Molecular Medicine

Characterization of Bridging Integrator 1 (*BIN1*) as a Potential Tumor Suppressor and Prognostic Marker in Hepatocellular Carcinoma

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It has been shown that bridging integrator 1 (*BIN1*) can interact with c-myelocytomatosis (*c-Myc*) oncoprotein in cancer. However, the role of *BIN1* in hepatocellular carcinoma (HCC) is not clear. In the present study, we investigated the expression and prognostic role of *BIN1* in primary HCC and evaluated the function of *BIN1* in hepatocarcinogenesis. Using real-time polymerase chain reaction and Western blot analysis, we found significantly decreased expression of *BIN1* in primary HCC tumor tissues (n = 42) compared with adjacent normal tissues and in HCC cell lines. Immunohistochemistry analysis also found decreased *BIN1* expression in HCC tumor tissues (n = 117). In clinicopathological analysis, loss of *BIN1* expression correlated significantly ($P < 0.05$) with differentiation scores and tumor size. Importantly, decreased expression of *BIN1* in tumors was found to be closely associated with a poor prognosis, and we conclude that *BIN1* was an independent prognostic factor in a multivariate analysis. In mechanistic studies, restoring *BIN1* expression in *BIN1*-null HCC cells significantly inhibited cell proliferation and colony formation and induced apoptosis of HCC cells. Furthermore, we found that *BIN1* overexpression could significantly suppress the motility and invasion of HCC cells *in vitro*. Our results indicate that *BIN1* may function as a potential tumor suppressor and serve as a novel prognostic marker in HCC patients. The *BIN1* molecule might play an important role in tumor growth, cell motility and invasion. Modulation of *BIN1* expression may lead to clinical applications of this critical molecule in the control of hepatocellular carcinoma as well as in early and effective diagnosis of this aggressive tumor.

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INTRODUCTION

Bridging integrator 1 (*BIN1*) maps to the long arm of human chromosome 2 (2q14) and encodes multiple tissue-specific isoforms of the Myc-interacting adaptor protein (1–4). *BIN1* expression is often found attenuated or even abolished in approximately 50% of the carcinoma cell lines as well as in several primary tumors, such as malignant melanoma, breast and prostate cancers, while its ectopic expression can inhibit cell proliferation and/or promote apoptosis (1,5–14). Notably, the effects of *BIN1* loss on cell growth and survival appear to be contingent on cell transformation (15–18). These studies suggest that *BIN1* has tumor suppressor features that are linked to cell death and differentiation decisions, and it may therefore be involved in neoplastic pathophysiology.

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and is a major cause of cancer-related death in many countries, especially in southern China, southeastern Asia and sub-Saharan Africa. The progression of HCC is a slow process that evolves through distinct stages associated with cumulative genomic alterations. Recent studies have shown that aberrant gene expression, including

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oncogene overexpression and tumor suppressor down-expression, is responsible for the development of HCC. However, the molecular pathogenesis of HCC still remains unclarified. In a previous study, a lack of *BIN1* expression was reported in a human HCC cell line, and overexpression of *BIN1* was shown to suppress tumor cell growth (1). These findings suggested that *BIN1* may play a critical role as a tumor suppressor in HCC. Nevertheless, the clinicopathological and prognostic significance of *BIN1* expression remains undefined in primary HCC. Furthermore, the functional role of *BIN1* in the pathogenesis and tumorigenicity of HCC is also unexplored.

In this study, we examined *BIN1* expression in primary HCCs and evaluated the relationship between *BIN1* expression and clinicopathological parameters of HCCs. Meanwhile, we investigated the prognostic value of *BIN1* for HCC patients. Furthermore, we evaluated the functional role of *BIN1* in the tumorigenesis of HCC by examining the *in vitro* proliferation, clone formation, cell cycle, apoptosis, motility and invasion of the *BIN1* transfected HCC cells.

MATERIALS AND METHODS

Cell Culture

Human HCC cell lines, HepG2 (well differentiated, low metastatic potential), Hep3B (well differentiated, low metastatic potential) and SK-Hep1 (poorly differentiated, high metastatic potential) were obtained from American Type Culture Collection (ATCC). Huh7 (well differentiated, low metastatic potential) was obtained from the RIKEN cell bank (Ibaraki, Japan). BEL-7402 (moderately differentiated, low metastatic potential) cells were obtained from the Committee of Type Culture Collection of the Chinese Academy of Sciences (CTCCCAS, Shanghai, China). Normal liver cell line (LO2) was also obtained from CTCCCAS. All cells were cultured in 5% CO_2 at 37°C in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS). The mRNA and protein were extracted from all cell lines in the same culture passage (subculture to three passages) for *BIN1* expression analysis.

Tissue Samples

Tissue samples, including HCC tumor tissues and adjacent noncancerous tissues (n = 42), were obtained from patients who had primary HCC surgical resection in the Sun Yat-sen University Cancer Center between 2007 and 2009. These patients did not receive any preoperative treatment, such as chemotherapy and radiotherapy. After surgical resection, the fresh tissues were frozen at –80°C and used for RNA extraction and real-time quantitative polymerase chain reaction (PCR) detection. Additional paraffin-embedded HCC samples (n = 117) were selected randomly from the patients who had primary HCC surgical resection in the Sun Yat-sen University Cancer Center between 1999 and 2001. These patients did not receive any pretreatment either. The serial 4-μm sections were cut and used for immunohistochemical detection. Cell histological types of tumor tissues were evaluated according to World Health Organization classification criteria. Postoperative follow-up visits were conducted by our outpatient department. Follow-up included clinical and laboratory examinations every 3 months for the first 2 years, every 6 months during the third to fifth years, and then annually for an additional 5 years or until patient death, whichever occurred first. Overall survival, which was defined as the time from the operation to the patient's death or the last follow-up, was used as a measure of prognosis. Before the study, written informed consent was obtained from each patient.

RNA Preparation and Protein Extraction

Total RNA from HCC tissues or cell lines was extracted using Trizol solution (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Total protein from HCC tissues or cell lines was extracted using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) according to the manufacturer's instructions. The RNA and protein samples were stored at –80°C until use.

Real-Time Quantitative Reverse Transcription–PCR

Total RNA (2 μg) was reverse transcribed, and first-strand cDNA was synthesized using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Beijing, China) according to the manufacturer's instructions. The resulting cDNA was used for real-time quantitative reverse transcription (RT)-PCR detection of *BIN1*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin was used as an internal control. For *BIN1*, the 5′ primer was 5′-ACAAC GACCT GCTGT GGATG G-3′, and the 3′ primer was 5′-CGTGA CTTGA TGTCG GGGAA CT-3′. For GAPDH, the 5′ primer was 5′-CTCCT CCTGT TCGAC AGTCA GC-3′, and the 3′ primer was 5′-CCCAA TACGA CCAAA TCCGT T-3′. For β-actin, the 5′ primer was 5′-TGACC CAGAT CATGT TTGAG-3′, and the 3′ primer was 5′-CGTAC AGGGA TAGCA CAG-3′. RT-PCR was performed as follows: 95°C for 10 min, one cycle, followed by 95°C for 30 s and 60°C for 60 s (45 cycles). After RT-PCR was finished, dissociation curves were analyzed, and the relative amount of product was calculated from the threshold cycles with the instrument's software (SDS 2.0). The experiments were performed for four times, and the statistical analysis was performed.

