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Biomarkers, Genomics, Proteomics, and Gene Regulation

Increased Expression of 14-3-3β Promotes Tumor Progression and Predicts Extrahepatic Metastasis and Worse Survival in Hepatocellular Carcinoma

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14-3-3β is implicated in cell survival, proliferation, migration, and tumor growth; however, its clinical relevance in tumor progression and metastasis have never been elucidated. To evaluate the clinical significance of 14-3-3β, we analyzed the association of 14-3-3β expression and clinicopathologic characteristics in primary and subsequent metastatic tumors of hepatocellular carcinoma patients. 14-3-3β was expressed abundantly in 40 of 55 (70.7%) primary tumors. Increased 14-3-3β expression in primary tumors predicted a higher 5-year cumulative incidence of subsequent extrahepatic metastasis, and multivariate analysis revealed 14-3-3β overexpression was an independent risk factor for extrahepatic metastasis. Patients with increased 14-3-3β expression in primary tumors had worse 5-year overall survival rates, and 14-3-3β overexpression was an independent prognostic factor on Cox regression analysis. Furthermore, stably overexpressed 14-3-3β enhanced hepatocellular carcinoma cell migration and proliferation and increased anchorage-independent cell growth. In addition, *in vivo* study in a nude-mice model showed tumor formation significantly increased with 14-3-3β overexpression. In conclusion, this is the first report to show that increased 14-3-3β expression is associated with subsequent extrahepatic metastasis and worse survival rates, as well as cancer progression of hepatocellular carcinoma. Thus, 14-3-3β may be a novel prognostic biomarker and therapeutic target in hepatocellular carcinoma. ***(Am J Pathol 2011, 179:2698–2708; DOI: 10.1016/j.ajpath.2011.08.010)***

Hepatocellular carcinoma (HCC) is considered a serious public health problem in endemic areas of hepatitis B or C viral infection, including Africa and Southeast Asia.[1] Despite recent progress in surgical and nonsurgical treatment, the prognosis for HCC patients still is dismal, in part because a substantial portion of the disease is discovered at an advanced stage when curative therapy is not possible.[2] Even patients with operable tumors show high rates of local recurrence or distant metastasis.[3,4] Therefore, investigating accurate prognostic biomarkers to identify patients at high risk of recurrence or metastasis is of utmost importance for developing preventive strategies to improve the outcomes of HCC patients.

14-3-3 proteins are a family of 28- to 33-kDa acidic polypeptides, and their sequences are highly conserved in eukaryotic organisms.[5] Seven 14-3-3 isoforms (β, ε, γ, η, σ, τ/θ, and ζ) have been identified in mammalian cells and found to have widely diverse intracellular functions.[5] In general, homodimerization or heterodimerization is required for 14-3-3 proteins to interact with and regulate associated intracellular proteins via phospho-serine/threonine binding activities.[6] Moreover, 14-3-3 proteins can regulate cell survival, proliferation, motility, and contribute to oncogenesis.[5,7]

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Some studies have shown isoform-specific expression of 14-3-3 proteins associated with various types of human malignancies. For instance, increased 14-3-3ζ expression promoted anchorage-independent cell growth in lung cancer cell lines,[8] and expression of 14-3-3ζ was associated with a worse disease-free survival rate and a high recurrence rate in human breast cancer.[9] The expression of six 14-3-3 isoforms was found to be increased in human lung cancer tissues.[10] In addition, 14-3-3β and 14-3-3η were found specifically expressed in human astrocytoma.[11,12] The expression frequency and levels of 14-3-3β and 14-3-3η were associated with human astrocytoma.[12] Moreover, increased expression of 14-3-3ε in breast cancer was shown in a proteomic study.[13] Intriguingly, 14-3-3σ (also known as stratifin) initially was considered a tumor-suppressor gene, and reduced 14-3-3σ expression was reported to increase the probability of metastasis in human nasopharyngeal carcinoma,[14] lung squamous cell carcinoma,[15] HCC,[16] and ovarian cancer.[17] In contrast, an increasing number of studies have indicated that the expression of 14-3-3σ was increased and associated with colorectal cancer myometrial invasion and lymph node metastasis.[10,18–21] Proteomic profiling analyses have suggested that 14-3-3σ is up-regulated in human cirrhosis-type gastric carcinoma cells[22] and human oral squamous cell carcinoma.[23] Moreover, a recent study showed that nuclear expression of 14-3-3σ was useful in prediction and was related to prognosis in patients with esophageal squamous cell carcinoma.[24]

Overexpression of 14-3-3β in NIH3T3 cells stimulated cell proliferation, anchorage-independent growth, and tumor formation.[25] This induced oncogenic transformation and cell growth was mediated by mitogen-activated protein kinase (MAPK)-dependent signal activation.[25] Forced expression of anti-sense 14-3-3β RNAs reduced the growth of rat hepatoma K2 cells *in vivo* and *in vitro*.[26] Furthermore, up-regulation of 14-3-3β expression was reported to be an effector in Kaposi's sarcoma and papillary thyroid carcinomas in mediating cell proliferation and tumor progression.[27,28] Increased 14-3-3β expression was observed in human lung cancer tissue,[10] and the expression of 14-3-3β was associated with the aggressiveness of human astrocytoma.[12] These results provide strong evidence that 14-3-3β plays a vital role in tumor growth and progression. However, no convincing clinical study of the association of 14-3-3β expression and tumor metastasis and progression in HCC has been reported. In this study, we investigated the association of 14-3-3β expression and clinicopathologic characteristics of HCC. We found 14-3-3β overexpression associated with distant metastasis and overall survival of HCC patients. We further performed the *in vivo* and *in vitro* experiments to show the role of 14-3-3β in HCC cell migration, proliferation, and tumor growth.

Materials and Methods

Patients and Clinical Specimens

Tissue samples were obtained from 55 HCC patients who underwent surgery for tumor resection or biopsy in Taichung Veterans General Hospital and retrospectively were enrolled (from January 1999 to December 2001) in this study. The mean follow-up period was 58.6 ± 28.6 months. Eighteen patients (32.7%) were shown by tissue analysis to develop metastasis 3 to 87 months after the diagnosis and surgery for primary HCC. The metastasis sites included bone (6 patients), abdominal and chest wall (5 patients), brain (2 patients), mesentery (1 patient), gall bladder (1 patient), peritoneum (1 patient), adrenal gland (1 patient), and retroperitoneum (1 patient). The paraffin-embedded surgical specimens composed of the primary and metastatic tumor and surrounding noncancerous liver parenchyma underwent pathology examination. Pathologic features, including Barcelona Clinic Liver Cancer staging,[29] and clinical outcomes were analyzed. This study was approved by the Institutional Review Board of Taichung Veterans General Hospital.

