

Received: 2018.01.17
Accepted: 2018.02.01
Published: 2018.03.26

MAP Kinase-Interacting Kinase 1 Promotes Proliferation and Invasion of Hepatocellular Carcinoma and Is an Unfavorable Prognostic Biomarker

Authors' Contribution:

Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

B **Xujing Wang***
B **Yongkun Wang***
B **Qiqi Zhang***
C **Huiren Zhuang**
A **Bo Chen**

Department of Hepatopancreatobiliary Surgery, East Hospital Affiliated to Tongji University in Shanghai, Shanghai, P.R. China

* These authors contributed equally to this work

Corresponding Author:

Bo Chen, e-mail: ipd1950825@126.com; chenbo7349@hotmail.com

Source of support:

This work was funded by the Pudong New District Science and Technology Development Fund (PKJ2015-Y15)

Background: Hepatocellular carcinoma (HCC) accounts for one of the most prevalent tumor types in the world. The MAP kinase-interacting kinase 1 (MNK1) functions downstream of MAP kinases such as p38 and ERK, and its potential role in cancer development is being uncovered. The aim of this study was to investigate the expression and function of MNK1 in HCC.


Material/Methods: Immunohistochemical staining and quantitative PCR were performed to explore the expression of MNK1 in both HCC tissues and adjacent normal liver tissues. Chi-square test, univariate analysis, and multivariate analysis were conducted to statistically evaluate clinical significance of MNK1 in HCC. Proliferation, migration, and invasion capacities of HCC cells were assessed after overexpressing or silencing MNK1.

Results: Both the RNA and protein levels of MNK1 were upregulated in HCC tissues compared to normal liver tissues. High expression of MNK1 was correlated with advanced tumor stage and poor overall survival. Moreover, MNK1 was identified as a novel independent prognostic factor for HCC patients. Cellular studies showed that MNK1 can enhance the proliferation, migration, and invasion capacities of HCC cells, thereby promoting tumor progression.

Conclusions: High expression of MNK1 is frequent in HCC tissues, which promotes tumor proliferation and invasion, and is correlated with a poor overall survival. Targeting MNK1 may be a novel direction for the drug development of HCC therapy.

MeSH Keywords: **Carcinoma, Hepatocellular • Neoplasm Invasiveness • Survival Analysis**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/909012>

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Background

Hepatocellular carcinoma (HCC) accounts for one of the most prevalent tumor types in the world [1,2]. Most HCC patients have a background of chronic liver cirrhosis induced by alcohol, steatohepatitis, or hepatitis B virus (HBV) infection [3]. Other epidemiological risk factors for HCC include obesity, diabetes, and metabolic diseases [4 5]. During the past decades, there have been great advances in the treatment of HCC including surgical improvement and drug therapies [6,7]. However, the overall clinical outcomes of HCC are far from satisfied [8,9]. For example, it was reported that more than 70% of patients have tumor recurrence within 5 years after surgical resection [10]. Moreover, prediction of HCC patients is not easy due to their distinct molecular expression patterns and biological behaviors. Therefore, identification of novel biomarkers at a molecular level is necessary to develop current criteria from clinical aspect.

Molecular markers with different levels can serve as prognostic biomarkers, such as mRNA, microRNA, and protein expression. More and more studies are now focused on post-translational modification, which plays critical roles in modulating protein functions and downstream signaling pathways. For example, the phosphorylation of Bcl2 [11], ubiquitination of beta-arrestin [12], and acetylation of histone H4 [13] have all been correlated with tumor progression. Correspondingly, these specific enzymes are determinants of upstream regulators. Here we focused on investigating the role of MAP kinase-interacting kinase 1 (MNK1) in HCC.

MNK1 functions downstream of MAP kinases, such as p38 and ERK, and its potential role in cancer development is being uncovered. The high expression of MNK1 and its prognostic role was identified in glioma [14], breast cancer [15], and ovarian cancer [16]. Furthermore, deficiency of MNK1 can delay glioma development in a mice model [17]. The inhibitor of MNK1 was also reported to suppress tumor progression of non-small cell lung cancer [18], melanoma [19], ovarian cancer [16], and prostate cancer [20]. However, currently there is no evidence about its expression pattern in HCC.