Western Blot

Western blot was performed to detect the protein levels of *BIN1* in paired clinical specimens from HCC patients and cell lines. Total protein (50 μg) was resolved using 12% sodium dodecyl–sulfate polyacrylamide gel (SDS-PAGE). After electrophoresis, the separated proteins were transferred to PVDF membranes. The membranes were blocked with 5% nonfat milk and incubated with primary polyclonal antibody against *BIN1* (Santa Cruz Biotechnology, Santa

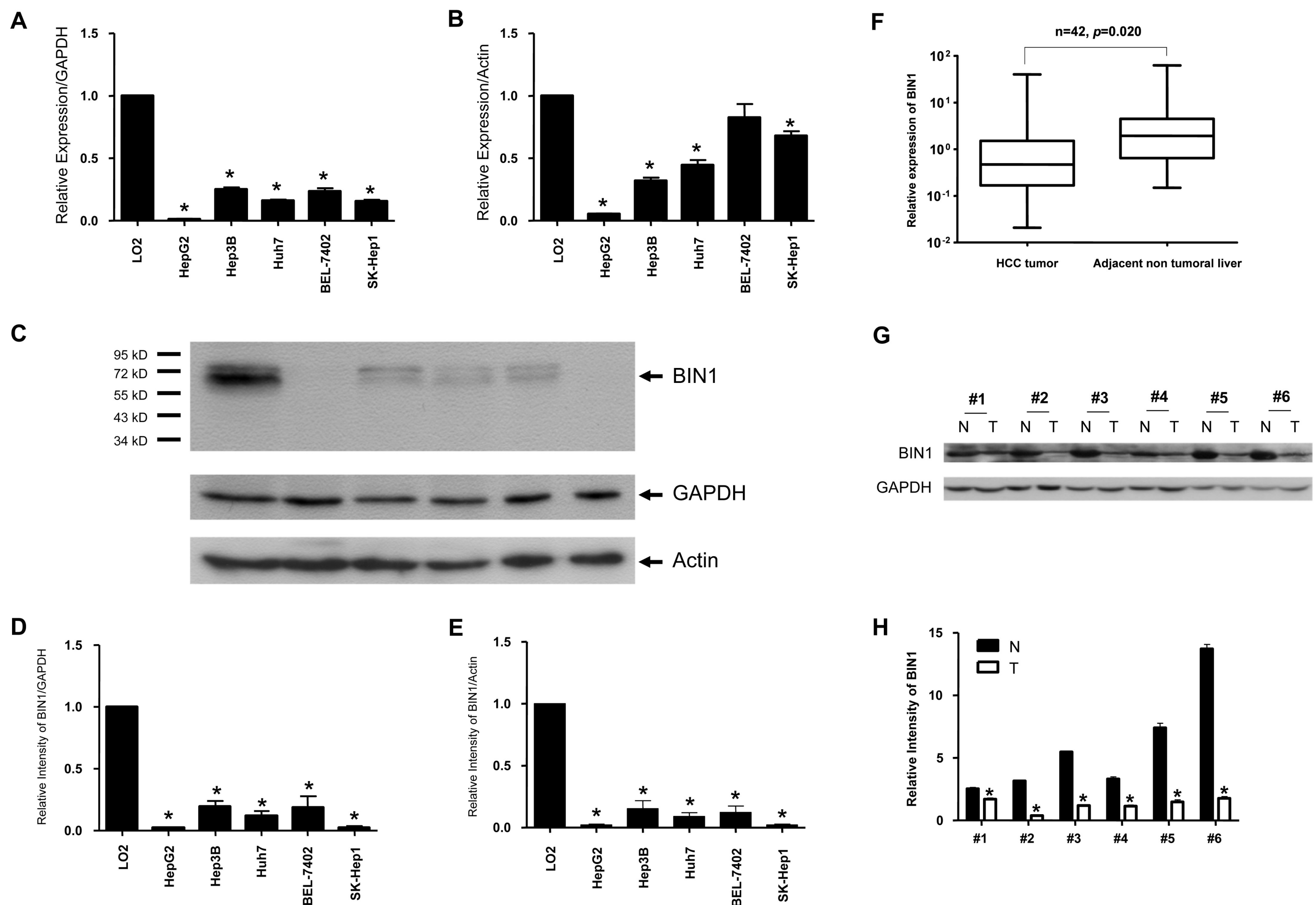

Figure 1. *BIN1* mRNA and protein expression were remarkably reduced in human HCC cell lines and in primary HCC surgical specimens assessed with real-time quantitative PCR and Western blot. (A, B) *BIN1* mRNA expression in human HCC cell lines. The normal liver cell line LO2 was used as a positive control. The *BIN1* mRNA expression level was normalized to GAPDH and LO2 or to β-actin and LO2. Experiments were performed four times (*$P < 0.05$ compared with LO2). (C) *BIN1* protein expression in human HCC cell lines. (D, E) Density of *BIN1* bands normalized to GAPDH and LO2 or to β-actin and LO2. Experiments were performed in three times (*$P < 0.05$ compared with LO2). (F) *BIN1* mRNA expression in human primary HCC tumor tissues and adjacent nontumor liver tissues (n = 42). The *BIN1* mRNA expression level was normalized to GAPDH. Experiments were performed four times. The difference in the expression levels was analyzed with the paired *t* test ($P = 0.02$). (G) *BIN1* protein expression in six representative human primary HCC tumor tissues (T) and adjacent nontumor liver tissues (N). (H) Density of *BIN1* bands normalized to GAPDH. Experiments were performed three times. *$P < 0.05$ compared T (tumor tissue) versus N (adjacent nontumor liver tissue). H: ■, N; □, T.

Cruz, CA, USA; at 1:250 dilution), GAPDH (Abcam, San Francisco, CA, USA; at 1:10,000 dilution) or β-actin (Proteintech Group, Chicago, IL, USA; at 1:10,000 dilution) overnight at 4°C. After washing, the membranes were probed with the horseradish peroxidase–conjugated secondary antibody and visualized with chemiluminescent system (Cell Signaling Technology, Danvers, MA, USA). The band intensity was measured using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as a loading control.

Immunohistochemical Staining

The HCC tissue sections were deparaffinized and rehydrated. Then, the sections were boiled in ethylenediaminetetraacetic acid (EDTA) (1 mmol/L; pH 8.0) for antigen epitope retrieval. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide. After rinsing, slides were incubated with a mouse polyclonal antibody against *BIN1* (Santa Cruz Biotechnology; at 1:100 dilution) overnight at 4°C. After washing, the slides were incubated for 30 min with horseradish peroxidase–conjugated secondary antibody (Envision™ Detection Kit,