Immunohistochemical Analysis

For immunohistochemistry analysis, an automatic immunostaining device and ultraView detection kit (Ventana XT Medical System, Tucson, AZ) was used to detect 14-3-3β expression in paraffin-embedded tissues by use of a primary antibody against 14-3-3β (1:800; Santa Cruz Biotechnology, Santa Cruz, CA). A negative control was prepared by the same staining procedure but not incubated with the primary antibody. The intensity of 14-3-3β protein staining was scored semiquantitatively by a Quick-score (Q-score) method based on intensity and heterogeneity.[30–34] Staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). For heterogeneity, the proportion of tumor cells positively stained with 14-3-3β was scored as follows: 0 (0%), 1 (1% to 25%), 2 (26% to 50%), 3 (51% to 75%), and 4 (76% to 100%). The Q-score of a given tissue sample was the sum of the intensity and heterogeneity scores and ranged from 0 to 7. The scoring of each sample was evaluated independently and blindly by 2 pathologists. A Q-score of 2 or greater was considered overexpressed or positive 14-3-3β expression, and a Q-score of less than 2 was considered normal or negative 14-3-3β expression. Some rare cases with less than 5% weakly stained specimens were considered negative expression.

Cell Culture, Stable Cells, and Reagents

Huh-7 human hepatoma cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone Thermo Fisher Scientific, Waltham, MA), 100 U/mL penicillin, and 100 U/mL streptomycin, in a humidified incubator with 5% CO_2 at 37°C. For stable transfection, 14-3-3β cDNA was amplified by PCR and then subcloned into the p3XFlag-CMV vector. Huh-7 cells were transfected with p3XFlag-CMV (control) and 14-3-3β cDNA (14-3-3β) by use of Polyjet transfection reagent (SignaGen Laboratories, Ijamsville, MD) according to the manufacturer's instructions. The transfected cells were screened with G418 (500 μg/mL; Biochrom AG, Berlin, Germany) for 4 weeks. Single colonies of stable clones

(at least 3 in each cell line) were maintained in DMEM with 10% FBS and 200 μg/mL G418. The established stable cell lines were confirmed by Western blot analysis of Flag expression. The Mek1/2 inhibitor U0126 was purchased from Sigma-Aldrich (St. Louis, MO).

Western Blot Analysis

Stable 14-3-3β overexpression and control cells were lysed in ice-cold radioimmunoprecipitation assay buffer (0.5 mol/L Tris-HCl, pH 7.4, 1.5 mol/L NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mmol/L EDTA; Millipore, Temecula, CA) containing cocktail protease inhibitors (Roche, Indianapolis, IN). Cell lysates were clarified by centrifugation at 16,100 $\times$ *g* for 20 minutes at 4°C. Protein concentrations were determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). In total, 20 μg protein of each sample was applied to a gradient SDS-PAGE gel and immunoblotted onto polyvinylidene difluoride membranes. The membranes were blocked for 1 hour in PBST (phosphate buffered saline with Tween 20, 0.1% Tween 20, 2.67 mmol/L KCl, 1.47 mmol/L KH_2PO_4, 137.93 mmol/L NaCl, 8.1 mmol/L Na_2HPO_4, pH 7.4) containing 5% nonfat dry milk. Membranes were incubated with primary antibodies against Flag (Sigma-Aldrich), Erk1/2, and phosphorylated-Erk1/2 (Cell Signaling, Danvers, MA) overnight and washed 3 times with PBST for 5 minutes. The membranes were immersed in PBST containing horseradish-peroxidase–conjugated secondary antibody for 1 hour and protein levels were determined by use of enhanced chemiluminescence reagents.

Immunofluorescence Staining

Stable 14-3-3β overexpression and control cells grown on glass coverslips were fixed in 2% paraformaldehyde for 15 minutes at 4°C. After washing, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and blocked with PBS containing 10% FBS at room temperature for 1 hour. Cells were incubated with 14-3-3β antibodies in PBS containing 1% FBS at 4°C overnight, then incubated with rhodamine-conjugated donkey anti-mouse secondary antibody in PBS containing 5% bovine serum albumin at room temperature for 2 hours. Samples were mounted in mounting medium (IBIDI, Martinsried, Germany), and images were analyzed using the Leica TCS SP5 Confocal Imaging System (Leica, Wetzlar, Germany).

Migration Assay

A Boyden chamber with Bio-coat cell migration chambers was used for cell migration assay (Becton Dickinson, Pont-de-Claix, France) as described previously.[35,36] Briefly, cells were trypsinized and suspended in 0.1% bovine serum albumin–DMEM and then 6 $\times$ 10^4 cells were added to the upper wells, which had 8-μm pores. Cells were allowed to migrate toward the bottom wells containing 100 μg/mL fibronectin (Becton Dickinson), transforming growth factor β (20 ng/mL), and 10% bovine serum albumin–DMEM for 20 hours. Cells remaining on the upper side were removed, and migrated cells on the bottom side were fixed and stained with 0.1% crystal violet containing 20% ethanol and 1% formaldehyde for 20 minutes. Cell migration was quantified by counting the total number of migrated cells in 3 inserts.

Cell Proliferation Assay

Cell proliferation was measured by MTT assay.[37,38] In brief, 1,500 cells were seeded onto the flat bottom of 96-well plates in 200 μL serum-free medium. After cells attached, the medium was replaced with fresh medium containing 10% FBS for incubation at the indicated times (0, 24, 48, 74 hours). MTT was added to each well and incubated at 37°C for 3 hours. Subsequently, the yellow MTT solution was removed and the produced formazan was solubilized by adding 200 μL dimethyl sulfoxide. Absorbance at 570 nm was measured with a reference wavelength of 690 nm.

Quantitative Real-Time PCR

Total RNA was extracted by use of the RNAspin Mini Kit (GE Health Care, Freiburg, Germany). cDNA was synthesized from 2 to 5 μg RNA by use of the oligo$(dT)_{18}$ primers and RevertAid First Strand cDNA Synthesis Kit (Fermentas Thermo Fisher Scientific, Waltham, MA). Quantitative real-time PCR involved using SYBR Green (Kapabiosystem, Woburn, MA) with specific oligonucleotide primers: E-cadherin, forward: 5′-CGAGAGCTACACGTTCACGG-3′, reverse: 5′-GGCCTTTTGACTGTAATCACAML-3′; cyclin A, forward: 5′-TGAACTACATTGATAGGTTCCTGT-3′, reverse: 5′-TGACTGTTGTGCATGCTGTGGTGC-3′; cyclin D, forward: 5′-GCCAACCTCCTCAACGACCGG-3′, reverse: 5′-GTCCATGTTCTGCTGGGCCTG-3′; cyclin E, forward: 5′-ATAATGCAGTCTGTGCAGAC-3′, reverse: 5′-GTTGTGTGCATCTTCATCAG-3′; Snail, forward: 5′-AATCGGAAGCCTAACTACAGCG-3′, reverse: 5′-GTCCCAGATGAGCATTGGCA-3′, from the AB 7900HT system (Applied Biosystems, Carlsbad, CA). Applied Biosystems Relative Quantification Manager Software version 1.2 was used to analyze the relative gene expression in each sample by the comparative cycle threshold (Ct) method. Gene expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase.

