Clinical Cancer Research

ShRNA-Targeted MAP4K4 Inhibits Hepatocellular Carcinoma Growth

An-Wen Liu[1,2], Jing Cai[2], Xiang-Li Zhao[1], Ting-Hui Jiang[1], Tian-Feng He[1], Hua-Qun Fu[2], Ming-Hua Zhu[3], and Shu-Hui Zhang[1]

Abstract

Purpose: Mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) is overexpressed in many types of cancer. Herein, we aimed to investigate its expression pattern, clinical significance, and biological function in hepatocellular carcinoma (HCC).

Experimental Design: MAP4K4 expression was examined in 20 fresh HCCs and corresponding nontumor liver tissues. Immunohistochemistry for MAP4K4 was performed on additional 400 HCCs, of which 305 (76%) were positive for hepatitis B surface antigens. The clinical significance of MAP4K4 expression was analyzed. MAP4K4 downregulation was performed in HCC cell lines HepG2 and Hep3B with high abundance of MAP4K4, and the effects of *MAP4K4* silencing on cell proliferation *in vitro* and tumor growth *in vivo* were evaluated. Quantitative real-time PCR arrays were employed to identify the MAP4K4-regulated signaling pathways.

Results: MAP4K4 was aberrantly overexpressed in HCCs relative to adjacent nontumor liver tissues. This overexpression was significantly associated with larger tumor size, increased histologic grade, advanced tumor stage, and intrahepatic metastasis, as well as worse overall survival and higher early recurrence rate. Knockdown of the *MAP4K4* expression reduced cell proliferation, blocked cell cycle at S phase, and increased apoptosis. The antitumor effects of MAP4K4 silencing were also observed *in vivo*, manifested as retarded tumor xenograft growth. Furthermore, multiple tumor progression–related signaling pathways including JNK, NFκB, and toll-like receptors were repressed by MAP4K4 downregulation.

Conclusions: MAP4K4 overexpression is an independent predictor of poor prognosis of HCC patients, and inhibition of its expression might be of therapeutic significance. *Clin Cancer Res; 17(4); 710–20. ©2010 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide, with more than 600,000 new cases diagnosed annually (1). The most frequent risk factors for HCC include chronic viral hepatitis, alcohol abuse, and dietary exposure to aflatoxins (2–5). Early HCC is potentially curable by resection, liver transplantation, or percutaneous treatment, and 5-year survival rates of up to 60% to 70% can be achieved in well-selected patients (6). Unfortunately, most patients are diagnosed at the advanced stage in developing countries where regular tumor surveillance has not been implemented and therefore precluded from the curative treatments. For patients with advanced HCC, the multikinase inhibitor sorafenib is available as a systemic therapy, offering a significant survival advantage (7, 8). Despite significant progress, advanced HCC still remains incurable. The identification of molecular markers and pathways that contribute to the aggressive phenotype of HCC is obviously essential for development of more effective, targeted therapies.

Mitogen-activated protein kinases (MAPK) are a family of conserved serine/threonine protein kinases that function to transmit extracellular signals into the cytoplasm. The different MAPKs can be divided into 3 main subgroups, that is, the extracellular signal-regulated protein kinase (ERK), c-*Jun* N-terminal kinase/stress-activated protein kinases (*JNK/SAPK*), and *p38* (9). MAPKs are commonly activated through phosphorylation cascades, and subsequently phosphorylate a number of effectors that contribute to the regulation of diverse cellular events, including differentiation (10, 11), proliferation (12, 13), survival (14), migration (15), and invasion (16). MAPK kinase kinase kinase isoform 4 (MAP4K4), a germinal centre protein kinase that belongs to the mammalian *STE20/MAP4K* family, is

Authors' Affiliations: [1]Department of Pathology, Yueyang Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China; [2]Department of Oncology, Second Affiliated Hospital, Nanchang University, Nanchang, China; and [3]Department of Pathology, Changhai Hospital and Institute of Liver Diseases, Second Military Medical University, Shanghai, China

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

A.W. Liu and J. Cai have contributed equally to this work.

Corresponding Author: Shu-Hui Zhang, Department of Pathology, Yueyang Hospital, Shanghai University of Traditional Chinese Medicine, No. 110 of Ganhe Road, Shanghai 200437, China. Phone: 86-021-65161782; Fax: 86-021-65161782. E-mail: zhangshuhui100@sohu.com

doi: 10.1158/1078-0432.CCR-10-0331

Translational Relevance

In this study, we showed that MAP4K4 is commonly upregulated in human hepatocellular carcinoma (HCC) specimens compared with adjacent nontumor liver tissues, and this upregulation is significantly associated with tumor size, tumor grade, and intrahepatic metastasis. Survival analyses have shown that high MAP4K4 expression serves as an independent predictor of poor prognosis in HCC patients. Downregulation of *MAP4K4* using RNA interference in HCC cell lines, HepG2 and Hep3B, significantly decreased cell proliferation and increased apoptosis. Moreover, *MAP4K4* silencing inhibited xenograft tumor growth in athymic nude mice. These findings suggest that *MAP4K4* may represent a potential therapeutic target for controlling HCC growth.

implicated in the activation of the *JNK/SAPK* pathway (17, 18). Recent studies (17, 19) have documented that MAP4K4 is overexpressed in many types of human cancer. The prognostic significance of MAP4K4 overexpression has been shown in patients with stage II pancreatic ductal adenocarcinoma (19). Silencing of MAP4K4 using small interfering RNAs (siRNA) causes reduced tumor cell motility in ovarian carcinoma-derived cell line, SKOV-3 (17). These findings suggest an involvement of MAP4K4 in cancer progression. However, little is known about its expression pattern, clinical relevance, and biological function in HCC.

Here, we showed that MAP4K4 is frequently overexpressed in HCC tissues as well as several HCC cell lines including HepG2 and Hep3B, and that this upregulation is significantly associated with worse prognosis of HCC patients. Short hairpin RNA (shRNA)-mediated silencing of the *MAP4K4* in HepG2 and Hep3B cells decreased cell proliferation, blocked cell cycle progression at S phase, and increased spontaneous apoptosis. *In vivo* studies further showed that *MAP4K4* silencing dramatically retarded xenograft tumor growth in nude mice. Using a quantitative real-time array, we found that the antitumor effects of MAP4K4 downregulation were likely associated with the inactivation of multiple tumor progression–related signaling pathways, including JNK, NFκB, and toll-like receptors (TLR). Together, our data highlight an important role for MAP4K4 in controlling HCC progression.