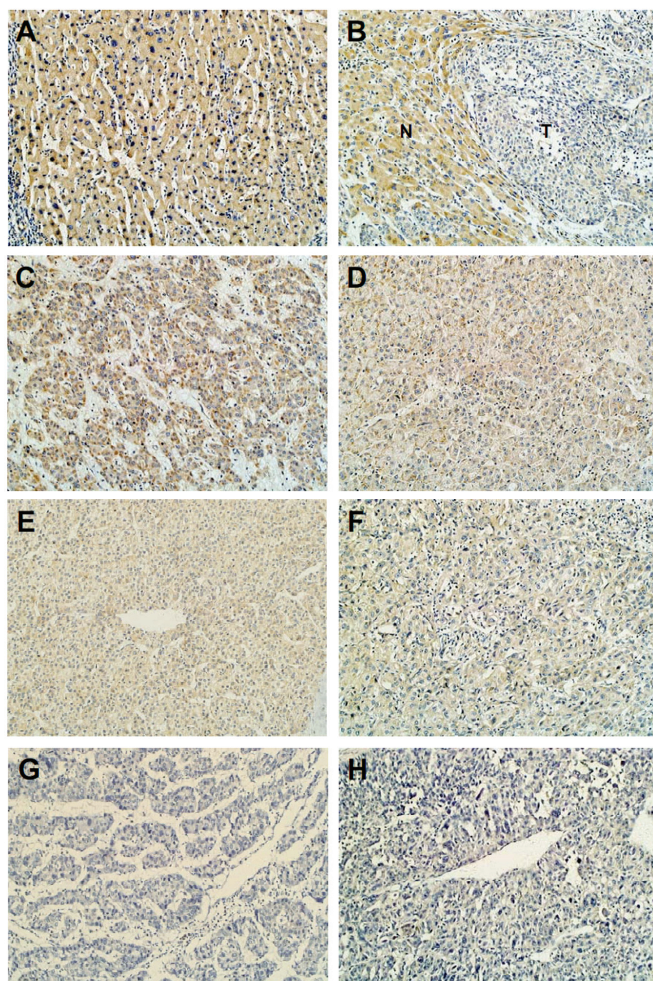

Figure 2. Decreased *BIN1* protein expression in primary HCC surgical specimens (n = 117) detected by immunohistochemistry. (A) Distant normal liver tissues. (B) Adjacent surrounding noncancerous tissues. (C, D) Well-differentiated HCC. (E, F) Moderately differentiated HCC. (G, H) Poorly differentiated HCC. N, nontumorous tissue; T, tumor tissue (original magnification 200×). Experiments were performed two times.

GK500705; Gene Tech, Shanghai, China) at room temperature. After this incubation, the slides were washed three times in PBS, and the antibody complexes were colored with diaminobenzidine (DAB) and then counterstained with hematoxylin. The total *BIN1* immunostaining score was calculated as the sum of the presence of positively stained tumor cells and the staining intensity. Briefly, the percent positive staining was scored as 0 (<5%, negative), 1 (5–25%, sporadic), 2 (25–50%, focal), or 3 (>50%, diffuse). The staining intensity was score as 0 (no staining), 1 (weakly stained), 2 (moderately stained) or 3 (strongly stained). The total immunostaining score was calculated with the value of percent positivity score × staining intensity score, which ranged from 0 to 9. We defined *BIN1* expression levels as follows: – (score 0–1), + (score 2–3), ++ (score 4–6) and +++ (score >6). All IHC results were evaluated by two independent pathologists who were unaware of the clinical data. For inconsistent evaluations of individual slides, both observers reviewed the slide again to obtain a consensus. On the basis of their *BIN1* immunostaining levels, the patients were divided into two groups: the *BIN1* low group (– and +) and the *BIN1* high group (++ and +++).

Expression Plasmid Construction and Transient Transfections

A eukaryotic expression plasmid of the human *BIN1* gene was constructed using a pcDNA3 vector (Invitrogen, China). We constructed *BIN1* cDNA expression vector on the basis of the sequence in NCBI (Accession number U68485). This *BIN1* mRNA does not contain the exon 12 (–12 isoform), and the sequence has normal tumor suppressor function. The empty vector was used as negative control. Huh7 and Hep-3B cells were cultured in six-well plates until they reached 85–90% confluence, and then transient transfections were performed using Lipofectamine 2000 (Invitrogen, China) according to the manufacturer instructions. At 48 h after transfection, gene expression was confirmed via Western blot analysis.

Tumor Suppressor Function of *BIN1*

Cell growth rate of *BIN1*-expressing cells and control cells or LO2 cells were detected with the MTS cell proliferation assay. Cells were seeded in 96-well plates at a density of 4×10^3 per well. The cell growth rate was detected using a cell proliferation MTS kit (Promega, Beijing, China) according to the manufacturer's instructions, and independent experiments were performed in triplicate. For the foci formation assay, 1×10^3 *BIN1*-expressing cells or control cells were plated in a six-well plate. After 10 d of culture, surviving colonies (>50 cells per colony) were counted with Giemsa staining. Colony-forming efficiency (CFE%) was defined as the ratio of the number of colonies formed in culture to the number of cells inoculated, and independent experiments were performed in triplicate.

Cell Cycle Assay

HCC cells transfected with the vector or *BIN1* were collected and washed twice with ice-cold PBS and fixed with ice-cold 75% ethyl alcohol at –20ºC for 4 h. The cells were washed and resuspended in 400 μL ice-cold PBS plus RNAase and in-

Table 1. Relationship between *BIN1* expression and clinicopathological features in HCC.

Clinicopathological variables	Number of each group	*BIN1* expression		P
		Low	High	
All cases	117	56	61	
Age (years)				0.790
< 50	60	28	32	
≥50	57	28	29	
Sex				0.019[a]
Male	101	44	57	
Female	16	12	4	
Tumor size (cm in diameter)				0.035[a]
<5	45	16	29	
≥5	72	40	32	
Histological differentiation				0.011[a]
Well	20	4	16	
Moderate	72	36	36	
Poor	25	16	9	
Liver cirrhosis				0.255
No	48	26	22	
Yes	69	30	39	
HBV				0.099
Negative	17	5	12	
Positive	100	51	49	
Serum AFP				0.065
<25 μg/L	30	10	20	
≥25 μg/L	87	46	41	
Recurrence (tumor appearing in 5 years)				0.725
No	86	42	44	
Yes	31	14	17	
Distant metastasis				0.0718
No	101	45	56	
Yes	16	11	5	

HBV, hepatitis B virus.
[a]$P < 0.05$.

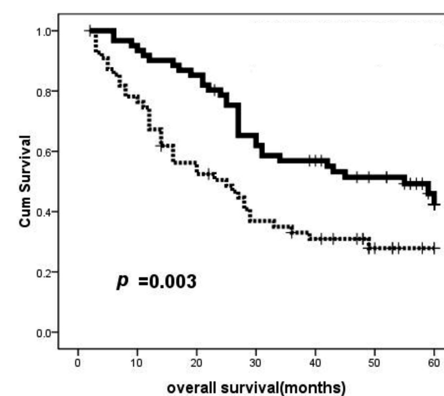

Figure 3. Correlation of overall survival with the expression of *BIN1* in 117 HCC patients. On the basis of the results of immunohistochemical staining, log-rank test revealed that HCC patients with low *BIN1* expression showed significantly poorer prognoses than patients with high *BIN1* expression (P = 0.003, Kaplan-Meier method). Experiments were performed two times. Cum, cumulative. ······, low *BIN1* group; ——, high *BIN1* group,

cubated in a 37°C water bath for 30 min. Then, the cells were stained with propidium iodide at 4°C in the dark for 30–60 min. The DNA content was analyzed using a flow cytometer (Beckman Coulter, Brea, CA, USA).

Apoptosis Assay

Transfected Huh7 or Hep-3B cells were collected and washed twice with ice-cold PBS and resuspended with 1× binding buffer before incubation with annexin V-FITC (Bestbio, Shanghai, China) according to the manufacturer's protocol. After 15 min of incubation at room temperature in the dark, propidium iodide was added, and the number of stained cells was analyzed using a flow cytometer (Beckman Coulter).