Anchorage-Independent Growth Assay

Anchorage-independent growth was assessed by soft-agar assay.[39,40] In brief, 5 $\times$ 10^3 cells were seeded onto 6-well plates with 2 mL complete DMEM containing 0.8% low-melting agarose (Lonza, Rockland, ME), then overlaid with 2 mL complete DMEM containing 0.4% low-melting agarose. Cells were overlaid with additional complete medium every 6 days. After 3 weeks, cells were stained with 0.005% crystal violet in 25% methanol. Colony numbers were counted and representative images were captured.

Knockdown of 14-3-3β by siRNA

Scramble siRNA (sc-37007) and small-interfering RNA (siRNA) targeting 14-3-3β (sc-29186) were purchased from Santa Cruz Biotechnology. For siRNA transfection experiments, 14-3-3β overexpressed and control cells were dissociated into single cells in suspension and plated on 6-well plates. Cells were transfected with scramble and 14-3-3β siRNA according to the manufacturer's protocol. Briefly, 14-3-3β and scramble siRNAs (30 pmol) were diluted in 500 μL DMEM and mixed with 5 μL Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). After 15 minutes' incubation at room temperature, the complexes were added to the cells with a final volume of 3 mL medium. Cells were harvested at the indicated time for further analysis. The efficiency of the 14-3-3β siRNA was confirmed by Western blot analysis of Flag expression.

Tumor Xenograft Experiments

BALB/c nu/nu nude mice (8 weeks old) were purchased from the National Laboratory Animal Center (National Science Council, Taiwan), and housed in microisolator cages at a specific pathogen-free facility. Control and 14-3-3β stable cells were trypsinized, washed, and resuspended in PBS. A total of 2×10^6 cells in 0.3 mL PBS were injected subcutaneously into the right flank of nude mice.[41,42] Tumor development was followed up in individual animals twice a week. Tumor volume was determined by sequential caliper measurements of length (L) and width (W) and calculated as $LW^2/2$. Mice were sacrificed at day 35, tumors were removed, and tumor weight was measured. The protocol of the *in vivo* experiments was approved by the Institutional Animal Care and Utilization Committee of Academia Sinica.

Statistical Analysis

One-way analysis of variance was used to analyze differences among clinicopathologic variables by 14-3-3β expression. Factors that influence extrahepatic metastasis were analyzed by multivariate logistic regression. The Wilcoxon signed-rank test was used to analyze the differences between primary tumors and matched metastatic tissues by 14-3-3β staining density. Kaplan-Meier curves were plotted, and the log-rank test was used to analyze time-related probabilities of metastasis, overall survival, and progression-free survival, and to calculate *P* value. Cox proportional hazard regression models were used to evaluate the impact of prognostic factors on survival. The Student's *t*-test was used to analyze differences among 2 groups. A *P* value of <0.05 was considered statistically significant, and a *P* value between 0.05 and 0.10 was considered marginally significant.

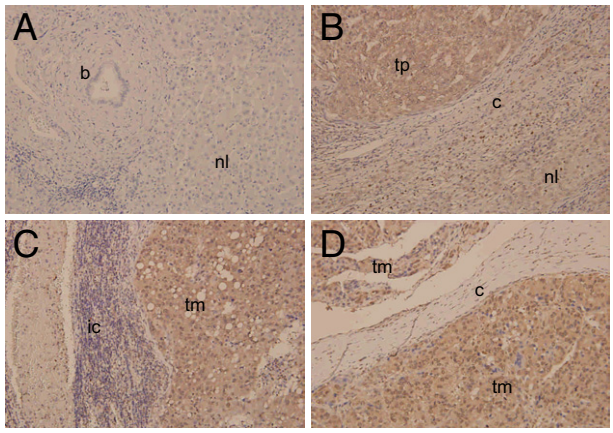

Figure 1. Immunohistochemical analysis of 14-3-3β in primary and metastatic HCC tissues. **A:** Negative control staining. Original magnification, ×200. **B:** 14-3-3β staining in representative primary HCC. Original magnification, ×200. Tumor cells were stained positively. **C:** Positive staining of 14-3-3β in representative metastatic HCC lesions in lung gallbladder (**D**). Original magnification, ×200. b, bile duct; c, capsule; ic, inflammatory cells; nl, normal liver; tm, metastatic tumors; tp, primary tumors.

Results

Overexpression of 14-3-3β in Primary and Metastatic HCC Tissues

To examine the expression of 14-3-3β in HCC, paraffin-embedded primary HCC tumors with surrounding noncancerous parenchyma from 55 patients and 18 matched extrahepatic metastatic tumors underwent immunohistochemical staining. 14-3-3β was not detectable or stained with background in noncancerous liver tissues (data not shown), and negative control slides were stained negatively (Figure 1A). The expression of 14-3-3β was increased in 40 (72.7%) of 55 primary HCC tumors, and noncancerous cells adjacent to tumors were stained negatively (Figure 1B and Table 1). Moreover, 14-3-3β was overexpressed in 18 matched metastatic HCC specimens, as illustrated representatively in a metastatic lesion in the lung (Figure 1C) and gall bladder (Figure 1D). Overexpression of 14-3-3β was not related significantly to most clinicopathologic characteristics but was associated, although weakly, with advanced Barcelona Clinic Liver Cancer stage ($P = 0.076$), tumor size ($P = 0.081$), presence of microvascular thrombi ($P = 0.058$), and high α-fetoprotein level ($P = 0.098$) (Table 1).

Association of 14-3-3β Overexpression and Extrahepatic Metastasis in HCC

Overexpression of 14-3-3β in primary HCC tumors was associated significantly with subsequent extrahepatic metastasis ($P = 0.002$) (Table 1). On multivariate analysis, overexpression of 14-3-3β was the only factor predicting distant HCC metastasis ($P < 0.001$) (Table 2). The cumulative probability of developing extrahepatic metastasis within 5 years was significantly higher in primary HCC with positive versus negative 14-3-3β expression (39.9% ± 7.9% versus 0.0%; $P = 0.008$) (Figure 2).

Table 1. Correlation of 14-3-3β Positivity with Clinicopathologic Characteristics in Primary Tumors of HCC Patients.