Materials and Methods

Cell culture and assay for proliferation, colony formation, and apoptosis

Cell culture, construction of shRNA-expressing stable cell lines, and assay for proliferation, colony formation, and apoptosis were performed as described in Supplementary Methods.

Quantitative reverse transcriptase polymerase chain reaction, Western blotting, and immunohistochemistry

Analysis of gene expression by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), Western blotting, and immunohistochemistry were conducted using standard procedures detailed in Supplementary Methods.

Patient cohorts

The study protocol was approved by the Ethics Committee of Second Military Medical University (Shanghai, China), and written informed consent was obtained from each patient. Tumor samples from resection specimens were collected from 2 consecutive cohorts of patients with HCC, who underwent surgical resection for the disease at Changhai Hospital and Institute of Liver Diseases (Shanghai, China) between August 2007 and October 2008 for cohort A and between January 2001 and December 2002 for cohort B. The patients of both cohorts were selected on the basis of (a) distinctive pathologic diagnosis of HCC, (b) receiving curative resection, defined as macroscopically complete removal of the neoplasm, and (c) availability of detailed clinicopathologic data. Patients with preoperative anticancer treatment or with evidence of other malignancies were excluded from the study. Cohort A consisted of 20 patients (14 men and 6 women; median age, 51 years; range, 29–76 years), from whom fresh tumor samples coupled with adjacent nontumor liver tissues were obtained for analysis of MAP4K4 gene expression. After removal at surgery, all the fresh tissues were cut into small pieces, snap frozen in liquid nitrogen immediately, and stored at −80°C until RNA or protein extraction. Cohort B comprised 400 patients (286 men and 114 women; median age, 50 years; range, 27–78 years), whose paraffin-embedded tumor specimens were available at the surgical pathology archive of Changhai Hospital, allowing for immunohistochemical analysis.

The clinicopathologic characteristics of both the study cohorts are summarized in Supplementary Table S1. In the following text, we focus on the description of the patients of cohort B, who were selected for further elucidation of the clinical significance of MAP4K4 expression. Of the 400 studied patients, 305 (76%) and 245 (61%) patients were positive for hepatitis B surface antigens (HBsAg) and hepatitis B e antigen (HBeAg), respectively. Liver cirrhosis was found in 275 (69%) cases. Seventy-eight patients (20%) showed alcohol abuse and 12 (3%) had non-alcoholic steatohepatitis. All the subjects were negative for hepatitis C virus RNA. Patients with large (>2 cm) tumors comprised 86%, and intrahepatic metastasis was seen in 62% patients. Tumors were staged according to the 2002 tumor-node-metastasis (TNM) classification system (20). A total of 85 (21%) patients were of stage I or II, and 315 (79%) of stage III or IV. Histologic grading of tumors was made according to the 2000 World Health Organization histologic classification of tumors of the liver and intrahepatic bile ducts (well, $n = 95$; moderate, $n = 236$; poor, $n = 69$). Of the 400

patients, 78 (19%) were lost to follow-up. The median follow-up time was 36 months (range, 6–66 months). By the end of follow-up, 249 patients (62%) died. Tumor recurrence was diagnosed on the basis of serum alpha-fetoprotein (AFP) elevation, imaging techniques including computed tomography, ultrasound, and magnetic resonance imaging, as well as histology.

Tumorigenicity in nude mice

For tumorigenicity assays, 4-week-old, male BALB/c nude mice were purchased from Experimental Animal Center of Shanghai, Shanghai, China. Mice were randomly divided into 2 groups ($n = 4$ for each group) to receive s.c. injection of 2×10^6 HepG2 transfectants expressing MAP4K4-A1 or scrambled control shRNAs. Tumor volumes were measured every 5 days with a caliper and calculated according to the formula: $0.5 \times \text{length} \times \text{width}^2$. At 30 days after the cell inoculation, mice were sacrificed, and all tumors were excised and processed for immunohistochemical staining. All experimental manipulations were undertaken in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, with the approval of the Scientific Investigation Board of the Second Military Medical University, Shanghai, China.

RT^2 Profiler PCR arrays

Human TLR Signaling Pathway RT^2 Profiler PCR Array (SABiosciences) was used to assess the molecular signaling pathways affected by MAP4K4 knockdown. This technique combines together the quantitative performance of real-time PCR and the multiple gene profiling capability of microarrays. All steps were done according to the manufacturer's protocol for the ABI PRISM7900 system (Applied Biosystems). Briefly, HepG2 transfectants expressing MAP4K4-A1 or scrambled shRNAs were seeded in 6-well plates at a density of 10^4 cells per well. At 72 hours after culture, cells were harvested and total RNA was isolated using TRIzol for real-time RT-PCR analysis. The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes and then 40 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 1 minute, and elongation at 72°C for 15 seconds. Data normalization was based on correcting all Ct values for the average Ct values of several constantly expressed housekeeping genes present on the array (21). Three independent experiments were performed with similar results, and the mean fold changes of gene expression are shown in Supplementary Table S2.

Statistical analysis

All statistical analyses were carried out using the SPSS.11 software. If not otherwise stated, mean of at least 3 independent experiments ± SD are shown. Using classic criteria (significance, 0.05; power, 0.8; 2-sided test), the sample size calculation revealed that 99 patients per group were required to detect an expected 1.5-times higher risk of death for the MAP4K4-H group than the MAP4K4-L group (22). Given the possibility of imbalance between the 2 groups, some more patients were included in the present study. Significance between controls and treatment samples was calculated by Student's t test. The relationship between MAP4K4 protein expression and clinicopathologic features of HCC was analyzed using the Pearson χ^2 test. Because early tumor recurrence (i.e., recurrence within 2 years after surgery) more likely occurs due to primary tumor dissemination rather than *de novo* carcinogenesis arising from cirrhotic liver (23), in this study we used early tumor recurrence as an endpoint. Time to recurrence was calculated from the date of surgery to the date of first documented recurrence; patients without recurrence within 2 years were censored. Another primary endpoint was overall survival, which was calculated from the date of diagnosis to the date of death or the date of last follow-up (if death did not occur). Overall survival and time to recurrence were calculated using the Kaplan–Meier method. The differences between patient groups were analyzed using the log-rank test. The prognostic significance of clinicopathologic factors was determined using univariate Cox regression analysis. Multivariate survival analysis was done with a Cox proportional hazards model. Statistical significance was assumed for a 2-tailed P value less than 0.05.