Wound-Healing Assay

Approximately 24 h after the beginning of the transfection, HCC cells or LO2 cells were seeded in a 12-well culture plate and allowed to grow to 90% confluence. A wound was then created on the cell monolayer using a pipette tip. The migration of cells toward the wound was monitored daily. Images were captured at regular time intervals. The experiment was done in triplicate.

Matrigel Invasion Assay

The matrigel invasion assay was performed using 24-well transwells consisting of polycarbonate membrane inserts with 8-μm pores (Corning, Shanghai, China), coated with a thin layer of 0.5 mg/mL Matrigel Basement Membrane Matrix (BD Biosciences, Bedford, MA, USA). HCC cells or LO2 cells (1×10^5) in 100 μL growth medium without FBS were added in the upper chamber, and 0.5 mL growth medium with 10% FBS was placed in the lower chamber. After incubation at 37°C for 48 h, the insert membranes were fixed with 75% methanol for 10 min. The cells remaining in the upper surface were removed with cotton-tipped swabs. The invaded cells on the lower surface of membranes were stained with 0.5% crystal violet. The stained cells at 10 random fields were counted under an inverted microscope. Each experiment was performed in triplicate.

Silencing *BIN1* Expression in LO2 Cells

To further investigate the tumor suppressor function of *BIN1*, the *BIN1* gene was silenced with siRNA. The siRNA sequences were as follows: *BIN1*-siRNA-1: 5′-GCGUC CAGAA UUUCA ACAAT T-3′; *BIN1*-siRNA-2: 5′-CCACU ACGAG UCCCU UCAAT T-3′; *BIN1*-siRNA-3: 5′-GCGUA GGUUU CUACG UCAAT T-3′; *BIN1*-siRNA-4: 5′-GCAAC ACCUU

CACGG UCAAT T-3′; negative control: 5′-UUCUC CGAAC GUGUC ACGUT T-3′.

The siRNAs were synthesized by GenePharma Company (Shanghai, China). A total of 400 pmol siRNA was transfected into 4 × 10^5 LO2 cells using Lipofectamine RNAi MAX reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. After that, proliferation, migration and invasion detection were then performed.

Statistical Analysis

The correlation between *BIN1* expression and clinical parameters were evaluated using χ^2 tests. Survival analysis was performed using the Kaplan-Meier method, and a log-rank test was used for comparison. Univariate and multivariate analyses were performed using the Cox proportional hazards regression model. The two-tailed unpaired Student *t* test was used to assess differences in cell growth rate, colony formation, apoptotic frequency and invading cell numbers between control vector- and *BIN1*-transfected HCC cells. All calculations were performed with SPSS 16.0. The results were considered significantly different when P was <0.05.

All supplementary materials are available online at www.molmed.org.

RESULTS

Expression of *BIN1* in HCC

We first examined *BIN1* expression status in HCC cell lines and primary HCC tissue samples with quantitative real-time PCR and Western blot analysis. All five HCC cell lines showed significantly (P < 0.05) decreased mRNA transcript levels of *BIN1*, as evidenced by quantitative real-time PCR analysis when using GAPDH as a loading control, compared with the normal liver cell line LO2 (Figure 1A). To verify this finding, we repeated this experiment using a different housekeeping gene, β-actin. As shown in Figure 1B, whereas the *BIN1* mRNA level of the BEL-7402 cell line was comparatively high, all the other 4 HCC cell lines revealed significantly (P < 0.05) lower *BIN1* levels compared with LO2. More importantly, Western blot analysis confirmed that *BIN1* protein expression levels were significantly (P < 0.05) decreased in HCC cell lines (Figures 1C, D, E) when using both GAPDH and β-actin as loading controls. In 42 paired primary HCC tissue samples, decreased mRNA transcript levels of *BIN1* were found in most tumor tissues (32/42) compared with adjacent nontumor tissues, as evidenced with quantitative real-time PCR analysis (P = 0.02, Figure 1F). This tendency was also verified at the protein level with Western blot analysis (Figures 1G, H).

From previous studies, the molecular cause of *BIN1* downregulation may be genetic alterations, including allelic deletions, of the *BIN1* gene in cancer cells. However, loss of heterozygosity (LOH) analysis showed that the frequency of *BIN1* LOH in primary cancer tumors was very low, suggesting that most losses were due to epigenetic causes (6,9,22). DNA methylation events that affect promoter activity of *BIN1* offer a potential mechanism for epigenetic alteration. However, CpG methylation analyses in breast and prostate tumors have demonstrated that this event was not responsible for the majority of cases of *BIN1* loss in cancer cells (22). Thus, the loss of *BIN1* may be due to other upstream epigenetically regulated factors (23).

Table 2. Univariate and multivariate analysis of overall survival in HCC.

	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
BIN1	0.490	0.304–0.790	0.003[a]	0.553	0.339–0.904	0.018[a]
Age	0.900	0.592–1.370	0.624			
Sex	0.679	0.341–1.353	0.271			
Tumor size	1.641	1.051–2.562	0.029[a]			
Histological grade	1.343	0.955–1.887	0.090			
Cirrhosis	0.782	0.517–1.183	0.244			
HBsAg status	1.123	0.623–2.023	0.700			
Serum AFP	1.721	1.036–2.861	0.036[a]	1.406	0.785–2.518	0.252
Recurrence	1.332	0.860–2.061	0.199			
Metastasis	1.522	0.859–2.696	0.150			

CI, confidence interval; HR, hazard ratio.
[a] P < 0.05.

Immunohistochemical Staining of *BIN1* in HCC Clinical Samples

To further investigate the relationship between *BIN1* expression and various clinicopathological characteristics, as well as the prognostic role of *BIN1* in HCC, 117 paraffin-embedded HCC tissue samples collected between 1999 and 2001 were used for immunohistochemical analysis. We found that *BIN1* expression was positive in distant normal liver tissues and adjacent nontumor liver tissues (Figures 2A, B), but its expression was decreased in tumor tissues compared with matched nontumor samples (Figure 2B). Furthermore, *BIN1* expression decreased in a stepwise fashion in well-differentiated to moderately differentiated to poorly differentiated HCC tumor tissues (Figures 2C–H). Decreased expression of *BIN1* (*BIN1* immunostaining levels of – and +) was found in 56 of 117 samples and correlated significantly with sex (P = 0.019), tumor size (P = 0.035) and histological differentiation (P = 0.011), but not with age, cirrhosis, serum alpha fetoprotein (AFP), hepatitis B surface antigen (HBsAg), metastasis or recurrence as seen in a χ^2 analysis (Table 1). In fact, we found that low expression of *BIN1* had a tendency to correlate with a higher metastasis rate of HCC despite no statistic difference (P = 0.0718, Table 1). Previous *BIN1* expression detection in HCC cell lines showed the

