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Functional and Clinical Evidence That TAZ is a Candidate Oncogene in Hepatocellular Carcinoma

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ABSTRACT

Transcriptional co-activator with PDZ-binding motif (TAZ) has been reported to be associated with carcinogenesis. However, the cellular function of TAZ in human hepatocellular carcinoma (HCC) remains elusive. In this study, an immunohistochemistry analysis revealed that the expression of TAZ in cancer tissue samples from 180 HCC patients was significantly higher than that in adjacent normal tissues. In addition, TAZ overexpression was significantly correlated with aggressive tumor characteristics such as tumor size, TNM stage, lymph node or distant metastasis, histological differentiation, and recurrent HCC ($P < 0.05$). The Kaplan–Meier test showed that TAZ-positive expression was related to a poor prognosis compared to TAZ-negative expression ($P < 0.05$). Furthermore, the expression level of TAZ was generally correlated with the invasiveness of cancer cells. The overexpression of TAZ in the Huh7 cell line, which endogenously expresses TAZ at low levels, significantly promoted cell proliferation, migration and invasion and inhibited apoptosis, whereas RNA interference-mediated knockdown of TAZ in the highly invasive cell line MHCC-97H significantly suppressed cell proliferation, migration and invasion in vitro and tumor formation in vivo. J. Cell. Biochem. 116: 2465–2475, 2015.

Taken together, these results suggest that TAZ plays an important role in the proliferation, apoptosis, migration and invasion of HCC cells and that the expression of TAZ in HCC patients is closely related to their prognosis. Thus, TAZ is a potential novel biomarker for predicting prognosis and guiding personalized therapeutic strategies.

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, representing 85% of liver cancers, which are the third leading cause of cancer-related death due to their poor prognosis [Block et al., 2010; Washburn and Halff, 2011]. In recent years, the incidence of HCC has been increasing in developed countries, although considerable progress has been made in diagnostic and therapeutic modalities [Luk and Liu, 2011]. The overwhelming majority of HCCs are caused by exposure to aflatoxins or infection with hepatitis B and C viruses. It has been shown that small-size HCCs can be cured by surgical resection or liver transplantation. Unfortunately, this disease is often diagnosed at an advanced stage, when conventional and effective treatment

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options become unavailable [Kudo, 2011; Lencioni and Crocetti, 2012]. Therefore, studying the molecular pathogenesis of HCC can guide our evaluation of diagnostic and prognostic markers for the treatment and prognosis of patients. Although many molecular markers, including alpha-fetoprotein (AFP), gamma-glutamyltransferase (GGT), insulin-like growth factor (IGF), and transforming growth factor (TGF), have been exploited for detecting HCC, these molecular markers lack sensitivity and specificity for evaluating the diagnosis and prognosis of HCC patients [Masuda and Miyoshi, 2011; Tanaka and Arii, 2011]. Thus, there is an urgent demand for research into novel molecular markers. The diagnosis and prognosis of HCCs with different biological behaviors will benefit from the identification of novel tumor markers with a higher specificity and sensitivity. This information will also be helpful for improving clinical strategies for treating HCC and the outcomes of this disease.