Results

MAP4K4 is overexpressed in HCC

We first determined the MAP4K4 expression in 20 pairs of fresh HCC and adjacent non-tumor samples by qRT-PCR and Western blot analyses. As illustrated in Figure 1A, the expression of MAP4K4 was significantly enhanced in HCC tissues both at mRNA and protein levels relative to non-tumor tissues. To further examine the frequency and the localization of MAP4K4 protein expression, we carried out an immunohistochemical analysis on additional, larger sample sets. Notably, MAP4K4-H was detected in 194 (48.5%) of 400 HCCs. Surrounding non-neoplastic liver tissues displayed less cytoplasmic staining for MAP4K4, whereas HCC tissues showed variable proportions of MAP4K4-positive cells that gradually increased with the grades of differentiation from well differentiated to poorly differentiated (Fig. 1B). Additionally, we also analyzed the MAP4K4 expression in several human HCC cell lines (HepG2, Huh7, Hep3B, and SMMU7721). MAP4K4 was found to be expressed at higher levels in HepG2 and Hep3B than in Huh7 and SMMU7721 cells (Fig. 1C). The results indicate that MAP4K4 is aberrantly upregulated in a subset of HCC tumors.

Increased MAP4K4 expression predicts poor prognosis in HCC patients

Next, we investigated whether MAP4K4 overexpression was associated with clinical characteristics or survival of HCC patients. A significantly higher proportion of patients in the MAP4K4-H group was positive for HBsAg ($P = 0.033$) and HBeAg ($P = 0.022$) compared with that in the MAP4K4-L group. High MAP4K4 expression was significantly correlated with tumor size ($P = 0.006$), histologic grade ($P < 0.001$), intrahepatic metastasis ($P = 0.002$), and tumor

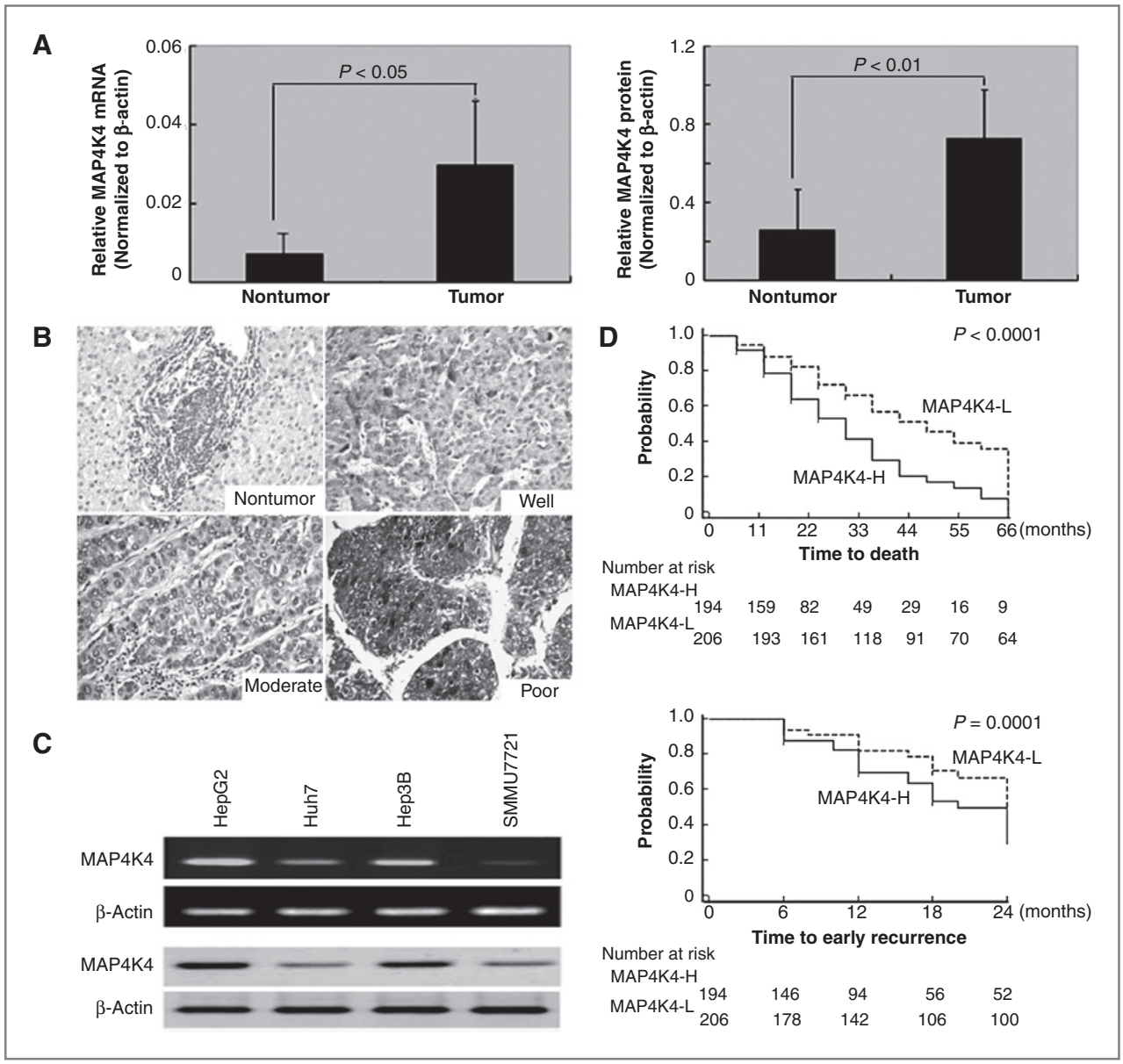

Figure 1. MAP4K4 expression and its association with survival in HCC patients. A, the relative *MAP4K4* mRNA (left) and protein (right) levels in human HCC tumors and adjacent non-tumor liver tissues were assessed by qRT-PCR and Western blot analysis, respectively. HCC tumors express significantly higher levels of MAP4K4 than non-tumor liver tissues. $n = 20$; error bars, SD. *P* values were calculated with Student's *t* test. B, representative immunostaining for MAP4K4 in non-neoplastic liver tissues, and in well, moderately, and poorly differentiated HCCs. Original magnification, ×400. C, *MAP4K4* mRNA (top) and protein (bottom) expression detected in several human HCC cell lines (HepG2, Huh7, Hep3B, and SMMU7721) by RT-PCR and Western blot analysis, respectively. D, Kaplan–Meier curves for time to death (top) and time to recurrence (bottom) by MAP4K4 expression in HCC patients. Patients whose tumors had high MAP4K4 expression (MAP4K4-H) showed significantly shorter overall survival and higher early recurrence rate than those with low MAP4K4 expression (MAP4K4-L). *P* values were obtained using a log-rank test.