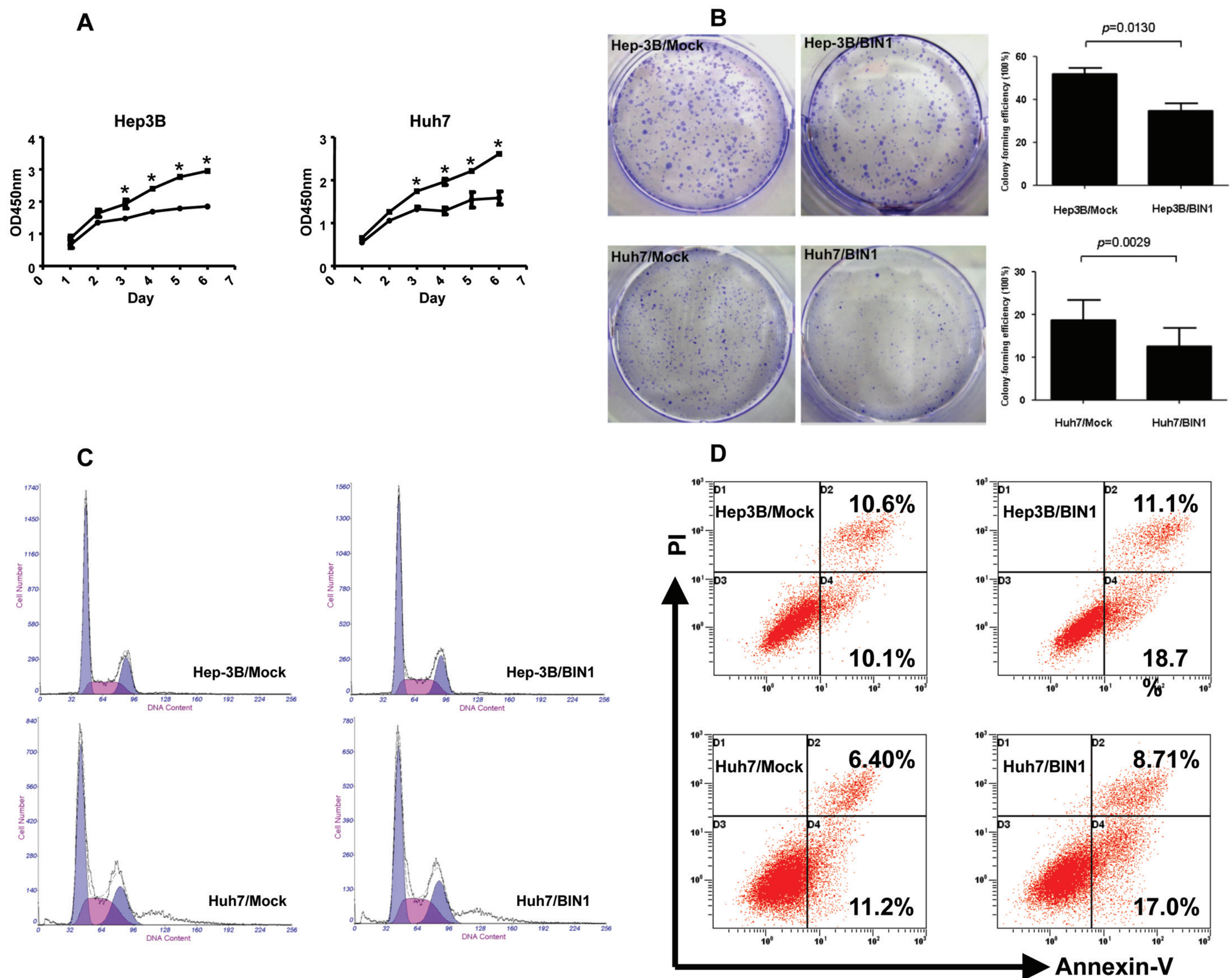

Figure 4. Tumor-suppressive role of *BIN1* in HCC cells Hep3B and Huh7. (A) MTS assay showing the suppressive effect of *BIN1* overexpression on the *in vitro* proliferation of HCC cell lines. Experiments were performed in triplicate. (B) Representative inhibition of colony formation in monolayer culture by *BIN1*. Right, quantitative analyses of foci numbers are shown as values of mean ± standard deviation. Experiments were performed in triplicate. *P* values were calculated using the Student *t* test. (C) The effect of *BIN1* on cell cycle in HCC cells. The cells were transfected with *BIN1* or control vector as in (A) and (B). After 48 h, the cells were collected for cell cycle distribution analysis. DNA content of *BIN1* vector– or control vector–transfected cells was detected by flow cytometry. Hep-3B/Mock panel: gap 1 (G1) = 54.831, synthesis phase (S) = 23.322, gap 2 (G2) = 24.841; Hep-3B/*BIN1* panel: G1 = 52.163, S = 26.885, G2 = 20.952; Huh7/Mock panel: G1 = 47.999, S = 30.405, G2 = 21.595; Huh7/*BIN1* panel: G1 = 47.341, S = 29.336, G2 = 23.323. (D) Apoptosis levels of *BIN1* vector– or control vector–transfected HCC cells were quantified with an annexin V and propidium iodide viability assay. The cells were transfected with *BIN1* or control vector as in (A), (B) and (C). After 72 h, the cells were collected for apoptosis analysis. The percentages of annexin V– or propidium iodide–positive cells were detected by flow cytometry. Experiments were performed in triplicate. A: ●, *BIN1*; ■, Mock.

same tendency, with results of immunohistochemical analysis of tumor tissues. Among the four-well or moderately differentiated and low-metastatic HCC cell lines, *BIN1* expression appeared in three cell lines (Hep3B, Huh7 and BEL-7402). Loss of *BIN1* expression was found in the poorly differentiated and high-metastatic cell line SK-hep1 (Figures 1A, C).

Survival Analysis

For the investigation of the prognostic value of *BIN1*, we analyzed the relationship between *BIN1* expression and patient survival. We found that the low *BIN1* ex-