Our study explored both the RNA and protein levels of MNK1 in HCC tissues and normal liver tissues for the first time. And we conducted statistical analyses to demonstrate its clinical significance. Importantly, we also performed cellular studies using human HCC cell lines to validate the cellular effects of MNK1 on the progression of HCC.

Material and Methods

Patients and samples

This study was in accordance with Declaration of Helsinki. This study was approved by the Ethics Committee of East Hospital Affiliated to Tongji University in Shanghai (China). Written informed consent was obtained from all participants before sample collection and usage. A total of 126 HCC patients were enrolled in the present study. All patients were treated with R0 curative surgical resection (no cancerous cell can be detected under light microscope on the resection margin) between January 2010 and January 2015 in our hospital. All diagnoses were based on histologically examination. None of the patients received any anti-tumor treatment before surgery. The median follow-up period was 31.0 months (range 3–72 months). Retrieved information included gender, age, hepatitis B surface antigen (HBsAg), tumor diameter, tumor number, pathological grade, and TNM stage. An additional 32 pairs of HCC samples and adjacent liver tissues were collected immediately after resection and stored in liquid nitrogen until further use.

Immunohistochemical (IHC) staining

We performed immunohistochemical (IHC) staining for MNK1 on 4- μ m sections from HCC and normal liver tissues as described previously by others [21,22]. Briefly, specimen sections were deparaffinized and rehydrated by using graded ethanol. Later, the slides were incubated with 3% H₂O₂ solution for 10 min to quench the endogenous peroxidase activity. Subsequently, sections were subjected to antigen retrieval in sodium citrate buffer (10 mM, pH 6.4) for 30 min at 98°C and then cooled to room temperature. After incubation with 5% goat serum for 30 min at 37°C to block nonspecific antigens, the slides were incubated with anti-MNK1 antibody (C4C1, Cell Signaling, 1:1000) or PBS at 4°C overnight. The next day, after washing the slides 3 times with PBS solution, horseradish peroxidase (HRP)-conjugated secondary antibody was added and incubated for 45 min at 37°C. Finally, slides were stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and observed under the microscope, followed by hematoxylin counterstaining.

IHC evaluation

The expression of MNK1 in tissues was assessed based on both the staining intensity and percentage of positive stained cells. For the staining intensity, we scored as followed: 1 (negative staining), 2 (lightly yellow), 3 (deep yellow), and 4 (yellow brown). For the percentage of positive stained cells, we classified as score 1, 0–25% positive tumor cells; 2, 25–50% positive tumor cells; 3, 50–75% positive tumor cells; and 4, 75–100% positive tumor cells. The final IHC score was calculated by

multiplying the 2 aforementioned scores (range, 1–16). All the results were reviewed by 2 independent pathologists.

Reverse transcription and real-time quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately 1 µg of total RNA was then reversely transcribed into cDNA, and subjected to RT-qPCR analysis with the following primers [16]:

MNK1 (F): 5'-GATTCCTCTGAGACTCCAAGTTAA-3',
MNK1 (R): 5'-ACGCTTCTTCTCCTCCTCTT-3',
GAPDH (F): 5'-GGGTGTGAACCATGAGAAGT-3',
GAPDH (R): 5'-GACTGTGGTCATGAGTCTCT-3'.

The results were normalized by GAPDH and calculated with ΔCT method [23].

Cell and transfection

HepG2 cells were purchased from ATCC (Shanghai, China). Cells were maintained in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were digested and expanded to 95% confluence.

Human originated MNK1 plasmid construct was obtained from William Hahn & Jean Zhao [24]. siRNAs were ordered from Sigma with the following sequences [16]: MNK1 siRNA: 5'-UGCCCAUCUCUAUAGGUUUUUU-3'; Control siRNA: 5'-AAACCUAAGUAGAGUGGACUU-3'. All the transfections were performed using Lipofectamine 2000 reagent at 40% cellular confluence according to the manufacturer's protocol.