Parameters	14-3-3β positivity (Q-score ≥2)% (n)	*P* value
Overall (*n* = 55)	72.7% (40)	
Age		NS
≥60 years (*n* = 28)	71.4% (20)	
<60 years (*n* = 27)	74.1% (20)	
Sex		NS
Male (*n* = 39)	71.8% (28)	
Female (*n* = 16)	75.0% (12)	
Histologic grade		NS
1 (*n* = 4)	50.0% (2)	
2 (*n* = 42)	73.8% (31)	
3 (*n* = 9)	77.8% (7)	
Types of surgery		NS
Core biopsy (*n* = 3)	100.0% (3)	
Wedge resection (*n* = 18)	55.6% (10)	
Segmentectomy (*n* = 28)	75.0% (21)	
Lobectomy (*n* = 6)	100.0% (6)	
Surgical margin		NS
Not available (*n* = 3)	100.0% (3)	
Free (*n* = 41)	70.7% (29)	
Involved (*n* = 11)	72.7% (8)	
BCLC staging		0.076
Not available (*n* = 3)		
Early (stage A1 to A4) (*n* = 29)	65.5% (19)	
Intermediate (stage B) (*n* = 21)	90.5% (19)	
Advanced (stage C) (*n* = 2)	100.0% (2)	
Tumor size		0.081
Not available (*n* = 2)	100.0% (2)	
≥5.0 cm (*n* = 12)	91.7% (11)	
<5.0 cm (*n* = 41)	65.9% (27)	
Tumor multiplicity		NS
Not available (*n* = 1)	100.0% (1)	
Single (*n* = 42)	69.0% (29)	
Multiple (*n* = 12)	83.3% (10)	
Capsular formation		NS
Not available (*n* = 11)	72.7% (8)	
Yes (*n* = 23)	78.3% (18)	
No (*n* = 21)	66.7% (14)	
Vascular thrombi		0.058
Not available (*n* = 3)	100.0% (3)	
Yes (*n* = 17)	88.2% (15)	
No (*n* = 35)	62.9% (22)	
Cirrhosis		NS
Not available (*n* = 6)	66.7% (4)	
Yes (*n* = 33)	72.7% (24)	
No (*n* = 16)	75.0% (12)	
Viral hepatitis		NS
Not available (*n* = 4)	50.0% (2)	
Hepatitis B (*n* = 28)	75.0% (21)	
Hepatitis C (*n* = 17)	70.1% (12)	
Both (*n* = 5)	80.0% (4)	
None (*n* = 1)	100.0% (1)	
α-Fetoprotein level		0.098
Not available (*n* = 7)	42.9% (3)	
≥ 80 ng/mL (*n* = 19)	89.5% (17)	
< 80 ng/mL (*n* = 29)	69.0% (20)	
Subsequent extrahepatic metastasis		0.002*
Yes (*n* = 18)	100.0% (18)	
No (*n* = 37)	59.4% (22)	

*$P < 0.05$.
BCLC, Barcelona Clinic Liver Cancer; NS, not significant; SD, standard deviation.

Table 2. Multivariate Analysis for Distant Metastasis in HCC Patients

Variables	*P* value
Histology grade (1 + 2: 3)	NS
Presence of cirrhosis (no: yes)	NS
14-3-3β expression (negative: positive)	<0.001*
Bulky tumor (≥5.0 cm: <5.0 cm)	0.095
Surgical margin (free: involved)	NS
Capsular formation (no: yes)	NS
Vascular thrombi (no: yes)	NS
BCLC staging (stage A: B to C)	NS

*$P < 0.05$.
BCLC, Barcelona Clinic Liver Cancer; NS, not significant.

Furthermore, the expression of 14-3-3β was increased significantly in metastatic HCC samples versus their primary tumors (11 with increased Q-score >2, 1 decreased, and 6 with no difference in Q-score; $P = 0.004$). These observations suggest a strong correlation of 14-3-3β expression with extrahepatic metastasis of HCC.

Association of 14-3-3β Overexpression with Patient Survival

After a mean follow-up period of 58.6 ± 28.6 months, 19 patients (34.5%) remained free of HCC, 21 patients (38.2%) had died of their disease, and 15 patients (27.3%) were still alive with disease recurrence and/or distant metastasis. Survival analysis revealed a significantly better overall survival rate in patients with negative versus positive 14-3-3β expression in primary HCC tumors (61.5% ± 7.8% versus 78.6% ± 11.0% for 5-year survival; $P = 0.046$) (Figure 3). However, the increased expression of 14-3-3β in primary tumors had no significant effect on progression-free survival in these HCC patients (data not shown). Furthermore, Cox proportional-hazard regression models revealed that overexpression of 14-3-3β significantly compromised the probability of overall survival with a hazard ratio of 3.135 (95% confidence interval, 1.103 to 9.804; $P = 0.043$) but had no

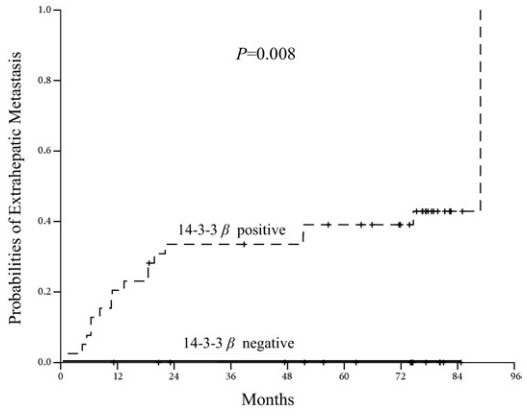

Figure 2. Kaplan-Meier analysis of 14-3-3β expression with metastatic risk of HCC patients. The 5-year cumulative probabilities of metastasis for 14-3-3β–negative patients was significantly lower than for 14-3-3β–positive patients (39.9% ± 7.9%; $P = 0.008$).

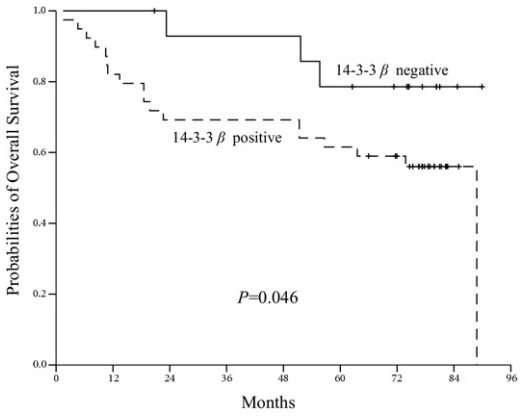

Figure 3. Kaplan-Meier analysis of 14-3-3β expression with overall survival of HCC patients. Patients with 14-3-3β positivity in primary tissues had a significantly worse 5-year overall survival rate than negative-stained tissues (61.5% ± 7.8% versus 78.6% ± 11.0%; $P = 0.046$).

effect on progression-free survival (Table 3). These results suggest that overexpression of 14-3-3β in primary tumors is an important predicting parameter of worse overall survival of HCC patients.