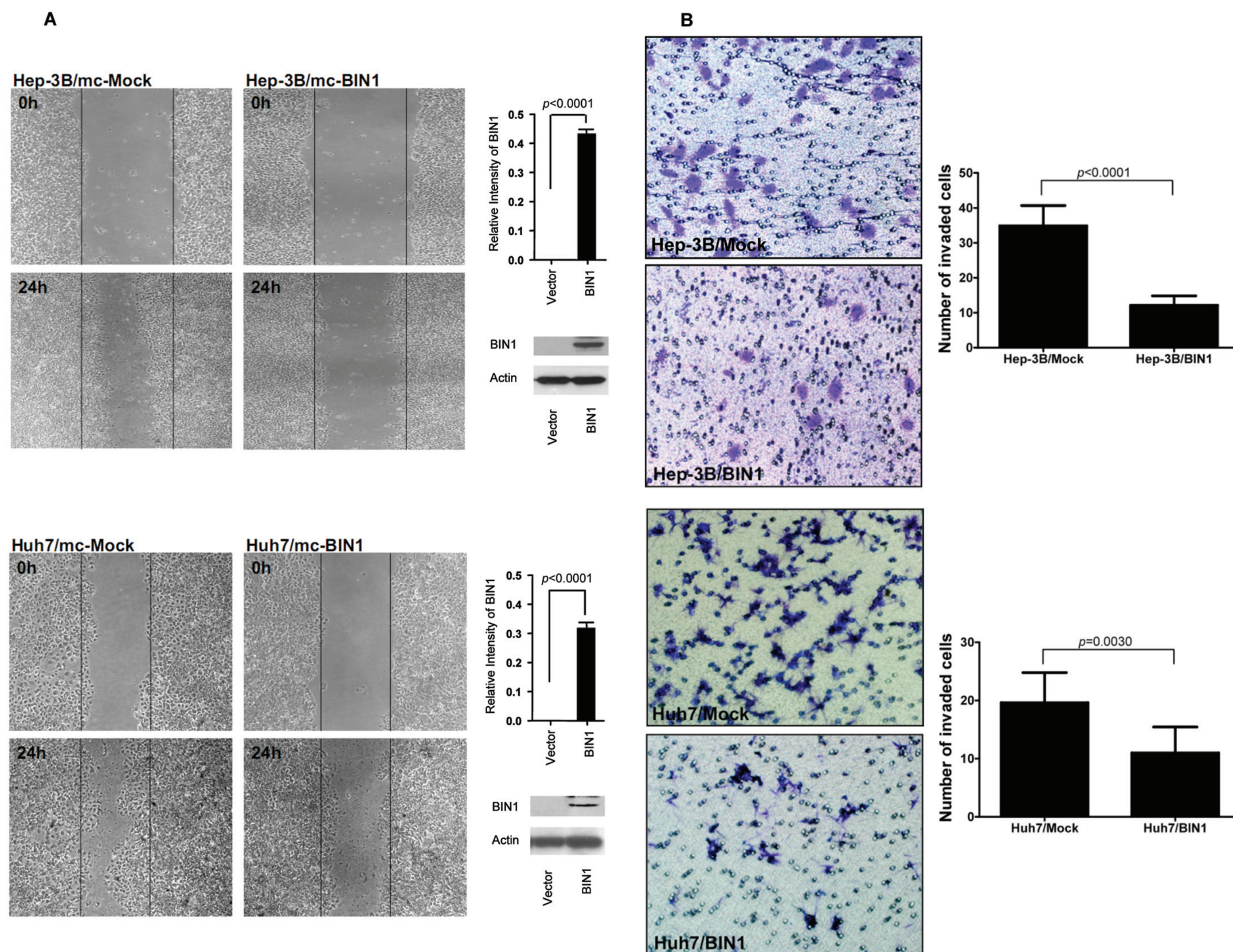

Figure 5. Enforced expression of *BIN1* inhibited HCC cell migration and metastatic capability. (A) The migration ability of *BIN1* vector– or control vector–transfected HCC cells was determined by wound-healing assay. The ability of Hep3B and Huh7 cells grown in a confluent monolayer to migrate into a linear wound created by a pipette tip was monitored for 24 h. *BIN1* overexpression was confirmed by immunoblotting and the density of each band was quantitated. (B) Cell invasion of HCC cells transfected with *BIN1* or control vector was evaluated with Matrigel assay. Invasive cells were stained, photographed and counted under the microscope. Right, quantitative analyses of stained cell numbers are shown as values of mean ± standard deviation. Experiments were performed in triplicate. *P* values were calculated using the Student *t* test.

pression groups showed a poorer prognosis compared to the high *BIN1* expression groups, as shown by the Kaplan-Meier survival analysis ($P = 0.003$, log-rank test; Figure 3). The univariate analysis showed that *BIN1* expression, tumor size and serum AFP were significant prognostic factors (Table 2). The multivariate analyses showed that only *BIN1* expression was identified as an independent risk factor for survival ($P = 0.018$, Table 2). These results indicated that *BIN1* could serve as prognostic marker for HCC.

Role of *BIN1* in Cell Proliferation, Clone Formation, Cell Cycle and Apoptosis in Hep3B and Huh7 Cell Lines

To assess the effects of *BIN1* on cell proliferation, the *BIN1* expression vector and the control vector were transfected into Hep3B and Huh7 cells, respectively. Both of these cell lines had previously shown low expression of *BIN1* (Figures 1A, C). The cell growth assay revealed that cell growth rates in *BIN1*-transfected HCC cell lines were significantly lower than those for control vector–transfected HCC cell lines (Figure 4A), but were similar to those for the normal liver cell line LO2 (Figure 6A). To

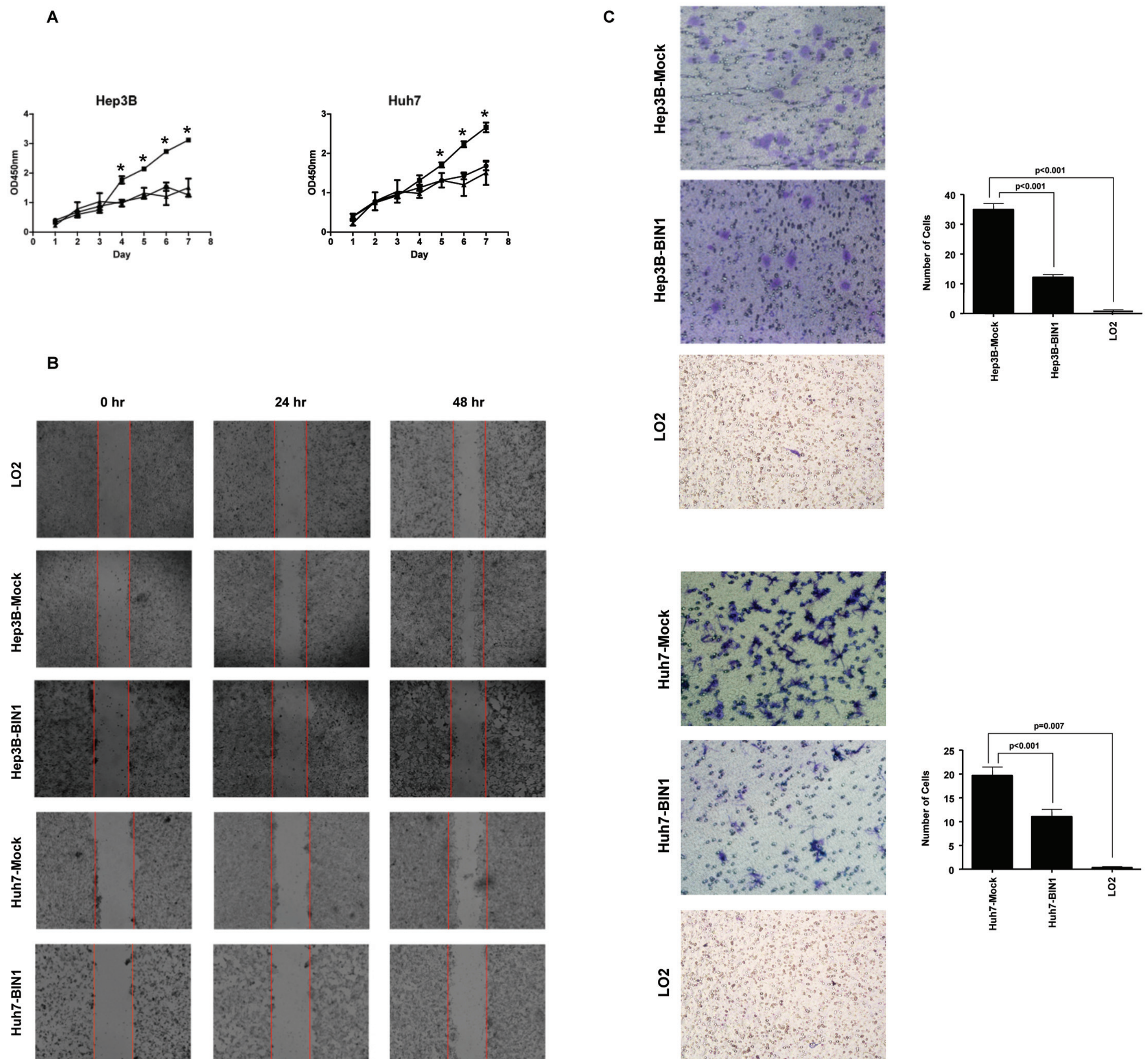

Figure 6. Analysis of proliferation, migration and invasion of HCC cell lines compared with normal liver cell line. (A) Proliferation detection in HCC cell lines and normal liver cell line. The growth rate of HCC cell lines with overexpressed *BIN1* was lower than the control but was similar to the normal liver cell line LO2 (*$P < 0.05$). (B) The migration ability of normal liver cells and *BIN1* vector– or control vector–transfected HCC cells was determined by wound-healing assay. The migration ability of HCC cells with overexpressed *BIN1* was lower than control and was similar to LO2. (C) Cell invasion of normal liver cells and HCC cells transfected with *BIN1* or control vector was evaluated with Matrigel assay. The invasion ability of HCC cells overexpressing *BIN1* was lower than control. LO2 did not show invasion. Experiments were performed in triplicate. A: ●, *BIN1*; ■, vector; ▲, LO2.