Western blot

Cultured cells were harvested and lysed with lysis buffer. The supernatant of lysate was collected by centrifuging at 13 000 rpm for 20 min. After quantification using a BCA kit, about 20 µg of total proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were firstly blocked with 5% non-fat milk for 1 hour at room temperature, and then incubated with specific antibodies (MNK1 and beta-actin) at 4°C overnight. The next day, membranes were subsequently incubated with corresponding secondary antibodies for 1 hour at room temperature. Finally, protein bands were visualized using an ECL western blotting kit (Thermo Fisher Scientific).

CCK-8 assay

To evaluate the effects of MNK1 on tumor cell proliferation, plasmid or siRNA transfected cells were seeded at 2×10^4 cells

per well in a 96-well plate and cultured in DMEM. At designated time points, the cell viability was assessed by a Cell Counting Kit (CCK)-8 (CK04-500, Dojindo, Japan) according to the manufacturer's instructions. Briefly, 10 µL of CCK-8 reagent was added into each well and incubated for another 4 hours at 37°C. Absorbance at 450 nm was then measured by a microplate reader, and the corresponding proliferation curves were plotted by Graphpad 5.0 Software. All experiments were performed in triplicate and repeated at least 3 times.

Transwell and matrigel-transwell assays

The Transwell chambers (BD Biosciences) were used to test the migration capacity of HepG2 cells [25]. Briefly, 5×10^4 cells were seeded into the upper chamber with 100 µL volume, and the lower chamber was supplied with 500 µL DMEM containing 20% FBS. After 24-hour incubation, cells migrated to the lower surface of membrane were fixed by methanol and stained with 0.1% crystal violet. The invasion analysis was quite similar with that of migration experiments, except pre-coating the Transwell chambers with Matrigel before seeding cells as described by others [26]. Both migration and invasion experiments were performed in triplicate and repeated at least 3 times.

Statistics

Statistical analyses were performed with SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). The associations between MNK1 staining and the clinicopathologic parameters of HCC patients were evaluated by chi-square tests. Kaplan-Meier model and log-rank test were conducted to test the effects of distinct variables on HCC survival. The factors with $P < 0.05$ in the univariate analyses were subjected into a multivariate analysis model to further validate their independent prognostic role. Data for the cellular experiments were presented with mean \pm SD, and differences between groups were determined by Student's *t*-test or one-way ANOVA test. Differences were considered statistically significant with $P < 0.05$.

Results

Patient information

Our cohort included 126 HCC patients (102 males and 24 females), and the median age was 48 years old (range, 32–74 years old). By testing the levels of HBsAg, 96 patients (76.2%) were diagnosed with HBV positive, and the other 30 patients (23.8%) with negative HBV infection. As for the tumor size, 53 patients (42.1%) possessed the largest tumor diameter ≤ 5 cm, while the other 73 patients (57.9%) suffered with a larger tumor size. Most of the cases (88 out of 126, 69.8%) showed