The Hippo pathway plays an important role in cell proliferation, organ size control, and cancer development and progression [Pan, 2010; Zhao et al., 2010; Chan et al., 2011]. YAP (Yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif) are both transcriptional co-activators that are inhibited by the Hippo pathway [Kanai et al., 2000; Lian et al., 2010]. When Hippo signaling is attenuated, the phosphorylation of YAP/TAZ is reduced, leading to its nuclear localization. Inside the nucleus, YAP/TAZ binds to various transcription factors to activate an array of target genes that are involved in cell proliferation, survival, and tissue growth. YAP was recently identified as a candidate oncogene in the chromosome 11q22 amplicon. YAP expression is elevated in a wide spectrum of human cancer cell lines and various primary tumors [Dong et al., 2007]. Overexpression of human YAP in nontransformed mammary epithelial cells induces epithelial-to-mesenchymal transition (EMT), suppresses apoptosis and promotes growth [Overholtzer et al., 2006; Yuan et al., 2008]. TAZ, which is a protein that is highly homologous to YAP, was initially identified based on its ability to interact with 14-3-3 proteins. TAZ also contains a conserved WW domain, a coil–coil domain, a transactivation domain, and a C-terminal PDZ-binding motif [Kanai et al., 2000]. It is involved in the development of multiple organs, including lung, fat, muscle, bone, limb, and heart tissues [Murakami et al., 2005, 2006]. TAZ also modulates mesenchymal stem cell differentiation by coactivating Runx2-dependent gene transcription while repressing PPARγ-dependent gene transcription [Hong et al., 2005]. Accumulating evidence suggests an important function of TAZ in cancer development [Lei et al., 2008; Cordenonsi et al., 2011]. Human YAP and TAZ are paralogous and show significant homology over their entire lengths (overall amino acid sequence identity, 46%; similarity, 60%). While YAP is conserved from *Drosophila* to humans, TAZ appears to be specific to vertebrates. In addition, there are reports showing that YAP may exhibit tumor-suppressing properties by interacting with and stabilizing the tumor suppressor p73 in the nucleus for proper execution of the cell death pathway to occur [Basu et al., 2003]. p73 is a member of the p53 family of transcription factors. p73 recruits YAP to promote apoptosis, which is different from its canonical role as an oncoprotein [Steinhardt et al., 2008]. Thus, the functions of YAP and TAZ are regulated through different mechanisms, and multiple pathways appear to converge on these transcriptional co-regulators that connect them to cellular signaling networks. YAP is overexpressed in HCC specimens, and overexpression of YAP promotes cell proliferation and survival in HCC cells [Zender et al., 2006]. However, there are significant functions of TAZ in HCC that remain unclear.

In this study, the expression level of TAZ was detected in 180 samples from patients with HCC. The relationship between TAZ expression and survival time during the 5-year follow-up period was evaluated. In addition, we regulated the expression level of TAZ in HCC cell lines both in vitro and in vivo to investigate the biological functions of TAZ in the proliferation and apoptosis of HCC cells. Our results provide explicit evidence of the functions of TAZ in HCC cells and indicate that TAZ may be a prognostic marker in HCC patients.

MATERIALS AND METHODS

CELL LINES

The normal liver cell line L-02 and the HCC cell lines Huh7, Hep3B, and HepG2 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). MHCC-97L and MHCC-97H cells were obtained from the Liver Cancer Institute of Fudan University (Shanghai, China). The cell lines were maintained in Dulbecco′s Modified Eagle′s Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Sijiqing Biological Engineering Materials Co., China) at 37°C under 95% air and 5% CO_2.

PATIENTS AND SAMPLES

HCC tissues and corresponding normal liver tissues were obtained previously from patients in our hospital (Xijing Hospital of the Fourth Military Medical University, China) with informed consent. The selection criteria were as follows: (a) confirmed by pathologic diagnosis, (b) without pre-operative anti-cancer treatment and distant metastases before liver transplantation, (c) curative liver transplantation, except for perioperative death, and (d) complete clinicopathological and follow-up data. A total of 180 patients were selected to elucidate the relationship between TAZ expression and the recurrence/invasive phenotype of HCC. All tumors were staged on the basis of the pTNM pathologic classification of the American Joint Committee on Cancer/International Union Against Cancer. Tumor differentiation was classified according to the Edmondson Grading System. To reveal the recurrence of HCC-related biomarkers through proteomics analysis, tissues were immediately snap frozen in liquid nitrogen after surgical resection and stored at −80°C. The pathologic features of these samples were examined via hematoxylin and eosin staining, and only >90% homogenous properties were included in the study. For validation analyses, 180 paraffin-embedded paired samples of fresh HCC tissues and tumor-adjacent normal liver tissues from 19 patients were obtained to compare TAZ expression levels. The follow-up procedures and diagnostic criteria of recurrence were in accord with uniform guideline as described previously [Zheng et al., 2008]. This study was approved by the Ethics Committee of the Fourth Military Medical University.