Regulation of Cell Migration and Proliferation by 14-3-3β Overexpression in HCC

To explore the effect of 14-3-3β on cancer cell migration, proliferation, and tumor progression, we established a stable HCC cell line with 14-3-3β overexpression. Huh-7 cells, determined to express a lower protein level of 14-3-3β (data not shown), were transfected with p3XFlag-CMV (control) and p3XFlag-14-3-3β (14-3-3β) vectors and selected by G418 for 4 weeks. Protein expression was validated by Western blot analysis of Flag in cells from 14-3–3β-Flag stably overexpressed clones (Figure 4A). Clone 1 of control cells and 14-3-3β stable cells were used for most further experiments, and the expression and the subcellular localization of 14-3-3β was confirmed by immunofluorescent confocal microscopy (Figure 4B). Expression of 14-3-3β was increased significantly and the majority of 14-3-3β protein was localized to the cytoplasm but partially to the cytoplasmic membrane or nucleus (Figure 4B). In addition, overexpression of 14-3-3β (stable clones 1 to 3) significantly induced cell migration in Boyden chamber experiments (Figure 4C).

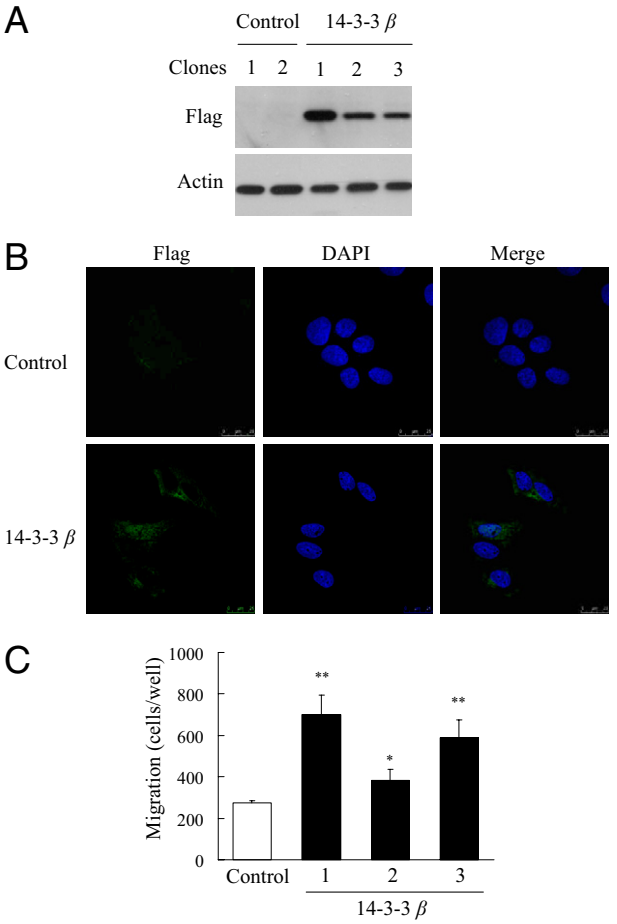

Figure 4. Overexpression of 14-3-3β increases HCC cell migration. **A:** Overexpression of 14-3-3β in stable HCC cells confirmed by Western blot analysis. Control, stable cell clones of empty vector (p3XFlag); 14-3-3β, stable clones of p3XFlag-14-3-3β. **B:** Overexpression of 14-3-3β and its subcellular localization examined by immunofluorescent confocal microscopy. Nuclei were stained with DAPI. Scale bar = 25 um. **C:** Cell migration ability was determined by Boyden chamber assay. Scale bars: mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, 14-3-3β versus control.

To investigate whether 14-3-3β plays an important role in cancer cell proliferation, we determined the mRNA levels of several cell-cycle regulatory factors and found the expression of cyclin A, cyclin D, and cyclin E were increased significantly in 14-3-3β–overexpressed cells (stable clones 1 to 3; Figure 5A). 14-3-3β overexpression inducing HCC cell proliferation was confirmed further by MTT assay (Figure 5B). Moreover, we determined an-

Table 3. Cox Proportional Hazard Regression Analysis for Survival in HCC Patients

Variables	Overall survival		Progression-free survival	
	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
14-3-3β expression (positive: negative)	3.135 (1.103 to 9.804)	0.043*	1.838 (0.311 to 10.870)	0.502
Types of surgery (wedge: wider resection)	1.232 (0.230 to 6.613)	0.807	0.950 (0.130 to 6.952)	0.959
Surgical margin (free: involved)	0.704 (0.191 to 2.599)	0.598	0.686 (0.179 to 2.621)	0.686
Capsular formation (no: yes)	0.883 (0.313 to 2.490)	0.814	0.522 (0.144 to 1.894)	0.522
α-Fetoprotein level (low: high)	0.322 (0.108 to 0.962)	0.049*	0.301 (0.086 to 1.056)	0.061
Cirrhosis (no: yes)	0.563 (0.205 to 1.547)	0.266	0.336 (0.079 to 1.433)	0.140
BCLC stage (A: B and C)	1.247 (0.447 to 3.301)	0.656	1.309 (0.355 to 4.823)	0.686

*$P < 0.05$.
BCLC, Barcelona Clinic Liver Cancer; CI, confidence interval; SE, standard error.