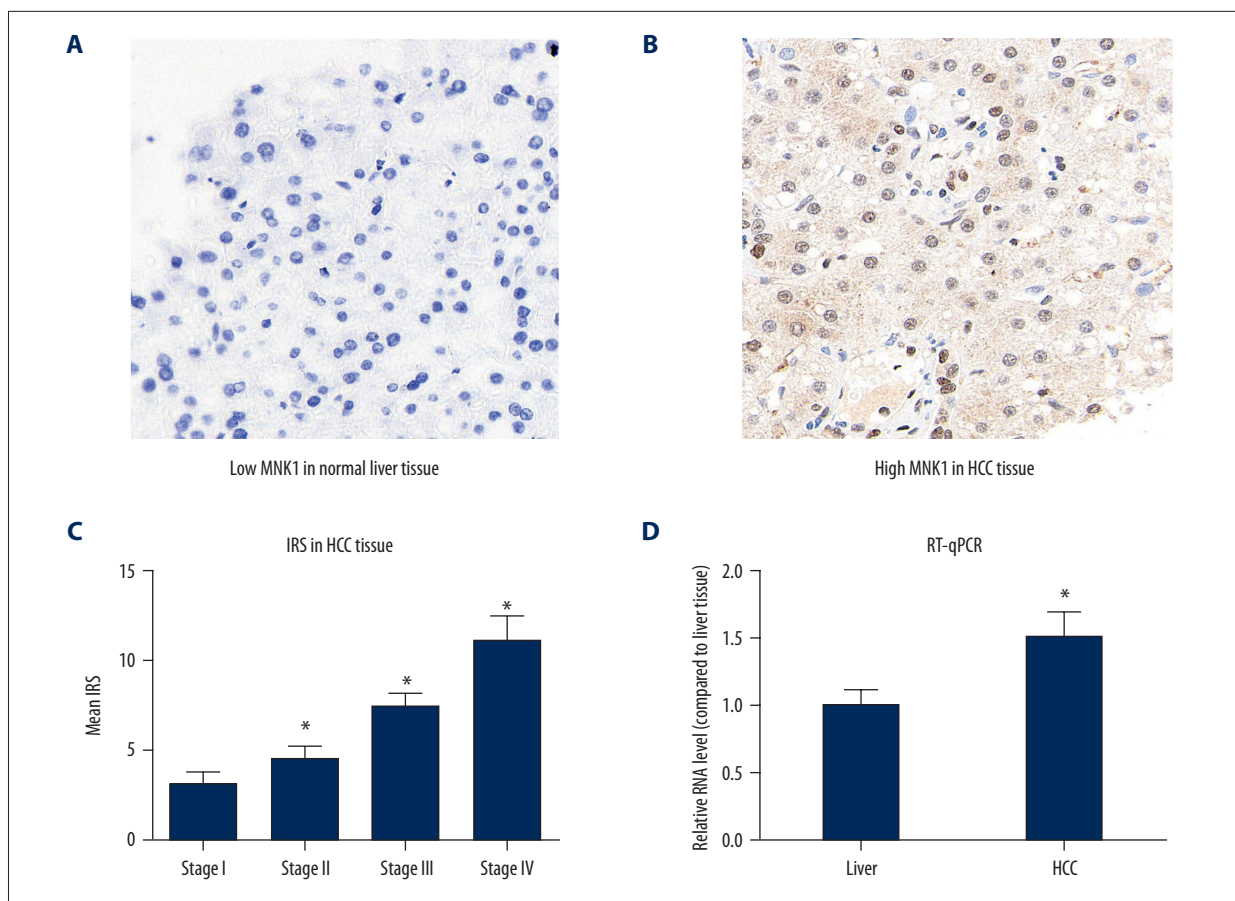


Figure 1. Expression of MNK1 in normal liver and hepatocellular carcinoma (HCC) tissues. **(A)** Representative immunohistochemical (IHC) expression of MNK1 in normal liver tissue. **(B)** Representative IHC expression of MNK1 in HCC tissue. **(C)** Comparison of average IHC scores of MNK1 in HCC patients with distinct TNM stages. **(D)** mRNA level of MNK1 in HCC tissues and adjacent non-tumor tissues were analyzed by qPCR, Data are mean \pm SD from 3 independent experiments (* $P < 0.05$).

single tumor site, while 38 patients (30.2%) showed multiple tumor sites. We also retrieved the pathological grade of all participants, 18 patients (14.3%) with grade 1, 76 patients (60.3%) with grade 2, and 32 patients (25.4%) with grade 3. Finally, more than half of the patients (65 out of 126, 51.6%) were diagnosed with TNM stage I or II.