IMMUNOHISTOCHEMISTRY

Immunostaining was performed with formalin-fixed, paraffin-embedded clinical tissue specimens. Briefly, the sections were dewaxed in xylene, rehydrated through a graded series of alcohols to water, and treated with hydrogen peroxide to quench endogenous peroxidase activity, and antigens were retrieved using a microwave. Subsequently, a blocking antibody (5% normal goat serum) was applied to the slides for 60 min at room temperature. Next, the slides were incubated overnight with an anti-TAZ antibody (sc-48805, Santa Cruz Biotechnology, USA) at a 1:50 dilution, then incubated with horseradish peroxidase-conjugated immunoglobulin for 30 min at room temperature. The results were visualized via reaction with diaminobenzidine and counterstaining with hematoxylin. Because the intensity of staining within each liver tissue specimen was mostly homogenous, the intensity of TAZ staining was semiquantitatively evaluated over five visually representative fields by two independent investigators without prior knowledge of the clinical pathologic data using the following previously described criteria: the expression of TAZ was evaluated based on the percentage of positive cells and the staining intensity. The percentage of positive cells was evaluated quantitatively and scored as 0 for staining of $\leq$1% of the total cells counted, 1 for staining of 2–25% of cells, 2 for staining of 26–50%, 3 for staining of 51–75%, and 4 for staining of $\geq$75% of the cells examined. The intensity was graded as follows: 0, no signal; 1, weak; 2, moderate; and 3, strong. A total "staining score" of 0–12 was calculated and was graded as negative (−, score 0–1), weak (+, score 2–4), moderate (++, score 5–8), or strong (+++, score 9–12) [Shi et al., 2010].

WESTERN BLOT ANALYSIS

Paired fresh HCC and tumor-adjacent normal liver tissues from 19 patients were obtained from the specimen library of the Division of Hepatobiliary and Pancreatic Surgery in our Hospital. Tissue samples were lysed in RIPA lysis buffer [50 mMTris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF]. The extracted proteins were then separated via SDS–PAGE and transferred to PVDF membranes. After being blocked by TBS-T buffer containing 5% non-fat powdered milk for 2 h, the membranes were immunoblotted overnight using a primary antibody against TAZ (1:200, Santa Cruz Biotechnology). An HRP-conjugated IgG antibody was used as the secondary antibody. β-actin (1:5000, Sigma–Aldrich, USA) was used as an internal control. Immunoreactive bands on the blots were visualized using an enhanced chemiluminescence reagent ECL kit (Beit Haemek, Israel). The intensity of individual bands was quantified by image analysis using NIH Image 1.62.

RETROVIRUS GENERATION AND INFECTION

Recombinant lentiviral vectors were constructed with Invitrogen's ViraPower^{TM} Lentiviral System. The cDNA of human TAZ was obtained from MCG clone 19891. Full-length TAZ was constructed via PCR using the MCG19891 clone and cloned into the vector pLenti6. Short hairpin RNAs (shRNA) against human TAZ were designed using a small interfering RNA design program and then subcloned into the EcoRI/Age I sites of the pLKO.1-TRC vector. The shRNA sequences specific for TAZ was as follows: 5′-AGGTACTTCCTCAATCACA-3′ (shTAZ). The scramble shRNA construct 5′-CCTAAGGTTAAGTCGCCCTCG-3′ (shScr) was used as a control. HEK-293T cells were transfected with the pLenti6-Cherry/TAZ, pLKO.1-Scramble/TAZ-shRNA, PAX2, and PMD2Glentiviral vectors using Lipofectamine2000 according to the manufacturer's instructions (Invitrogen). After 48 h, the lentiviral supernatants were collected, filtered (0.45 μm size filter; Millipore, USA), and added to the target cells in the presence of 5 μg/ml of polybrene (Sigma–Aldrich), followed by incubation for 6–8 h. Two rounds of infection were performed. After infection, the cells that survived the treatment were selected. Stable cell lines were established by selecting transduced cells in 2 μg/ml Blasticidin (Sigma–Aldrich) or 3 μg/ml puromycin (Sigma–Aldrich) for 4 weeks.