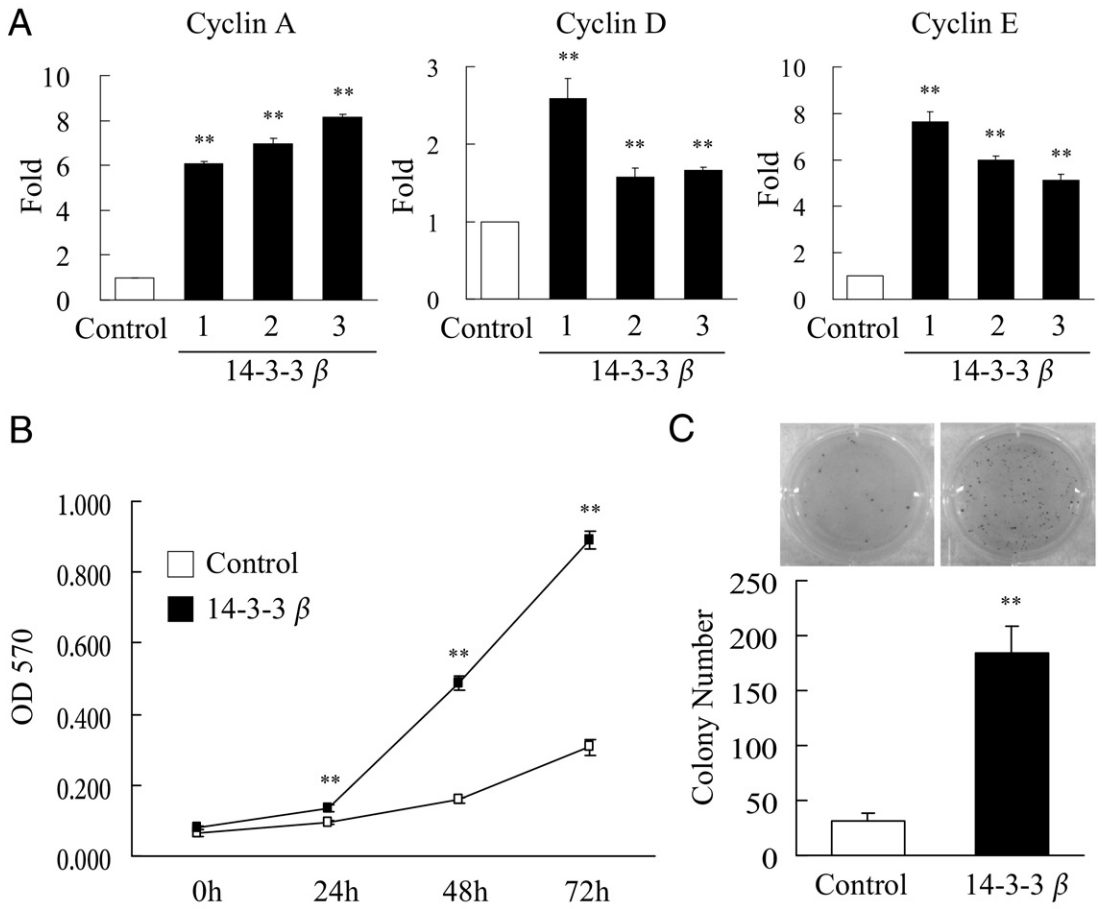

Figure 5. Overexpression of 14-3-3β promotes cell proliferation. **A:** Expression of cyclin A, cyclin D, and cyclin E in control and 14-3-3β stable cells determined by quantitative real-time PCR. **B:** Cell proliferation determined by MTT assay. **C:** Anchorage-independent growth was determined by soft-agar assay. Representative photographs are shown (**upper panel**). Quantitative analysis of total colony number in each culture well from 3 separate experiments (**bottom panel**). Scale bars: mean ± SD. $^{**}P < 0.01$, 14-3-3β1 versus control.

chorage-independent growth with 14-3-3β overexpression by soft-agar assay. 14-3-3β–overexpressed cells significantly increased the colony numbers formed in the soft agar as compared with control cells (Figure 5C). These results suggest that 14-3-3β induces HCC cell proliferation and promotes anchorage-independent growth.

To further confirm the role of 14-3-3β on HCC cell proliferation and migration, we performed the knockdown experiments by using siRNA. Control and 14-3-3β–overexpressed cells were transfected with scramble or 14-3-3β siRNAs and expression of 14-3-3β was determined by Western blot analysis. 14-3-3β siRNA significantly reduced the expression level of Flag-tagged 14-3-3β (Figure 6A). 14-3-3β siRNA suppressed HCC cell proliferation and cell migration was confirmed by MTT assay (Figure 6B) and Boyden chamber experiments (Figure 6C), respectively.

Effect of MAPK on 14-3-3β–Induced HCC Cell Proliferation and Migration

To investigate whether activation of MAPK was involved in 14-3-3β–induced HCC cell proliferation and migration, we pretreated cells with U0126, a Mek1/2 inhibitor, and determined the expression level of Erk1/2 phosphorylation by Western blot analysis. Phosphorylated Erk1/2 was enhanced significantly in 14-3-3β–overexpressed HCC cells (Figure 7A). U0126 completely abrogated phosphorylation of Erk1/2 in both control and 14-3-3β cells (Figure 7A). We next determined the effect of U0126 on 14-3-3β–induced HCC cell growth and migration by MTT and Boyden chamber assays. The increased effects of cell proliferation and migration by 14-3-3β were abolished significantly by U0126 (Figure 7, B and C). These results suggest that 14-3-3β promotes HCC progression through activation of a MAPK signal pathway.

Effect of 14-3-3β Overexpression on in Vivo *Tumor Growth*

The *in vivo* xenograft model was used to test the effect of 14-3-3β on HCC tumor growth. Control and 14-3-3β–overexpressed Huh-7 cells were injected into nude mice for 5 weeks, and tumor volumes and tumor weights were determined. Tumors sizes were significantly larger in nude mice with 14-3-3β overexpression than in control