stage ($P = 0.012$; Table 1). The patients in the MAP4K4-H group had a shorter median overall survival (30 versus 48 months, $P < 0.0001$) than those in the MAP4K4-L group (Fig. 1D). The early recurrence rate was significantly ($P = 0.0001$) higher in the MAP4K4-H group than in the MAP4K4-L group (Fig. 1D). A total of 12 clinicopathologic variables were included in the univariate analysis to study their correlations with patients' survival, including sex, age, serum AFP level, serum HBsAg, serum HBeAg, tumor size, histologic grade, liver cirrhosis, capsule, intrahepatic metastasis, tumor stage, and MAP4K4 expression. As shown in Table 2, serum HBsAg ($P = 0.036$ and 0.008), serum HBeAg (0.025 and 0.007), intrahepatic metastasis ($P = 0.043$ and 0.032), tumor stage ($P = 0.026$ and 0.013), and MAP4K4

Table 1. Clinicopathologic factors and MAP4K4 expression in hepatocellular carcinomas ($n = 400$) on the basis of immunohistochemistry

Variables	*n*	MAP4K4-L ($n = 206$)	MAP4K4-H ($n = 194$)	*P*
Sex				
Male	286	141	145	0.163
Female	114	65	49	
Age				
<50	184	86	98	0.079
≥50	216	120	96	
Serum AFP level, μg/L				
<20	137	71	66	0.952
≥20	263	135	128	
Serum HBsAg				
Positive	305	148	157	**0.033**
Negative	95	58	37	
Serum HBeAg				
Positive	245	115	130	**0.022**
Negative	155	91	64	
Tumor size				
≤2cm	57	39	18	**0.006**
>2cm	343	167	176	
Histologic grade				
Well differentiated	95	77	18	**<0.001**
Moderately differentiated	236	120	116	
Poorly differentiated	69	9	60	
Liver cirrhosis				
Absent	125	60	65	0.345
Present	275	146	129	
Tumor capsule				
Intact	79	41	38	0.957
Absent or not intact	321	165	155	
Intrahepatic metastasis				
Absent	126	74	42	**0.002**
Present	274	132	152	
TNM stage				
I + II	85	54	31	**0.012**
III + IV	315	152	163	

NOTE: Significant *P* values are marked in bold.

expression ($P = 0.019$ and 0.018) were significantly associated with the overall survival and early recurrence, respectively. The multivariate survival analysis revealed that MAP4K4 expression was a predictor of overall and early recurrence ($P = 0.019$ and 0.018, respectively) independent of serum HBsAg and HBeAg status, intrahepatic metastasis, and tumor stage (Table 3).

Targeted reduction of MAP4K4 results in decreased growth and enhanced apoptosis of HepG2 cells *in vitro*

To explore the biological significance of MAP4K4 in HCC, we specifically knocked down its expression in HepG2 cells using RNA interference (RNAi). This HCC cell line was chosen because of its high abundance of MAP4K4. The transfection of HepG2 cells with MAP4K4-A1 shRNA caused an approximately 75% downregulation of *MAP4K4* transcript levels compared with control shRNA-transfected cells (Supplementary Fig. S1A). Moreover, the amounts of MAP4K4 protein were considerably reduced in cells expressing MAP4K4-A1, showing efficient knockdown (Supplementary Fig. S1B). Similar results were observed in MAP4K4-A3–producing cells, although the extent of MAP4K4 downregulation was smaller. However, MAP4K4 expression levels were little affected by the presence of MAP4K4-A2 shRNA.

We next studied the impact of *MAP4K4* silencing on HepG2 cell proliferation *in vitro*. The results of the MTT assay showed that downregulation of MAP4K4 by

Table 2. Univariate analysis of overall survival and early recurrence in patients with hepatocellular carcinoma ($n = 400$)

	Overall survival		Early recurrence	
	HR (95% CI)	***P***	**HR (95% CI)**	***P***
Sex				
Male	1.00		1.00	
Female	1.23 (0.72–2.61)	0.453	1.42 (0.83–2.57)	0.327
Age				
<50	1.00		1.00	
≥50	1.30 (0.40–3.90)	0.67	1.12 (0.45–2.37)	0.625
Serum AFP level, μg/L				
<20	1.00		1.00	
≥20	1.12 (0.47–2.59)	0.944	1.46 (0.78–2.54)	0.379
Serum HBsAg				
Negative	1.00		1.00	
Positive	1.62 (1.03–2.71)	**0.036**	1.94 (1.23–4.57)	**0.008**
Serum HBeAg				
Negative	1.00		1.00	
Positive	2.19 (1.35–7.07)	**0.025**	2.64 (1.61–6.53)	**0.007**
Tumor size, cm				
≤2	1.00		1.00	
>2	0.60 (0.30–1.30)	0.224	0.73 (0.39–1.22)	0.127
Histologic grade				
Well	1.00		1.00	
Moderate	1.35 (0.53–9.17)	0.896	1.32 (0.64–7.35)	0.538
Poor	1.32 (0.74–7.59)	0.514	1.36 (1.87–15.9)	0.571
Liver cirrhosis				
Absent	1.00		1.00	
Present	0.95 (0.46–1.95)	0.890	1.28 (0.58–3.02)	0.753
Tumor capsule				
Intact	1.00		1.00	
Absent or not intact	1.40 (0.70–2.80)	0.304	1.57 (0.74–2.52)	0.429
Intrahepatic metastasis				
Not observed	1.00		1.00	
Observed	2.40 (1.00–5.90)	**0.043**	1.86 (1.21–3.74)	**0.032**
TNM stage				
I + II	1.00		1.00	
III + IV	2.63 (1.24–6.90)	**0.026**	3.54 (1.94–6.32)	**0.013**
MAP4K4 expression				
Low (≤10%)	1.00		1.00	
High (>10%)	2.98 (1.05–7.71)	**0.019**	4.12 (1.72–8.30)	**0.018**

NOTE: Significant *P* values are marked in bold.