TUMORIGENESIS IN NUDE MICE

BALB/C athymicnude mice (male, 4 weeks of age) from the Laboratory Animal Research Center of the Fourth Military Medical University (Xi'an, China) were used in compliance with the regulations of the Animal Ethics Committee of the Fourth Military Medical University of the People's Liberation Army. ResuspendedMHCC-97H cells (5×10^6 cells in 200 μl) were separately infected with either shRNA-Scramble or shRNA-TAZ, injected into the left flank or the thoracic mammary fat pad of the mice. Each experimental group contained five mice. Tumor volume was monitored every 3 days for a total of 21 days. Tumor size was calculated based on Vernier caliper measurements of the length and width of the lesions using the following formula: $0.5 \times \text{length} \times \text{width}^2$. The growth curve was then derived from these data. Four weeks later, the mice were sacrificed, and their primary tumors were removed for further histological examination.

REAL-TIME PCR ANALYSIS

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) and 1 mg of total RNA was reverse transcribed to obtain first strand cDNA using an RNA-PCR kit (Fermantas, Lithuania) following the manufacturer's instructions. The resulting cDNA was used for real-time RT-PCR using a SYBRGreen PCR Master Mix kit. PCR was performed in a 25 ml volume containing 1 ml cDNA, 300 mM of primer and 12.5 ml of 2 × PCR master mixes under the following conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1min. The primers used were as follows: TAZ, 5′-GAAAATCACCACATGGCAAGACCC-3′ (sense) and 5′-TTACAG CCAGGTTAGAAAGGGCTC-3′ (antisense); β-actin, 5′-GATCATTGCTCCTCCTGAG C-3′ (sense) and 5′-TGTGGACTTGG-GAGAGGACT-3′ (antisense). The data were normalized to the amount of β-actin mRNA, and the values are represented as the mean ± SD. of 2-ΔΔCt in a duplicateassay.

CELL PROLIFERATION ASSAY

The cells were seeded into 96-well plates at a cell density of 1×10^4/well. Viable proliferating cells were detected using the MTT assay at different times (24, 48, 72, and 96 h) in five wells each time. MTT was dissolved and sterilized in PBS at 5 mg/ml, and 20 μl was added to each well. The plate was then incubated at 37°C for 4 h before the medium was removed. Approximately 150 μl of DMSO was added to each well, and the plate was gently rocked for 10 min to dissolve the

dark blue MTT crystals. Cell viability was expressed as the optical density (OD), which was measured using a multiscanner reader (TECAN-spectra mini Grodig, Austria) at a wavelength of 490 nm. Cell growth curves were drawn based on the average absorbance at 490 nm obtained from three independent experiments. The percent inhibition was calculated using the following formula: % inhibition $= 1 -$ (absorbance test/absorbance control) $\times$ 100%.

CELL APOPTOSIS ANALYSIS

Cells were collected, washed twice with PBS and incubated in the dark for 15 min with binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$), Annexin V-FITC 200 mg/ml; BD Pharmingen, USA) and propidium iodide PI, 1 mg/ml; Sigma–Aldrich). The fluorescence of Annexin V-FITC and PI was measured via flow cytometry using an Epics Elite apparatus. The data were analyzed using CellQuest software (Becton Dickinson, USA).

CELL MIGRATION ASSAY

Cells were plated in 6-well plates and grown to confluence. The monolayer was wounded by scratching with a sterile 200 μl pipette tip, lengthwise along the chamber. After wounding, the cells were washed twice with PBS and cultured at 37°C for 12 h. Images were captured immediately after cell wounding (0 h) and 12 h after cell wounding. The wound width was measured using Open Lab software.