MNK1 was upregulated in HCC tissues

We first measured the protein expression of MNK1 in both HCC tumor tissues and adjacent normal liver tissues by IHC strategy. Accordingly, MNK1 showed negative or light staining in normal liver tissues (Figure 1A), while positively stained in HCC tissues which predominantly located in the cell nucleus (Figure 1B). By analyzing the IHC scores among HCC patients in different stages, we found that MNK1 showed increased expression levels in patients with advanced stages (Figure 1C). In addition, we tested the RNA levels of MNK1 in fresh-frozen tissues, which consistently revealed an elevation level in HCC tissues than that in normal liver tissues (Figure 1D).

MNK1 high expression was positively correlated with HCC progression

To better explore the clinical relevance of MNK1 in HCC patients, we classified cases into low MNK1 expression (IHC score ≤ 6) and high MNK1 expression (IHC score > 6) groups. The chi-square test was then enrolled to compare the differences of MNK1 in patients with distinct clinicopathological phenotypes (Table 1). No correlation between MNK1 and age, gender, HBsAg, or pathological grade was observed. However, a higher MNK1 level was positively associated with larger tumor size ($P < 0.001$), multiple tumor locations ($P = 0.018$), and advanced TNM stages ($P = 0.013$). The positive correlations between MNK1 and unfavorable tumor features indicated that MNK1 may function as a tumor promoter in HCC progression.

Table 1. Correlations between MNK1 protein expression and features of HCC patients.

Variables	Cases (n=126)	LOXL2 expression		P value
		Low (n=46)	High (n=80)	
Sex				0.911
Feale	24	9	15	
Male	102	37	65	
Age (years)				0.886
≤50	75	27	48	
>50	51	19	32	
HBsAg				0.374
Negative	30	13	17	
Positive	96	33	63	
Tumor size				<0.001*
≤5 cm	53	35	18	
>5 cm	73	11	62	
Tumor number				0.018*
1	88	38	50	
≥2	38	8	30	
Pathological grade				0.115
Grade 1	18	10	8	
Grade 2	76	23	53	
Grade 3	32	13	19	
TNM stage				0.013*
Stage I-II	61	29	32	
Stage III-IV	65	17	48	

* Statistically significant by chi-square test.

MNK1 was an independent prognostic factor for the overall survival of HCC patients

We next aimed to test the clinical significance of MNK1 in HCC. The overall survival curves were plotted by Kaplan-Meier method (Figure 2). According to our data, patients with larger tumor size ($P=0.003$), advanced pathological grade ($P=0.002$) or later TNM stages ($P=0.004$) showed poorer overall survival (Table 2). Importantly, HCC patients with higher MNK1 expression also showed poorer prognoses compared to those with lower MNK1 level (mean overall survival 30.9 ± 2.3 vs. 44.2 ± 3.0 months, $P=0.002$). In contrast, HCC clinical outcomes seemed to be little affected by patients' age, gender, HBV infection, or tumor numbers.

The multivariate Cox regression analysis was subsequently performed, which showed no significantly independent effect of tumor size or pathological grade on HCC survival (Table 3). The

advanced TNM stages, consistent with data from other groups, served as an independent risk factor for patients' survival (HR=2.116, 95%CI 1.136–3.940, $P=0.018$). Moreover, our data identified a significant role of MNK1 expression in help predicting HCC prognosis (HR=3.007, 95%CI 1.691–4.600, $P=0.001$).

MNK1 enhances HCC proliferation, migration, and invasion

Since the clinical statistics demonstrated a tumor-promoting role of MNK1 in HCC progression, we next performed cellular experiments to investigate its underlying mechanisms. A human HCC cell line, HepG2, was transfected with MNK1-siRNA and MNK1 plasmids, respectively (Figure 3A). As a consequent, cells transfected with MNK1-siRNA showed an attenuated proliferation pattern, while cells overexpressing MNK1 showed an enhanced proliferation ability (Figure 3B). Similarly, both the migration (Figure 3C) and invasion (Figure 3D) processes