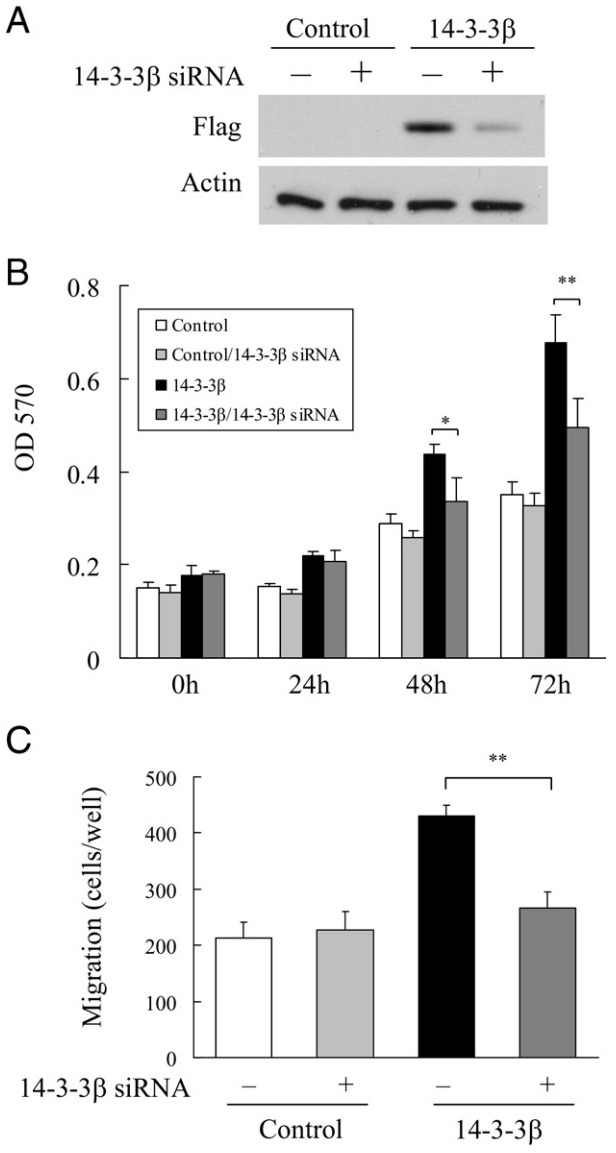

Figure 6. Knockdown 14-3-3β expression by siRNA abolishes 14-3-3β–induced cell proliferation and migration. **A:** 14-3-3β siRNA reduced 14-3-3β expression was determined by Western blot analysis of Flag. Cells were transfected by scramble or 14-3-3β siRNA for 48 hours. **B:** 14-3-3β siRNA abrogated 14-3-3β–induced HCC cell proliferation was determined by MTT assay. **C:** Cell transfected with scramble or 14-3-3β siRNA for 48 hours and migration ability was determined by Boyden chamber assay. Scale bars: mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

mice (Figure 8A). 14-3-3β overexpression significantly increased tumor volume (Figure 8B) and tumor weight as compared with control cells (Figure 8C). These results suggest that increased 14-3-3β expression promotes *in vivo* HCC tumor growth.

Discussion

14-3-3β has been implicated in cell survival, proliferation, migration, and tumor growth, but its clinical relevance in terms of tumor progression and metastasis has never been elucidated. In this study, we provide strong clinical evidence that increased 14-3-3β expression is related significantly to subsequent extrahepatic metastasis and overall survival in HCC patients. Notably, none of the patients with negative 14-3-3β expression in primary HCC tissues has shown distant metastasis. On multivariate analysis, 14-3-3β overexpression was an independent predicting factor of HCC metastasis. Furthermore, significantly increased 14-3-3β expression in metastatic HCC tissues implied a potential biological role in the development of distal metastatic lesions. Thus, 14-3-3β may be a critical regulator of HCC metastasis. In addition, increased 14-3-3β expression in primary tissues was associated significantly with poor overall survival of HCC patients. Interestingly, overexpression of 14-3-3β was not associated significantly with other clinicopathologic variables in HCC, which suggests that 14-3-3β may not contribute to early HCC carcinogenesis but may have an

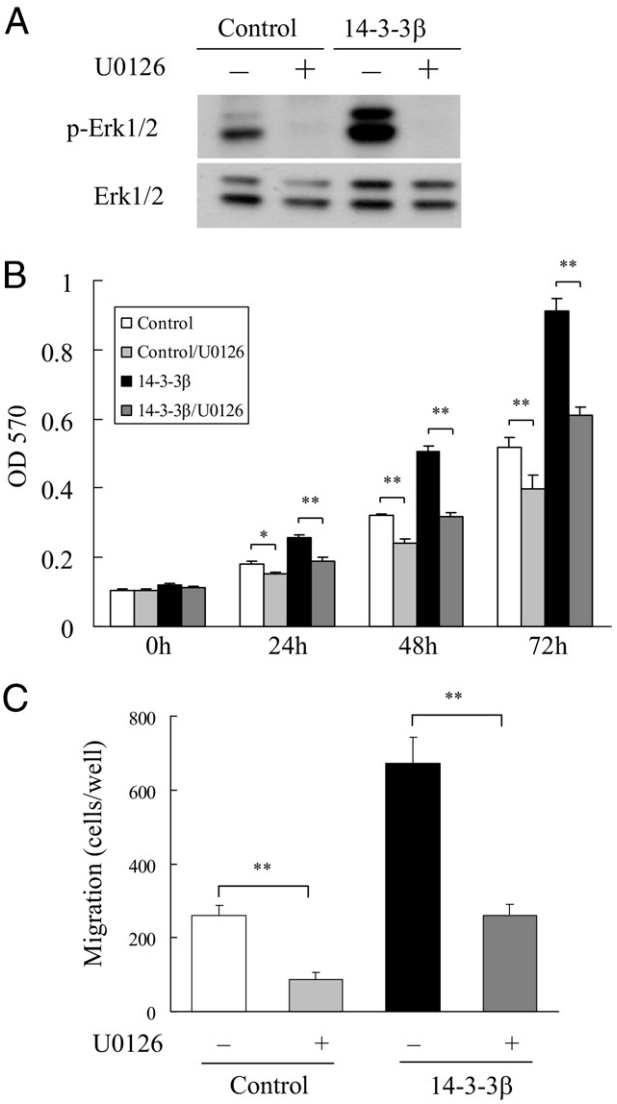

Figure 7. Effect of MAPK on 14-3-3β–induced cell proliferation and migration. **A:** Control and 14-3-3β–overexpressed cells were treated with 10 μmol/L U0126 for 1 hour. Expression of Erk1/2 and phosphorylated-Erk1/2 were determined by Western blot analysis. **B:** U0126 abrogated 14-3-3β–induced cell proliferation was determined by MTT assay. **C:** U0126 suppressed cell migration was determined by Boyden chamber assay. Scale bars: mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