MAP4K4-A1 and -A3 significantly reduced the proliferation rate of HepG2 cells compared with cells expressing MAP4K4-A2 or control shRNA ($P < 0.01$; Fig. 2A). Colony formation assay further showed that MAP4K4-A1–mediated and MAP4K4-A3–mediated knockdown of MAP4K4 resulted in a substantial decrease in the HepG2 cell growth in comparison with scrambled shRNA-expressing cells ($P < 0.05$; Fig. 2B). However, MAP4K4-A2 did not significantly affect the number of colonies. Cell cycle analysis revealed that MAP4K4 silencing caused a considerable inhibition of cell cycle progression, leading to a selective accumulation of cells in the S phase compared with control shRNA transfectants (Fig. 2C; Supplementary Table S3). Accompanying with S phase arrest, a characteristic sub-G_1 peak, indicative of DNA fragmentation and a hallmark of apoptosis, was seen in cells with downregulated MAP4K4. Quantification of apoptosis by Annexin V/PI double labeling indicated that a remarkably higher apoptotic index was detected in MAP4K4-A1 and -A3 transfectants relative to control cells (Fig. 2D;

Table 3. Multivariate analysis of overall survival and early recurrence in patients with hepatocellular carcinoma ($n = 400$)

	Overall survival		Early recurrence	
	HR (95% CI)	***P***	**HR (95% CI)**	***P***
Serum HBsAg				
Negative	1.00		1.00	
Positive	1.59 (1.09–6.64)	**0.043**	2.69 (1.34–8.01)	0.033
Serum HBeAg				
Negative	1.00		1.00	
Positive	2.35 (1.40–5.95)	**0.029**	2.57 (1.64–6.33)	0.017
Intrahepatic metastasis				
Not observed	1.00		1.00	
Observed	1.94 (1.23–4.41)	**0.037**	2.39 (1.35–6.01)	0.023
TNM stage				
I + II	1.00		1.00	
III + IV	2.37 (1.31–7.52)	**0.026**	2.33 (1.94–6.04)	0.025
MAP4K4 expression				
Low (≤10%)	1.00		1.00	
High (>10%)	2.41 (1.38–5.14)	**0.014**	2.54 (1.56–6.66)	**0.006**

NOTE: Significant *P* values are marked in bold.

Supplementary Table S3). By contrast, the delivery of MAP4K4-A2 had no significant influence on cell apoptosis. The knockdown of MAP4K4 expression was also conducted in Hep3B cells, another HCC cell line with high endogenous levels of MAP4K4. Similar results were achieved, that is, decreasing cell proliferation and increasing cell apoptosis by MAP4K4 downregulation (Supplementary Fig. S2). These findings collectively highlight an important role for MAP4K4 in regulation of HCC cell growth and survival.

Downregulation of MAP4K4 retards tumor growth *in vivo*

To get more insight into the relevance of MAP4K4 silencing *in vivo*, an equal number of HepG2 transfectants producing MAP4K4-A1 or control shRNA were injected s.c. into the left flank of nude mice. Consistent with the *in vitro* studies, *MAP4K4* downregulation by MAP4K4-A1 significantly slowed the xenograft tumor growth compared with the control shRNA ($P < 0.01$; Fig. 3A). Expression of MAP4K4 in the xenografts was determined by immunohistochemistry. Strong cytoplasmic MAP4K4 staining of cells in the control xenografts was observed (Fig. 3B). In contrast, the MAP4K4-A1 transfectant-derived tumor xenografts displayed a considerably lower proportion of MAP4K4-positive cells.

MAP4K4 regulates multiple signaling pathways associated with tumor progression

Having identified *MAP4K4* as a potential oncogene in HCC, we next sought to investigate its downstream signaling pathways using the human *TLR* signaling pathway PCR array. This array was chosen because it can profile the expression of multiple genes related to the *TLR*, *NFκB*, and *JNK/p38* signaling pathways, which contribute to HCC progression (24–27). The most differentially expressed genes whose expression changed 2-fold or more in MAP4K4-A1–producing HepG2 cells compared with control shRNA-expressing cells are summarized in Supplementary Table S4. Among the 54 genes, 53 (98.1%) were downregulated and only 1 (1.9%) was upregulated. The downregulated genes included *MAP3K1*, *MAP2K4*, *MAPK8 (JNK)*, *FOS*, *ELK1*, *NFκB1*, *NFκB2*, *TLRs*, *MyD88*, and *TRAF6*, suggesting an important role for *MAP4K4* in the regulation of the *JNK*, *NFκB*, and *TLR* signaling pathways. To confirm the qRT-PCR array data, we performed Western blot analysis in HepG2 cells harboring MAP4K4-A1 or control shRNA. Although the total protein amounts of JNK and NFκB were little affected by MAP4K4-A1, phosphorylation of these 2 proteins was significantly disrupted in MAP4K4-A1 transfectants relative to the control cells (Fig. 3C). In contrast, the phosphorylation levels of ERK1/2 or p38 MAPK were comparable between the MAP4K4-A1–expressing and control shRNA-expressing cells, suggesting that MAP4K4 selectively regulates the JNK rather than ERK1/2 or p38 MAPK pathway. Additionally, we found that the expression of TLR5, TLR4, MyD88, and TRAF6 was also downregulated in MAP4K4-A1–transfected cells (Fig. 3C). These data suggest that in HCC, multiple tumor progression–related signaling pathways including JNK, NFκB, and TLRs were coordinated by MAP4K4.