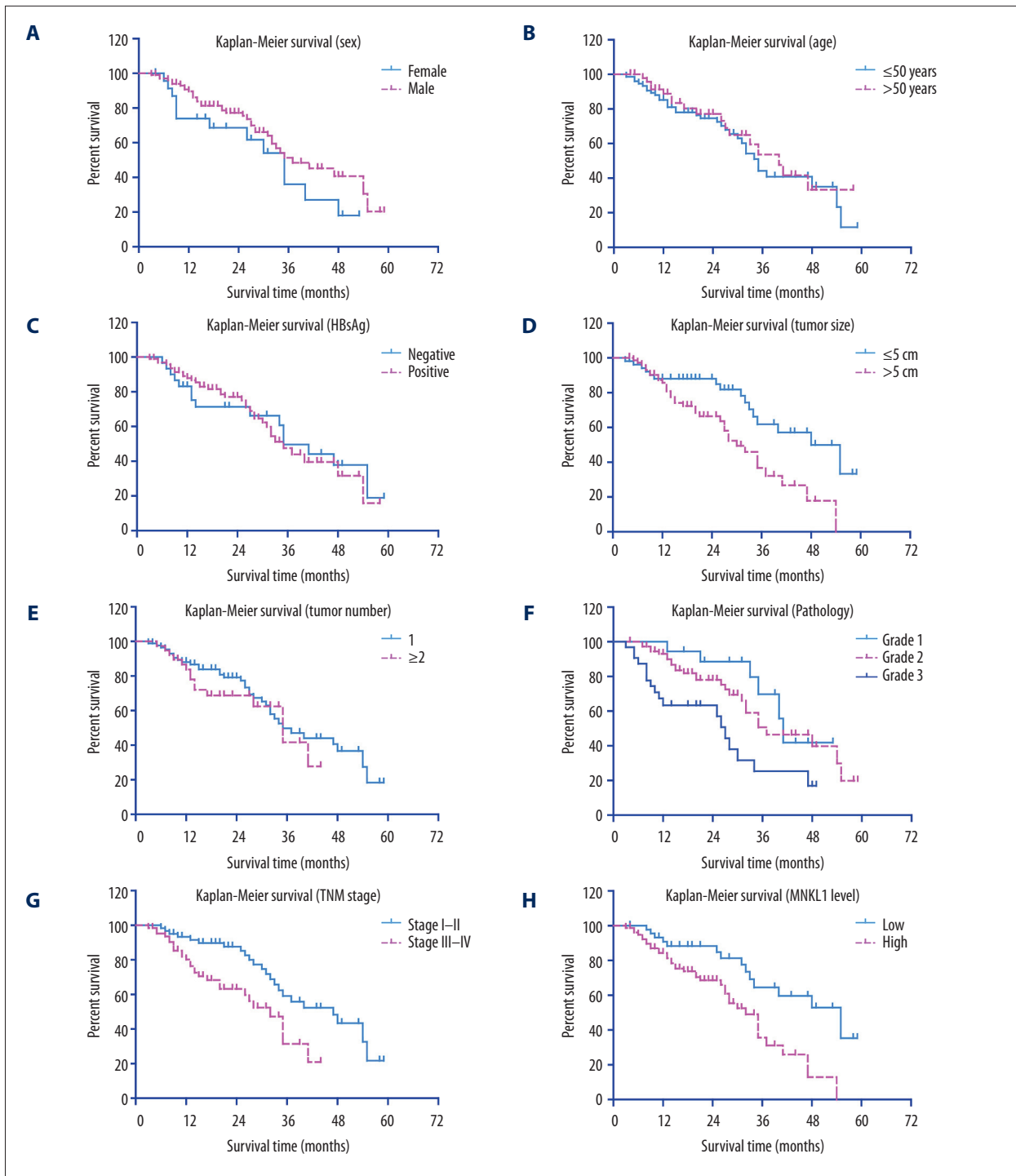


Figure 2. Kaplan-Meier analyses of overall survival. Kaplan-Meier curves showed the correlations between overall survival of hepatocellular carcinoma patients by: (A) gender; (B) age; (C) HBV infection; (D) tumor size; (E) tumor number; (F) pathological grade; (G) TNM stage; and (H) MNK1 protein level. * indicates $P < 0.05$ by log-rank test.