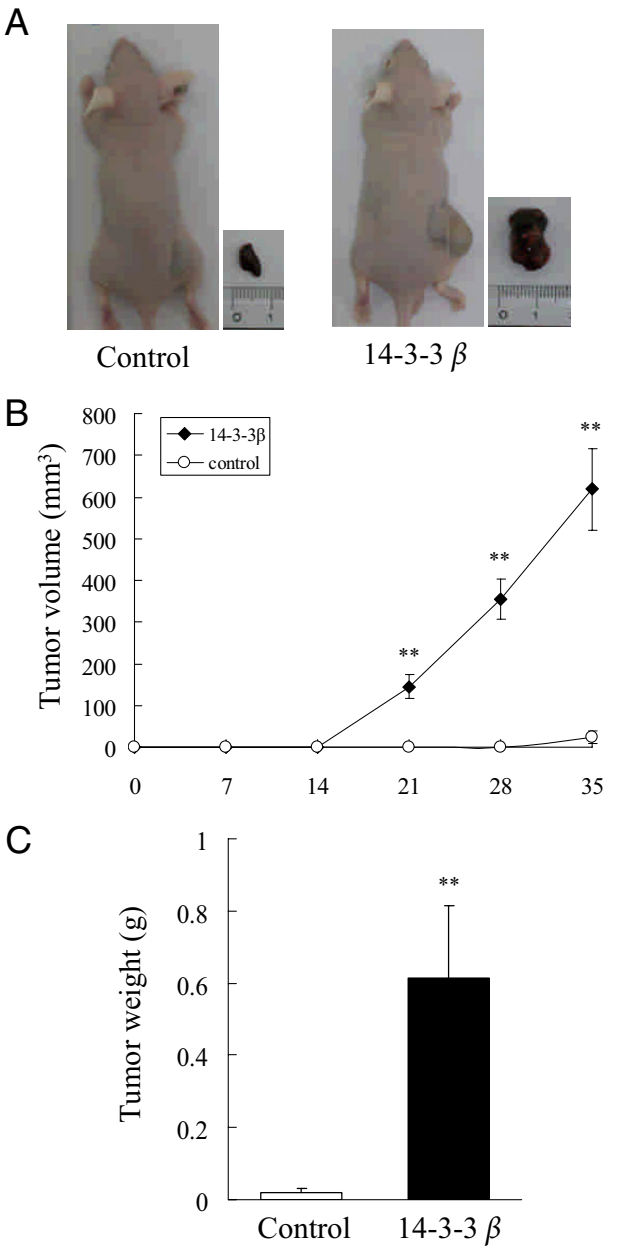

Figure 8. Overexpression of 14-3-3β promotes *in vivo* tumor growth. **A:** Representative photographs of nude mice and developed tumors injected with control (**left panel**) and 14-3-3β overexpressed (**right panel**) stable cells at day 35. Overexpression of 14-3-3β significantly increased *in vivo* tumor growth according to the results of calculated tumor volume (**B**) and tumor weight (**C**). Scale bars: mean ± SEM. $^{**}P < 0.01$.

important role in promoting advanced tumor progression, especially metastasis. In support of these data, our *in vitro* and *in vivo* xenograft studies provide additional evidence that 14-3-3β is tightly involved in the advanced development of HCC. Forced overexpression of 14-3-3β induced HCC cell migration and increased cell proliferation, as well as tumor growth. Thus, 14-3-3β may be a potential biomarker for distant metastasis and poor survival with HCC.

Our recent studies discovered that the expression of 14-3-3ε and 14-3-3γ also was increased in a substantial portion of HCC tissues and was associated with distant metastasis and overall survival.[33,34] These results, along with the dimerization property of 14-3-3 proteins, suggests that 14-3-3β, 14-3-3ε, and 14-3-3γ potentially form heterodimers to interact with and regulate their broad-spectrum downstream signal effectors in HCC tissues. These 14-3-3 isoforms may express and work synergistically to promote HCC cell migration/invasion, proliferation, and tumor metastasis. Our findings provide a better understanding of the isoform-specific expression profile of 14-3-3 proteins in distinct tumor tissues. Further work is ongoing to investigate the synergism of 14-3-3 isoforms and their mechanistic interacting factors to control advanced HCC metastasis and progression.

14-3-3β has been shown to bind with phosphorylated Raf-1 and to activate Raf-1 kinase activity.[43,44] Thus, 14-3-3β may contribute to control cell proliferation and differentiation by regulating Raf-1 and its downstream signal pathway factors. An earlier study showed that overexpression of 14-3-3β promotes cell proliferation and oncogenic transformation.[25] Forced 14-3-3β overexpression induced MAPK activity and stimulated NIH3T3 cell growth, anchorage-independent growth, and tumor formation in nude mice.[25] These induced effects of cell proliferation could be abolished by expression of a dominant-negative mutant of MEK1.[25] In addition, a novel 14-3-3β–interacting protein, 14-3-3β interactant 1 (FBI1), was identified by yeast two-hybrid screening.[45] 14-3-3β bound to and formed a complex with FBI1 and transcriptionally suppressed the expression of MAPK phosphatase-1.[45] 14-3-3β was thus considered a transcriptional repressor to promote tumorigenicity and metastasis.[45] Moreover, 14-3-3β was reported to regulate the mammalian target of rapamycin pathway by interacting with and suppressing the activity of the tumor-suppressor tuberous sclerosis complex 2.[46,47] Interaction and sequestration of the tuberous sclerosis complex 2 by 14-3-3β reduced the binding activity of tuberous sclerosis complex 2 with the mammalian target of rapamycin complex, thereby enhancing mammalian target of rapamycin activity and its subsequent signal pathways to control cell proliferation. These results provide insight into the molecular mechanisms of 14-3-3β controlling cell signaling to enhance cancer cell growth.

14-3-3β deregulation was suggested to be associated negatively with tumor growth,[26] and our current work shows that 14-3-3β overexpression promotes HCC cell growth and migration. However, the clinical significance of 14-3-3β expression with HCC tumor metastasis has never been shown. 14-3-3β was found to be associated with the integrin β1 cytoplasmic domain during the early stage of cell migration, and overexpressed 14-3-3β enhanced cell spreading and migration of NIH3T3 cells.[48] Results from a further study suggested that 14-3-3β–regulated cell spreading and migration requires a functional amphipathic groove.[49] In addition, our earlier study revealed focal adhesion kinase, a regulator of nonreceptor tyrosine kinase to control cell adhesion and migration, was overexpressed in HCC tumors.[32] Thus, 14-3-3β may combine with integrin β1 and focal adhesion kinase to form a complex to induce HCC cell migration and tumor metastasis. Moreover, 14-3-3β stimulated

Ras-related C3 botulinum toxin substrate 1 (Rac1)-p21–activated kinase signaling to regulate Akt-mediated cytoskeletal organization, lamellipodia formation, and fibronectin matrix assembly.[50] Taken together, these results have revealed an important role of 14-3-3β in controlling cell migration/invasion and metastasis by affecting integrins or cytoskeletal-regulated signaling pathways.

14-3-3 proteins, including 14-3-3β, exert antiapoptotic activity by binding with the proapoptotic Bad protein.[6,51–54] 14-3-3 proteins interacts with phosphorylated Bad, sequesters Bad in the cytosol, and prevents its mitochondrial translocation, thereby suppressing the mitochondria-mediated apoptosis pathway.[6,51–54] Furthermore, 14-3-3β was reported to be the binding partner of serine/arginine protein-specific kinase 2.[55] 14-3-3β bound to Akt-phosphorylated serine/arginine protein-specific kinase 2 to protect it against nuclear translocation, protein degradation, and apoptotic cell death.[55] Moreover, Akt-mediated phosphorylation was found to trigger 14-3-3β–dependent re-localization of S-phase kinase-associated protein 2 to the cytosol to contribute to tumorigenesis.[56]

In conclusion, our study provides strong clinical evidence that 14-3-3β is an important and independent factor predicting subsequent distant metastasis and worse survival for patients with operable HCC. Our results imply that 14-3-3β is involved in advanced disease progression in HCC. Therefore, 14-3-3β may be a potential biomarker to identify HCC patients at high risk of metastasis. New therapeutic strategies or drugs aimed at 14-3-3β or other 14-3-3 isoforms and potential targets might be developed for these patients.

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