further explore the tumor-suppressive effect of *BIN1*, clone formation was tested. The results demonstrated that the efficiency of clone formation was significantly ($P = 0.013$ for Hep3B, $P = 0.0029$ for Huh7) inhibited in *BIN1*-transfected HCC cells compared with control vector–transfected HCC cells (Figure 4B). To confirm the tumor suppression func-

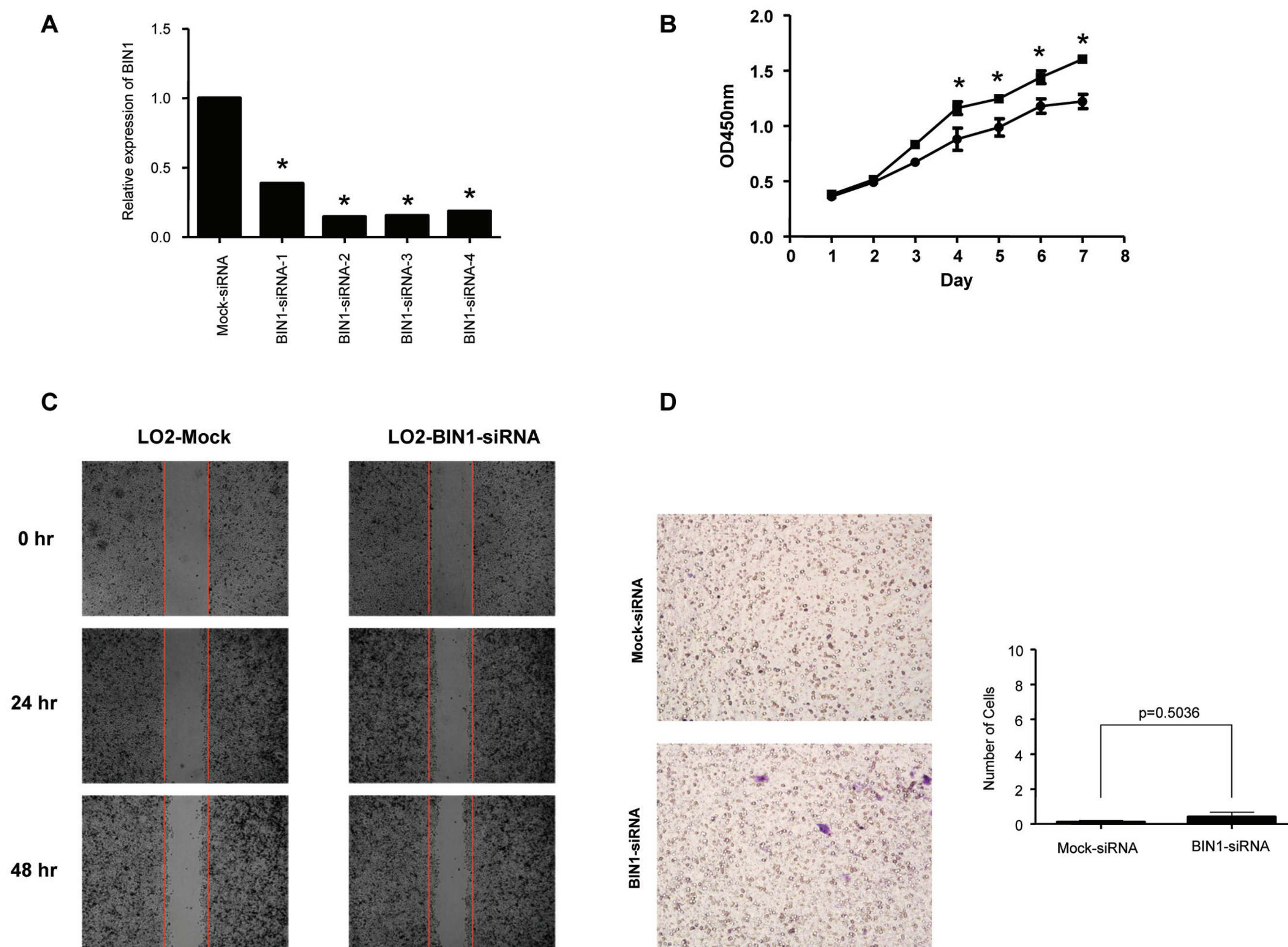

Figure 7. Evaluation of proliferation, migration and invasion of LO2 transfected with control siRNA or *BIN1*-siRNA. (A) *BIN1* mRNA detection in LO2 cells tranfected with control siRNA or *BIN1*-siRNA. All four *BIN1*-siRNAs silenced *BIN1* expression effectively. *$P < 0.05$ compared with Mock-siRNA. We then randomly selected number 3 *BIN1*-siRNA for the subsequent experiments. (B) Proliferation detection of LO2 cells transfected with control siRNA or *BIN1* siRNA. LO2 cells transfected with *BIN1*-siRNA showed a higher growth rate than control. Experiments were performed four times (*$P < 0.05$ compared with Mock-siRNA). (C) Migration detection of LO2 cells transfected with control siRNA or *BIN1* siRNA. LO2 cells transfected with *BIN1*-siRNA showed a minor difference compared with the control. (D) Invasion detection of LO2 cells transfected with control siRNA or *BIN1* siRNA. LO2 cells transfected with *BIN1*-siRNA did not alter the invasion ability. B: ●, Mock-siRNA; ■, *BIN1*-siRNA.

tion of *BIN1*, we silenced the *BIN1* expression in the LO2 cell line with siRNA. We found that silencing *BIN1* expression in LO2 significantly ($P < 0.05$) enhanced the cell growth rate compared with mock siRNA treatment (Figure 7B).

We proceeded by exploring the potential mechanism(s) underlying tumor cell growth inhibition by *BIN1*. We determined cell cycle distributions of control- versus *BIN1* vector–transfected HCC cells with flow cytometry. As showed in Figure 4C, restored *BIN1* expression did not significantly change the G1, S or G2 level of HCC cells, both in Hep3B and Huh7 cells. We then used the annexin V-FITC binding assay to explore the effects of *BIN1* on apoptosis in HCC cells. Analysis of the proportion of apoptotic cells on the basis of flow cytometry revealed that apoptosis levels in HCC cells transfected with the *BIN1* vector were significantly ($P = 0.0094$ for Hep3B, $P = 0.0145$ for Huh7) higher than those in cells transfected with the control vector (Figure 4D). This outcome was true for Hep3B: 29.8% (11.1% + 18.7%) versus 20.7% (10.6% + 10.1%), as well as for Huh7: 25.71% (8.71% + 17.0%) versus 17.6% (6.4% + 11.2%). These results thus suggest that *BIN1* may play an important proapoptotic role in HCC development.