Table 2. Overall survival of HCC patients.

Variables	Patients (n=126)	Overall survival months		P value
		Median	Mean ±S.D.	
Sex				0.144
Female	24	35.0	30.9±3.8	
Male	102	37.0	38.3±2.3	
Age (years)				0.581
≤50	75	35.0	35.9±2.5	
>50	51	40.0	38.2±3.3	
HBsAg				0.910
Negative	30	35.0	36.8±3.9	
Positive	96	35.0	36.3±2.4	
Tumor size				0.003*
≤5 cm	53	48.0	43.2±3.0	
>5 cm	73	30.0	31.1±2.4	
Tumor number				0.376
1	88	35.0	37.9±2.3	
≥2	38	35.0	30.5±2.5	
Pathological grade				0.002*
Grade 1	18	41.0	41.8±3.3	
Grade 2	76	37.0	38.9±2.7	
Grade 3	32	27.0	25.9±3.2	
TNM stage				0.004*
Stage I-II	61	47.0	41.6±2.5	
Stage III-IV	65	32.0	28.0±2.0	
MNK1 expression				0.002*
Low	46	55.0	44.2±3.0	
High	80	32.0	30.9±2.3	

* Statistically significant by log-rank test.

Table 3. Multivariate Cox analysis for prognostic factors of HCC patients.

Variables	HR	95% CI	P value
Tumor size (>5 cm vs. ≤5 cm)	1.832	0.884–3.795	0.103
Pathological grade (grade 3 vs. 1/2)	1.611	0.732–3.545	0.236
TNM stage (stage III/IV vs. I-II)	2.116	1.136–3.940	0.018*
MNK1 expression (high vs. low)	3.077	1.691–4.600	0.001*

* Statistically significant by Cox regression model. HR – hazard ratio; CI – confidence intervals.