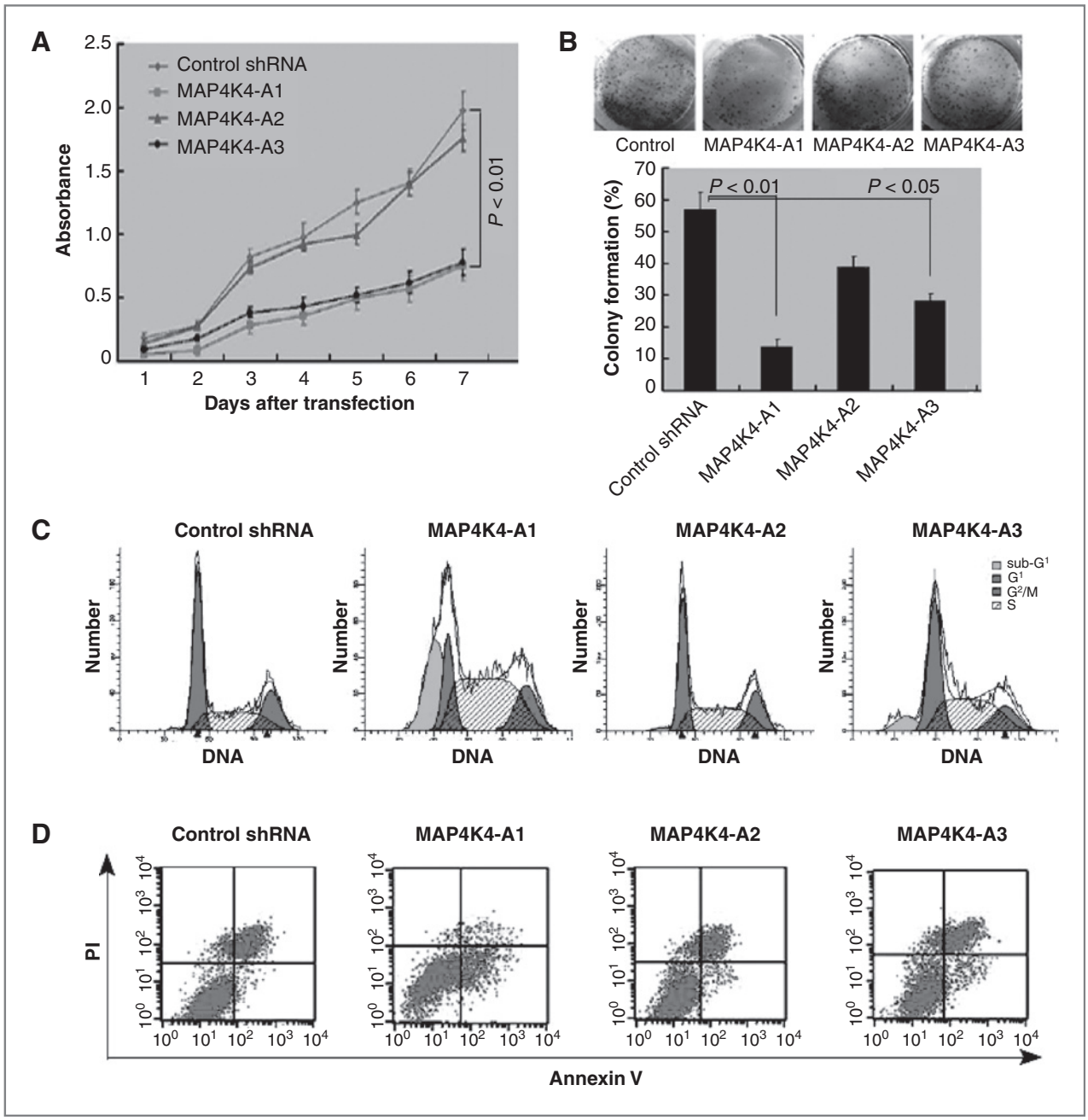

Figure 2. ShRNA-mediated silencing of MAP4K4 in HepG2 cells. A, assessment of cell proliferation by the MTT assay. HepG2 cells expressing MAP4K4-A1 or MAP4K4-A3 shRNAs showed a significantly reduced proliferation rate compared with those harboring MAP4K4-A2 or scrambled control shRNAs ($P < 0.01$). $n = 6$; error bars, SD. B, colony formation assay. Top, representative dishes of HepG2 cells expressing indicated shRNAs. Bottom, quantitative analyses of colony numbers. P values were calculated with Student's t test. C, analysis of cell cycle distribution of HepG2 cells expressing indicated shRNAs. Representative flow cytometry histograms of PI-stained cells are shown. D, representative flow cytometry dot plots of HepG2 cells expressing indicated shRNAs. Early apoptotic cells (Annexin V-FITC positive, PI negative) were increased in MAP4K4-A1–producing and MAP4K4-A3–producing cells compared with MAP4K4-A2 or control transfectants.

Discussion

Recent studies have shown that MAP4K4 levels are elevated in many solid neoplasms, including colon cancer, lung cancer, ovarian cancer, kidney cancer, and brain cancer (17, 28). MAP4K4 overexpression has been reported to be associated with worse prognosis and to serve as a prognostic marker for stage II pancreatic ductal adenocarcinomas (19). These findings imply an oncogenic role for MAP4K4; however, little is known about its expression