CELL INVASION ASSAY

In vitro invasion assays were performed using 24-well transwell units with Matrigel-coated polycarbonate filters (Corning Costar, USA). Cells were seeded in the upper chamber of the transwell at a density of 1×10^5 cells in 500 μl of serum-free medium, while the bottom chamber was filled with 200 μl of medium containing 10% FBS. After 24 h of incubation, the transwells were fixed with methanol for 15 min and stained with gentian violet for 10 min. The cells in the upper chamber were removed using a cotton swab, and cells that had invaded through the Matrigel to the other side of the filter were manually counted. These experiments were performed in triplicate, and the presented data represent the average number of cells from three filters.

STATISTICAL ANALYSIS

Student's *t*-test was used to compare two groups of data represented as the mean ± SD. All data on the clinicopathologic variables of HCC patients in the study were evaluated with SPSS version 17.0 software. Kaplan–Meier curves were calculated for the survival probability, and differences were assessed using the log-rank test. Differences were considered significant at $P < 0.05$.

RESULTS

TAZ EXPRESSION IN HCC AND ITS CORRELATION WITH PROGNOSIS

The expression of TAZ was generally localized to both the nucleus and the cytoplasm of HCC cells, with most TAZ expression being found in the nucleus. TAZ protein expression in the HCC specimens was significantly increased compared to that in normal liver tissues. In addition, the TAZ expression level increased from well-differentiated to poorly differentiated tumors (Fig. 1A: a-d). As shown in Fig. 1B and Table I, 180 HCC patients were subdivided into the following two subgroups based on the expression levels of TAZ:66showed no to weak expression (36.67%; ±), while114 displayed moderate to strong expression (63.33%; ++/+++). The correlations between the expression level of TAZ and patient characteristics, such as gender, age, tumor size, tumor number, tumor differentiation, TNM stage, lymph node or distant metastasis, recurrent HCC, HBV, and HCV, were investigated. TAZ protein expression was significantly correlated with tumor size, TNM stage, lymph node or distant metastasis, histological differentiation, and recurrent HCC (Table 1). Analysis of overall survival using the Kaplan–Meier method revealed that the prognosis of 180 HCC patients showing high or moderate TAZ expression was significantly worse than that of patients displaying no or weak TAZ expression (Fig. 1B). Thus, TAZ expression may be useful for predicting the overall survival of HCC patients.

To further confirm these findings, Western blot analysis was performed in 19 freshly obtained HCC tissues and adjacent normal tissues. The quantitative analysis of results showed that cancer tissues tended to show higher TAZ expression compared to the adjacent normal tissues in 12 samples (0.76 ± 0.03 vs. 0.40 ± 0.04, $P < 0.01$), which was consistent with the results of the immunohistochemical analysis. However, no significant changes were observed in the remaining seven samples. These findings indicate that TAZ has potential oncogenic properties in hepatocarcinogenesis.

TAZ EXPRESSION IN HCC CELL LINES

TAZ protein expression was significantly higher in the HCC tumor tissues than that in normal liver tissues. We also detected the expression of TAZ at both the transcriptional and translational levels in current HCC cell lines. As shown in Figure 2, we found that TAZ expression was significantly higher in the HCC cell lines than that in the normal liver cell lines. In addition, TAZ was expressed at varying levels in all of the examined HCC cell lines. Among the examined cell lines, high levels of TAZ were detected in the MHCC-97H and MHCC-97L cells, whereas moderate levels were observed in HepG2 and Hep3B cells, and Huh7 cells and normal liver cells (L-02 cells) express low levels of TAZ. Significantly, the highly metastatic HCC cell lines (MHCC-97L and MHCC-97H) exhibited high expression levels of TAZ. Two (HepG2 and Hep3B) of the three weak metastatic potential HCC cell lines [Cui et al., 2006] expressed moderate levels of TAZ, while Huh7 expressed low levels of TAZ. These results suggest that TAZ may participate in governing the invasiveness of HCC cells.