Previous studies demonstrated that *BIN1* might affect cancer cell proliferation via multiple mechanisms. For example, *BIN1* can functionally interact with Myc through its Myc binding domain (MBD) and inhibit Myc activation by recruiting the transcriptional repres-

sor to the promoter of *BIN1*, resulting in inhibition of malignant cell proliferation (2). However, MBD deletion only partly relieved suppression, underscoring the importance of MBD-independent mechanisms for some types of growth inhibition by *BIN1*, because the N-terminal BAR-C region of *BIN1* was also required to inhibit Myc transformation (2). Furthermore, other domains of *BIN1*, such as U1 and SH3, are required to suppress E1A or mutant p53 transformation. Thus, *BIN1* could regulate malignant cell proliferation through Myc-independent as well as Myc-dependent mechanisms (2).

BIN1 mediates apoptosis of transformed primary cells also through the c-myelocytomatosis (*c-Myc*) pathway (15). *BIN1* was not proapoptotic when overexpressed in primary cells by itself, but it instead provides a conditional linkage between *c-Myc* and death effector machinery (15,16). Thus, *BIN1* might also regulate the HCC cell proliferation and apoptosis through the *c-Myc* pathway.

Role of *BIN1* in Cell Migration and Invasion in HCC Cells

Finally, we assessed the role of *BIN1* in HCC cell migration and invasion using wound-healing and Matrigel assays, respectively. The wound-healing assay revealed that the ability of cell migration was decreased when *BIN1* was overexpressed, both in Hep3B and Huh7 cell lines (Figure 5A). In addition, *BIN1* overexpression significantly inhibited cell invasion of HCC cells ($P <$ 0.0001 and $P = 0.003$ for Hep3B and Huh7 cells, respectively; Figure 5B). LO2 did not show the invasion abilities in our experiment (Figure 6C). Interestingly, we found that, while the abilities of migration of HCC cell lines with overexpressed *BIN1* were similar to LO2 (Figure 6B), the invasion of the *BIN1*-expressing HCC cells was higher than LO2, but significantly ($P < 0.05$) lower than HCC (see Figure 6C). However, silencing *BIN1* expression in LO2 showed minimal difference, both in cell migration and invasion (Figures 7C, D).

DISCUSSION

Previous studies have shown that *BIN1* is a potential candidate tumor suppressor involved in HCC. However, clinical implications remain undefined because of the lacking studies of human HCC tumor samples. At the same time, relative functional study of *BIN1* in HCC cells is also unclear. In the present study, we evaluated the correlation of *BIN1* expression level and the clinical outcome of HCC patients by using the primary HCC tissue samples (n = 117) for the first time. Furthermore, using an *in vitro* cell model, we also investigated the tumor suppressor role of *BIN1* in HCC cells in detail. We found that the expression of *BIN1* was significantly downregulated in 76% of the primary HCC tumors and in the entire HCC cell lines tested in this study, both at the mRNA level and at the protein levels. Tissue immunohistochemistry analysis also showed that decreased expression of *BIN1* was found in 48% of the primary HCC samples, and downregulated expression of *BIN1* significantly correlated with larger tumor size and poorly differentiated HCC. Together, these results suggest that *BIN1* could be as a tumor suppressor, and alternation of this molecule may play an important role in the tumorigenesis of HCC.

In a mouse model, *BIN1*-null mice developed poorly differentiated tumors, characterized by reduced tubule formation, high mitotic indices and high degrees of nuclear pleomorphism (19). In human lung and lung adenocarcinoma tissues, Chang *et al.* found strong *BIN1* expression in normal bronchial epithelia and stage I tumors. However, in case of stage II–IV lung adenocarcinoma, its expression was apparently reduced (20). This pattern was also seen in breast and prostate tumors (6,7). Our results are consistent with these studies and have suggested that the *BIN1* expression might suppress the differentiation of HCC cells. As a result, the loss of *BIN1* expression promotes HCC development.

Importantly, we found that patients with low *BIN1* expression revealed a significantly ($P = 0.003$) shorter overall survival than patients with high *BIN1* expression. Furthermore, we showed that *BIN1* expression was an independent prognostic risk factor for HCC patients, suggesting that decreased *BIN1* expression might help the characterization of HCC patients with a poor prognosis. Therefore, *BIN1* may serve as a novel prognostic marker in HCC cancer patients.

Because of extremely low expression of *BIN1* in Hep3B and Huh7 HCC cell lines, we transfected full-length *BIN1* into these cells to further observe the functional role of this molecule in HCC. Enforced introduction of the *BIN1* gene significantly suppressed cell proliferation and decreased clone formation in soft agar. In addition, silenced *BIN1* expression enhanced the normal liver cell line LO2 growth rate. These studies have thus provided additional experimental evidence that *BIN1* plays an important role in suppressing the development of HCCs. This result was consistent with the clinicopathological findings that decreased expression of *BIN1* in HCCs was associated with larger tumor size, indicating that the loss of *BIN1* may facilitate rapid HCC tumor growth.

To explore the potential mechanism for *BIN1* to function as a tumor suppressor in HCC, we monitored cell cycle changes in HCC cells. Surprisingly, overexpression of *BIN1* did not significantly affect the DNA content in HCC cells or cell cycle distribution compared with control cells, suggesting that *BIN1* may play a minor role in the HCC cell cycle. However, we found that *BIN1* is able to promote apoptosis of HCC tumor cells. This adds to the previous findings reported in melanoma, breast cancer, neuroblastoma and astrocytoma cells (5,6,13). It is known that *BIN1* may interact with *c-Myc* and help mediate *c-Myc*–induced apoptosis (1–4,16). Our findings that restoring *BIN1* expression induced apoptosis in HCC cells suggest that *BIN1* may inhibit the proliferation of HCC cells and their colony formation through a proapoptotic pathway.

Our additional mechanistic studies showed that overexpression of *BIN1* could inhibit the migration and invasion of HCC cells. These data suggest that the loss of *BIN1* may favor the metastasis of HCC. These findings are consistent with our clinicopathological analysis results, which demonstrated that positive expression of *BIN1* had a tendency to correlate with a lower metastasis rate of HCC. Previous studies have reported that *BIN1* is linked to metastatic potential in neuroblastoma because *BIN1* expression is frequently lost in metastatic neuroblastoma cell lines (21), and there was a strong trend toward lower *BIN1* expression in metastatic neuroblastoma compared with localized NB (9). Similar results were also found in breast and prostate cancer studies (7,10). Thus, inhibiting the metastatic ability of tumor cells may represent another mechanism involved in the role of *BIN1* as a tumor suppressor in HCC. However, for the normal liver cell line LO2, silencing *BIN1* expression did not enhance the abilities of invasion. Together, our results suggest that single loss of *BIN1* expression may be a prerequisite, but is not sufficient to alter the metastatic ability of cells.

CONCLUSION

In summary, in the present study, we presented evidence that *BIN1* expression is significantly decreased in surgically excised HCC patient specimens as well as in HCC cell lines. We also showed that decreased *BIN1* expression correlates with the degree of differentiation of HCC and predicts poor prognosis in HCC patients. Furthermore, using an *in vitro* model, we demonstrated that ectopic *BIN1* expression could inhibit the growth, colony formation and invasion abilities of HCC cells. Of note, these results are consistent with our clinicopathological association study. Our findings thus indicate that *BIN1* may be a novel prognostic marker for HCC patients, and it plays an important suppressive role in HCC development and progression.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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