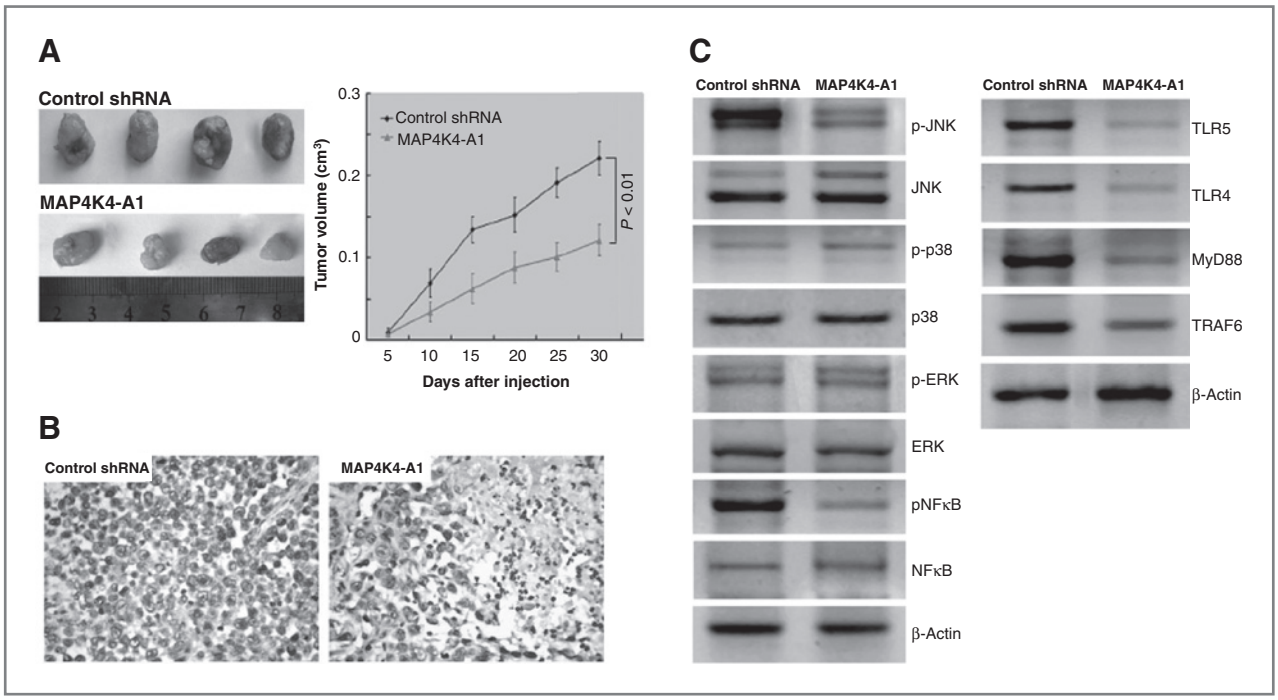

Figure 3. Downregulation of MAP4K4 inhibits the growth of HepG2-derived xenografts in athymic nude mice. HepG2 transfectants (2×10^6) expressing MAP4K4-A1 or scrambled control shRNAs were inoculated s.c. into immunodeficient mice. Tumor volumes were measured every 5 days. At 30 days after the cell inoculation, mice were sacrificed and tumors were collected. A, left, macrographic images of the HepG2 tumor xenografts from each mouse ($n = 4$ per group). Right, growth curves of the tumor xenografts. error bars, SD. *P* values were calculated with Student's *t* test. B, representative sections of the MAP4K4-A1 or control shRNA expressing HepG2 tumor xenografts stained by anti-MAP4K4 antibody. Original magnification, ×400. C, MAP4K4 knockdown influences the expression and activation of multiple genes. MAP4K4-A1 or control shRNA expressing HepG2 transfectants were subjected to Western blot analysis of MAP4K4. Representative images of 3 independent experiments with similar results are shown. Left, silencing of MAP4K4 decreases the phosphorylation of JNK and NFκB protein, but not ERK or p38 protein. Right, the protein amounts of the TLR5, TLR4, MyD88, and TRAF6 are reduced in MAP4K4-A1–expressing cells relative to control cells.

pattern and biological significance in HCC, one of the most common malignancies worldwide. In this study, we showed that MAP4K4 expression determined by qRT-PCR and Western blotting is upregulated in HCCs relative to adjacent nontumor liver tissues. Immunohistochemical analysis performed in a large cohort of archival HCC specimens revealed that high MAP4K4 expression is significantly correlated with tumor size, histologic grade, and intrahepatic metastasis. Most interestingly, MAP4K4 overexpression predicts poor prognosis of HCC patients. To extend our clinical studies and investigate its biological function, we generated stable MAP4K4-downregulated cell lines in HepG2 cells by delivery of its specific shRNA. Knockdown of endogenous MAP4K4 attenuated *in vitro* proliferation of HepG2 cells and reduced the growth of HepG2-derived xenografts in mice. These data strongly suggest that MAP4K4 is a key player in HCC progression.

The anticancer effects of MAP4K4 downregulation are associated with inhibition of cell cycle progression and induction of apoptosis. *MAP4K4* silencing caused a cell cycle arrest at the S phase coupled with increased apoptosis in HepG2 cells. This phenomenon coincides with inactivation of several tumorigenesis-related signaling pathways, including *JNK*, *NFκB*, and *TLRs*. Despite lack of direct evidence, the antitumor effects of *MAP4K4* depletion is likely mediated through the *JNK* pathway. This hypothesis is supported by several indirect observations. First, similar to MAP4K4, JNK expression is elevated in human HCCs (25, 29). Second, sustained JNK activity contributes to hepatocarcinogenesis. Inhibition of JNK by pharmacologic inhibitors or siRNAs reduces HCC cell growth and sensitizes cells to apoptosis *in vitro* and *in vivo* (24, 25, 29, 30). Third, our data and previous studies (17, 18) show that *MAP4K4* regulates the JNK activation. In contrast to JNK, the other 2 MAPK pathways, ERK and p38, are not affected by MAP4K4 inhibition, implying a unique mediator role for JNK in MAP4K4 signaling transduction.

Besides *JNK*, another 2 *MAP4K4*-repressed pathways, *TLR* and *NFκB*, are also involved in tumorigenesis (26, 27). *NFκB* has been shown to link inflammation to cancer (31–33). In different contexts, *NFκB* displays opposing roles in carcinogenesis. Inhibition of NFκB activity by chemical reagents has yielded anti-inflammatory and anticancer effects (34). However, aberrant inactivation of NFκB through deletion of its essential modulator (NEMO, NF-κB essential modulator) triggers chronic inflammation and spontaneous HCC development (31). Likewise, TLRs, key players in innate immunity to pathogens and

ubiquitously expressed on cancer cells, also play a complex role in regulating inflammation and tumorigenesis (35, 36). Given the regulatory role in the activation of the TLR and NFκB pathways, we assume that MAP4K4 might represent a promising therapeutic target for chronic liver inflammation and associated cancer progression. Indeed, the anti-inflammatory activities of *MAP4K4* depletion have been documented by a recent study, in which orally delivered siRNA targeting macrophage *MAP4K4* suppresses systemic inflammation (37).