TAZ OVEREXPRESSION PROMOTES THE PROLIFERATION, MIGRATION AND INVASION OF HCC CELLS AND INHIBITS THE APOPTOSIS OF HCC CELLS

To examine the functional consequences of TAZ expression in HCC cells, we overexpressed TAZ via lentiviral infection in Huh7 cells, which exhibit low endogenous levels of TAZ (Fig. 3A). Cell proliferation was measured by the MTT assay. TAZ significantly promoted the growth of Huh7 cells compared to the control. Moreover, the data from the MTT assay showed that there was no significant difference between the parental (Control) and

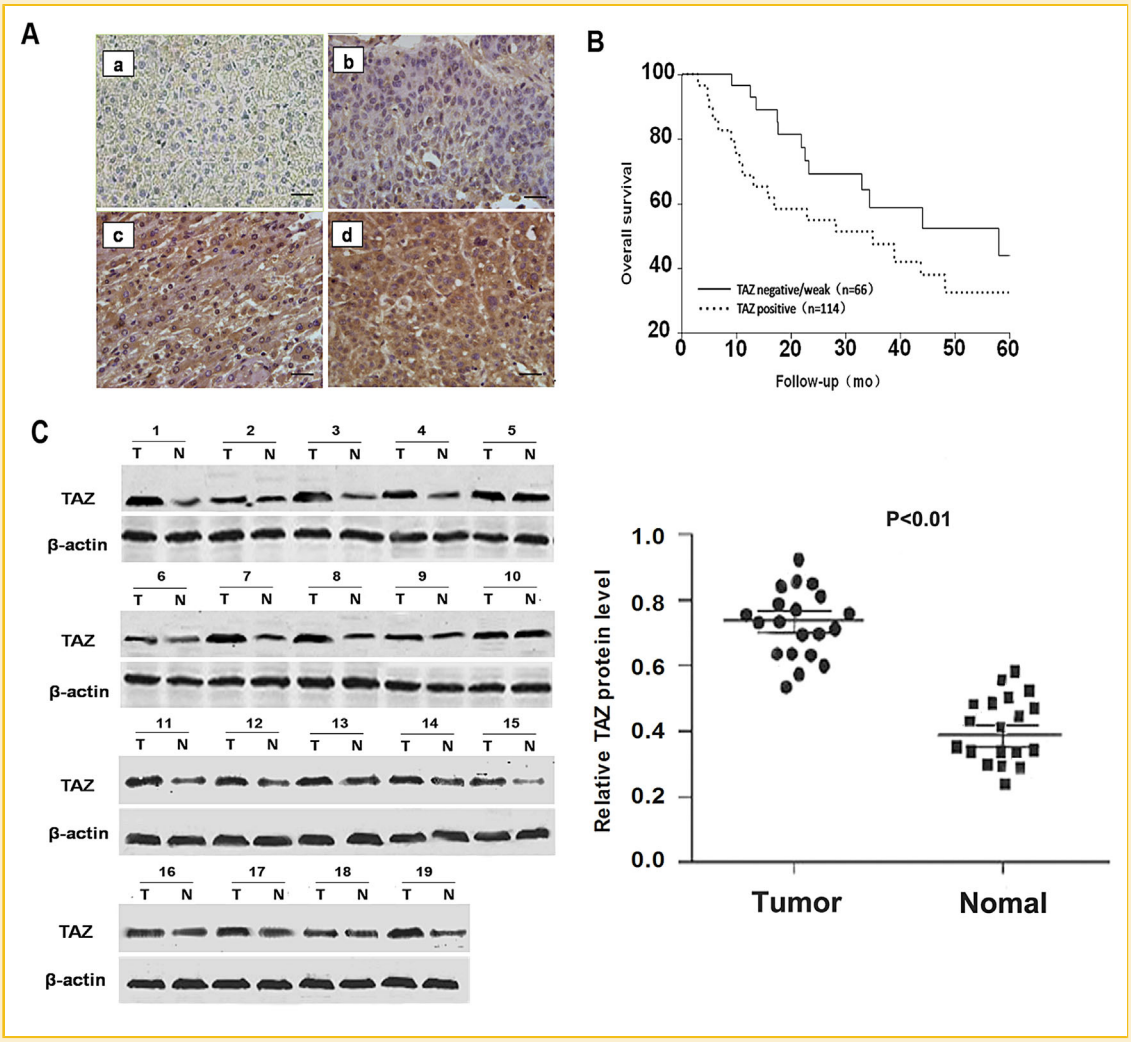

Fig. 1. Expression of TAZ in liver tissues and survival analysis of HCC patients in relation to TAZ expression patterns. (A) Immunohistochemistry of liver tissues from HCC patients showing TAZ staining. Nonneoplastic liver cells showing negative staining (a) of TAZ in hepatocytes and HCC cells showing weak positive staining (b), moderate positive staining (c), and strong positive staining (d) of TAZ in the liver of HCC patients. (Magnification 400×). (B) The overall survival rates of 180 patients with HCC were compared between the TAZ-positive and TAZ-negative (weakly positive and negative staining) groups. Curves were estimated using the Kaplan–Meier method ($P < 0.05$). (C) The protein expression level of TAZ was examined via Westernblot analysis in HCC liver tissues (T) and adjacent non-cancerous liver tissues (N) ($n = 19$). β-actin was used as an internal control.

Cherry-transduced Huh7 cells (Fig. 3B). We conducted flow cytometry analysis to study the effects of TAZ expression on cell apoptosis (Fig. 3C). The apoptosis rate in Huh7 cells infected with TAZ was significantly lower than the rates of the Control and Cherry cells. Through the wound-healing assay, we compared the migration of Huh7 cells expressing TAZ (Fig.3D). The