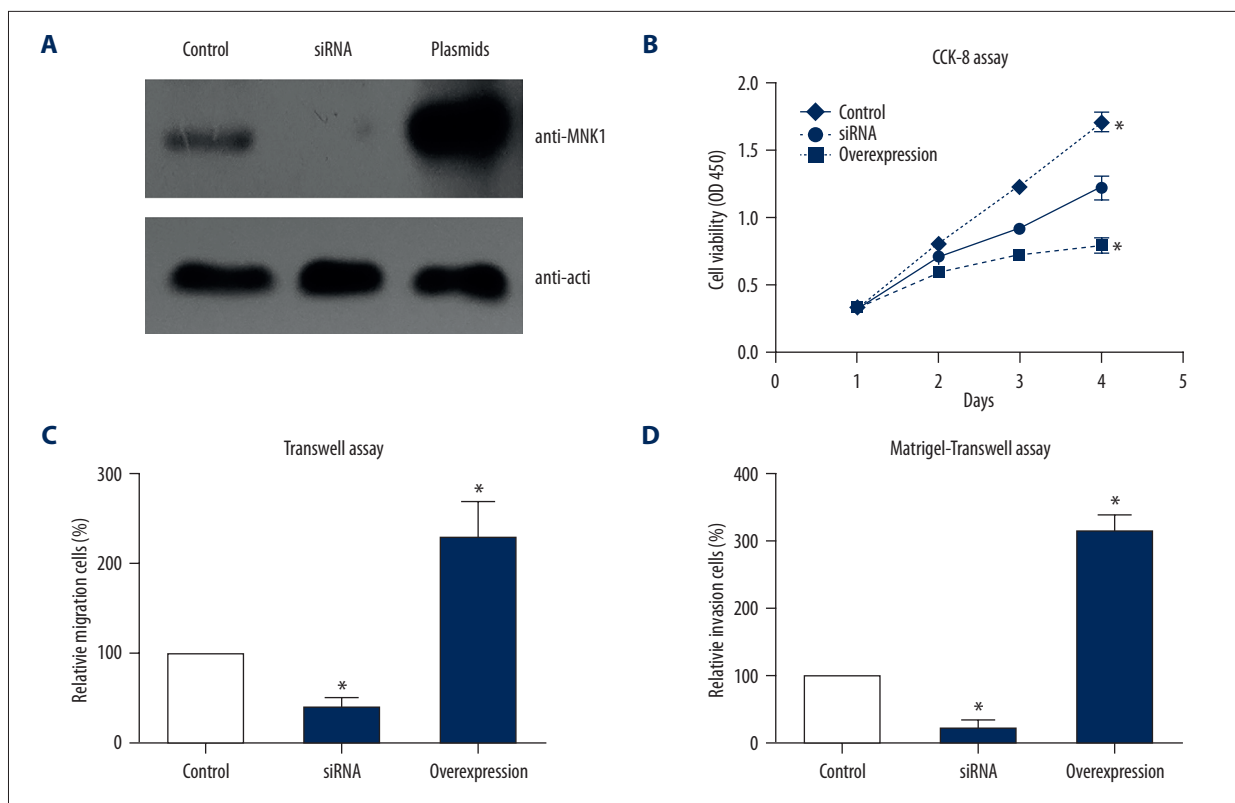


Figure 3. MNK1 promotes the proliferation and invasion of HepG2 cells. (A) Transfection efficiency of pcDNA-MNK1 plasmids and MNK1-siRNA in HepG2 cells were validated by western blot. (B) The CCK-8 assay showed that overexpression of MNK1 enhanced the proliferation of HepG2 cells. (C) Transwell assay showed that overexpression of MNK1 up-regulated the migration capacity of HepG2 cells. (D) Matrigel-Transwell assay confirmed the oncogenic role of MNK1 in promoting the invasion process of hepatocellular carcinoma cells. * Indicates $P < 0.05$ by Student's *t*-test compared with control group.

of HCC cells were inhibited in those silencing MNK1. On the other hand, MNK1 overexpression promoted HCC cell migration and invasion (both $P < 0.05$).

Discussion

MNK1 is regulated by the p38 and ERK MAP kinases. Till now, the only acknowledged substrate of MNK1 has been the eukaryotic initiation factor 4E (eIF4E), which can be phosphorylated by MNK1/2 on its Ser209 site [16]. More and more attention is now focusing on MNK1/2 due to their potential functions in modulating tumorigenesis and tumor progression. Regarding the aspect of signaling pathway, MNK1 can be activated by its upstream Ras/Raf/MAPK signals, and subsequently phosphorylate and activate eIF4E [27], which will induce an enhanced transcription of certain tumor related genes [28].

Besides the oncogenic role, inhibition of MNK1 was also reported to sensitize anti-tumor therapies. For example, MNK1-eIF4E signaling can regulate the different response to arsenic trioxide therapy towards glioma [29]. Similarly, MNK1 inhibitor

showed a synergic effect when combined with gemcitabine in impeding invasion and proliferation of pancreatic cancer cells [30]. Some other MNK1 inhibitors, such as CGP57380 and BAY 1143269, were also reported with anti-tumor potentials [16,31,32]. Although accumulating evidence revealed the tumor-promoting role and drug potential of MNK1, there have been no studies reported on its expression in HCC.

For the first time, our study showed that MNK1 was upregulated in HCC tissues compared to normal liver tissues. By analyzing its relationship with clinicopathological features, we draw the conclusion that MNK1 functions as a tumor promoting protein in HCC development. Furthermore, univariate and multivariate analyses verified that a high MNK1 protein level can serve as an independent unfavorable factor for the overall survival of HCC patients. Finally, cellular studies were conducted to explore the underlying mechanisms of MNK1 in promoting HCC progression. According to our results, MNK1-overexpression can directly enhance the proliferation, migration, and invasion capacities of HCC tumor cells, which is consistent with its tumor-promoting role in other solid tumor types. Therefore, our data also implied that MNK1 is a potential therapeutic target in HCC treatment.

Conclusions

High expression of MNK1 is frequent in HCC tissues, which promotes tumor proliferation and invasion, and is correlated with a poor overall survival.

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Conflict of Interest

None.