In conclusion, we identified a frequent overexpression of MAP4K4 in HCCs. This overexpression is an independent predictor of adverse prognosis, and contributes to tumor growth through coordinating several signaling pathways involved in cancer progression, thereby providing a potential therapeutic target for HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported in part by grants from National Nature Science Foundation of China (No.81072020), the Shanghai Natural Science Foundation of China (No. 08ZR1419600) and Key Research Foundation of Shanghai Municipal Education Commission of China (No. 08ZZ123) to S.H. Zhang, and from the Jiangxi Natural Science Foundation of China (No. 2009GZY0161) to A.W. Liu.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 6, 2010; revised July 4, 2010; accepted August 25, 2010; published OnlineFirst December 30, 2010.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.
2. Block TM, Mehta AS, Fimmel CJ, Jordan R. Molecular viral oncology of hepatocellular carcinoma. Oncogene 2003;22:5093–107.
3. Evans AA, Chen G, Ross EA, Shen FM, Lin WY, London WT. Eight-year follow-up of the 90,000-person Haimen City cohort: I. Hepatocellular carcinoma mortality, risk factors, and gender differences. Cancer Epidemiol Biomarkers Prev 2002;11:369–76.
4. Schütte K, Bornschein J, Malfertheiner P. Hepatocellular carcinoma–epidemiological trends and risk factors. Dig Dis 2009;27:80–92.
5. Wogan GN. Aflatoxins as risk factors for hepatocellular carcinoma in humans. Cancer Res 1992;52:2114s–8s.
6. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907–17.
7. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008;359:378–90.
8. Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 2009;10:25–34.
9. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001;22:153–83.
10. Eriksson M, Leppä S. Mitogen-activated protein kinases and activator protein 1 are required for proliferation and cardiomyocyte differentiation of P19 embryonal carcinoma cells. J Biol Chem 2002;277:15992–6001.
11. Aouadi M, Bost F, Caron L, Laurent K, Le Marchand Brustel Y, Binétruy B. p38 mitogen-activated protein kinase activity commits embryonic stem cells to either neurogenesis or cardiomyogenesis. Stem Cells 2006;24:1399–406.
12. Roux PP, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 2004;68:320–44.
13. Barr RK, Bogoyevitch MA. The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs). Int J Biochem Cell Biol 2001;33:1047–63.
14. Lee TJ, Sartor O, Luftig RB, Koochekpour S. Saposin C promotes survival and prevents apoptosis via PI3K/Akt-dependent pathway in prostate cancer cells. Mol Cancer 2004;3:31.
15. Su CM, Lu DY, Hsu CJ, Chen HT, Huang CY, Yang WH, et al. Glial cell-derived neurotrophic factor increases migration of human chondrosarcoma cells via ERK and NF-kappaB pathways. J Cell Physiol 2009;220:499–507.
16. Wendt MK, Schiemann WP. Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-beta signaling and metastasis. Breast Cancer Res 2009;11:R68.
17. Collins CS, Hong J, Sapinoso L, Zhou Y, Liu Z, Micklash K, et al. A small interfering RNA screen for modulators of tumor cell motility identifies MAP4K4 as a promigratory kinase. Proc Natl Acad Sci USA 2006;103:3775–80.
18. Machida N, Umikawa M, Takei K, Sakima N, Myagmar BE, Taira K, et al. Mitogen-activated protein kinase kinase kinase kinase 4 as a putative effector of Rap2 to activate the c-Jun N-terminal kinase. J Biol Chem 2004;279:15711–14.
19. Liang JJ, Wang H, Rashid A, Tan TH, Hwang RF, Hamilton SR, et al. Expression of MAP4K4 is associated with worse prognosis in patients with stage II pancreatic ductal adenocarcinoma. Clin Cancer Res 2008;14:7043–9.
20. Sobin LH, Wittekind C, editors. TNM classification of malignant tumors (UICC International Union Against Cancer). 6th ed. New York: Wiley-Liss; 2002p. 81–3.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–8.
22. Schmoor C, Sauerbrei W, Schumacher M. Sample size considerations for the evaluation of prognostic factors in survival analysis. Stat Med 2000;19:441–52.
23. Bruix J, Sherman M. Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. Hepatology 2005;42:1208–36.
24. Mucha SR, Rizzani A, Gerbes AL, Camaj P, Thasler WE, Bruns CJ, et al. JNK inhibition sensitises hepatocellular carcinoma cells but not normal hepatocytes to the TNF-related apoptosis-inducing ligand. Gut 2009;58:688–98.
25. Hui L, Zatloukal K, Scheuch H, Stepniak E, Wagner EF. Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. J Clin Invest 2008;118:3943–53.
26. Arsura M, Cavin LG. Nuclear factor-kappaB and liver carcinogenesis. Cancer Lett 2005;229:157–69.
27. Mencin A, Kluwe J, Schwabe RF. Toll-like receptors as targets in chronic liver diseases. Gut 2009;58:704–20.
28. Wright JH, Wang X, Manning G, LaMere BJ, Le P, Zhu S, et al. The STE20 kinase HGK is broadly expressed in human tumor cells and can modulate cellular transformation, invasion, and adhesion. Mol Cell Biol 2003;23:2068–82.
29. Chang Q, Zhang Y, Beezhold KJ, Bhatia D, Zhao H, Chen J, et al. Sustained JNK1 activation is associated with altered histone H3 methylations in human liver cancer. J Hepatol 2009;50:323–33.

30. Aderca I, Moser CD, Veerasamy M, Bani-Hani AH, Bonilla-Guerrero R, Ahmed K, et al. The JNK inhibitor SP600129 enhances apoptosis of HCC cells induced by the tumor suppressor WWOX. J Hepatol 2008;49:373–83.
31. Beraza N, Malato Y, Sander LE, Al-Masaoudi M, Freimuth J, Riethmacher D, et al. Hepatocyte-specific NEMO deletion promotes NK/NKT cell- and TRAIL-dependent liver damage. J Exp Med 2009;206:1727–37.
32. Inokuchi S, Aoyama T, Miura K, Osterreicher CH, Kodama Y, Miyai K, et al. Disruption of TAK1 in hepatocytes causes hepatic injury, inflammation, fibrosis, and carcinogenesis. Proc Natl Acad Sci U S A 2010;107:844–9.
33. Melisi D, Niu J, Chang Z, Xia Q, Peng B, Ishiyama S, et al. Secreted interleukin-1alpha induces a metastatic phenotype in pancreatic cancer by sustaining a constitutive activation of nuclear factor-kappaB. Mol Cancer Res 2009;7:624–33.
34. Muriel P. NF-kappaB in liver diseases: a target for drug therapy. J Appl Toxicol 2009;29:91–100.
35. Wolska A, Lech-Marańda E, Robak T. Toll-like receptors and their role in carcinogenesis and anti-tumor treatment. Cell Mol Biol Lett 2009;14:248–72.
36. Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. Nat Rev Cancer 2009;9:57–63.
37. Aouadi M, Tesz GJ, Nicoloro SM, Wang M, Chouinard M, Soto E, et al. Orally delivered siRNA targeting macrophage Map4k4 suppresses systemic inflammation. Nature 2009;458:1180–4.