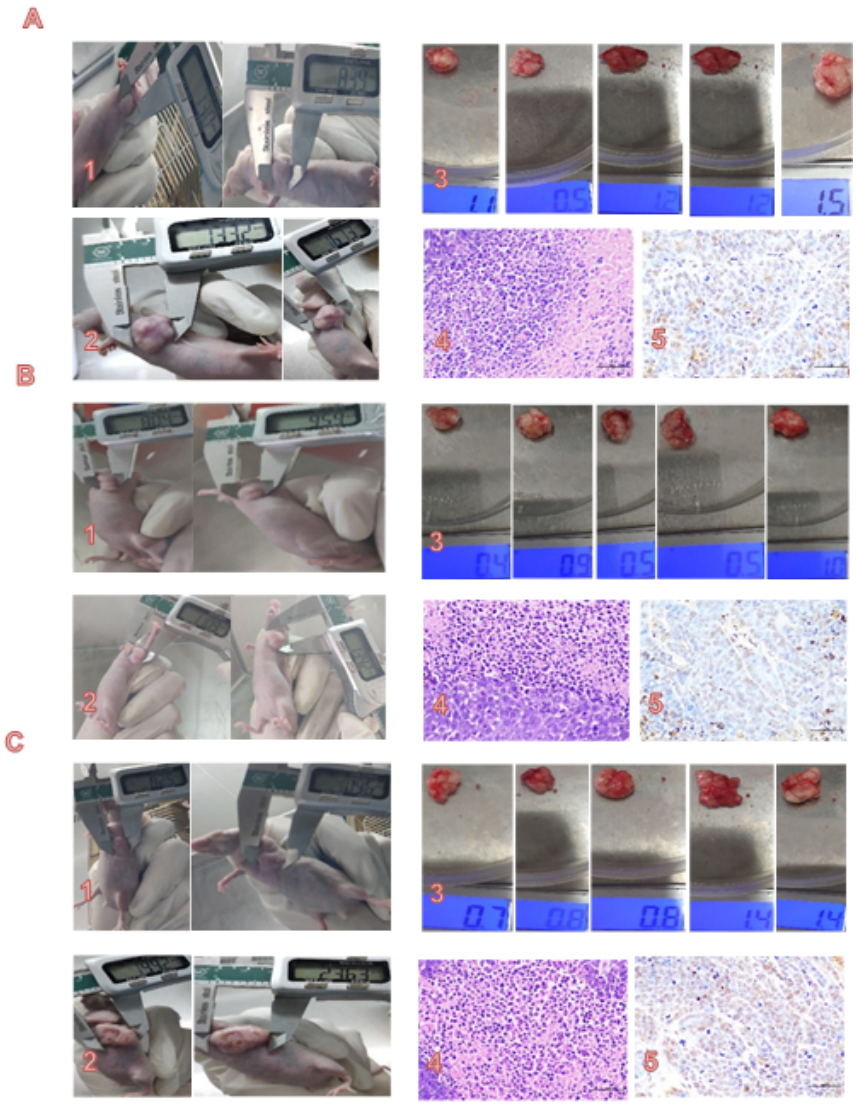

Figure 5

The tumor-formation assay in nude mice. A: Blank group B: NEK6 group C: NC-control group 1: The calculation of tumor volume when they reached about 0.6 mm3 2: The calculation of tumor volume before the nude mice were sacrificed. 3: The weight of tumors. 4: HE staining ×40. 5: TUNEL staining ×40.

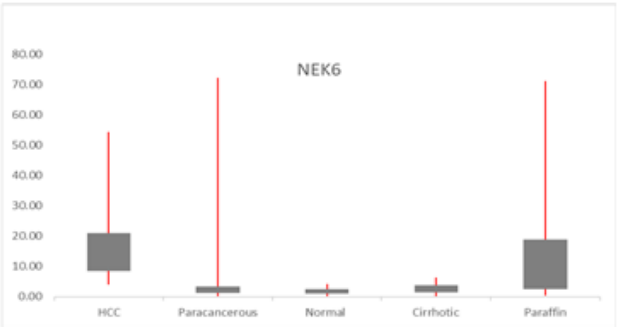

Figure 6

Expression of NEK6 in different liver tissues. (Median (P25, P75)) The value of NEK6 expression in each group were 16.49 (8.36, 20.88), 2.06 (1.12, 3.31), 1.47 (0.96, 2.41), 2.47 (1.53, 3.79) and 5.93 (2.57, 18.83), respectively.

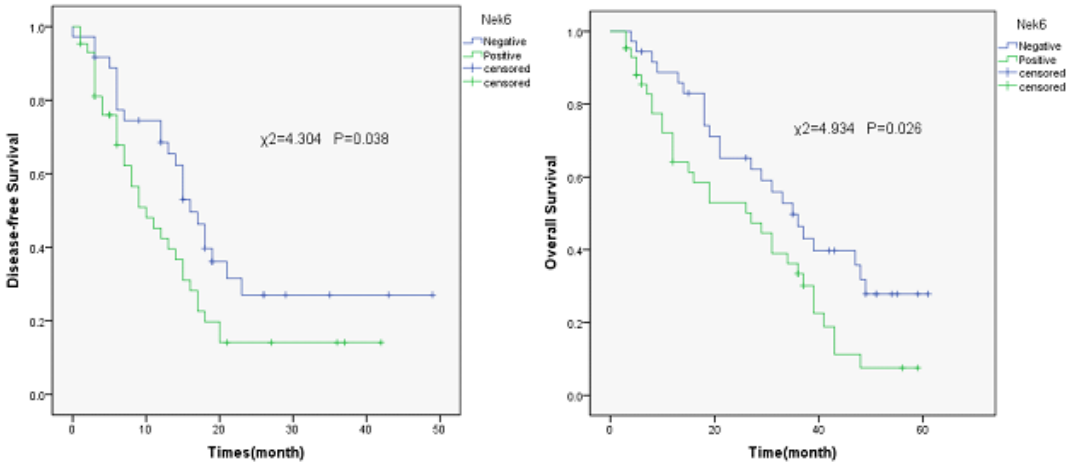

Figure 7

The disease-free survival (DFS) and overall survival (OS) of Nek6 Overexpression group and Control group.