wound healing achieved by migrating cells was imaged at 0–12 h. The mobility of TAZ-overexpressing cells was found to be significantly enhanced. The area of the wound was significantly reduced by the migrating TAZ-overexpressing cells. The invasiveness of these cells was independently assessed by Transwell assays (Fig. 3E). The invasiveness of TAZ-expressing cells was significantly increased compared to the parental and Cherry-transduced Huh7 cells. These results suggest that up-regulated TAZ expression may promote the proliferation, migration and invasion of HCC cells, and inhibit their apoptosis.

TAZ DOWN-REGULATION INHIBITS THE PROLIFERATION, MIGRATION AND INVASION OF HCC CELLS AND INDUCES APOPTOSIS

To further confirm the role of TAZ in HCC cells, a lentivirus-containing short-hairpin RNA targeting TAZ was employed to down-regulate endogenous TAZ expression in MHCC-97H cells (shTAZ-MHCC-97H). A scramble shRNA sequence was used as a negative control (shScr-MHCC-97H). Infection with lentivirus expressing shTAZ appeared to reduce the expression of TAZ in MHCC-97H cells effectively, whereas no significant change was observed in MHCC-97H cells infected with lentivirus expressing shScramble (Fig. 4A). When the growth curves of these cell lines were compared, the curves for shTAZ-MHCC-97H cells were significantly lower than those for Control and shScr-MHCC-97H cells (Fig. 4B). When we examined the effect of TAZ on cell apoptosis, we found that

TABLE I. Clinicopathologic Features of HCC in Relation to the TAZ Expression Pattern

Variable	TAZ protein expression		*P* value
	Negative/weak	Positive	
Sex			0.414
Male	53	97	
Female	13	17	
Age (y)			0.439
<55	32	63	
≥55	34	51	
Tumor size (cm)			0.019
Small (<5)	46	58	
Large (>5)	20	56	
TNM stage			0.025
I–II	31	34	
III–IV	35	80	
Edmondson grade			0.033
I–II	32	31	
III–IV	34	83	
Lymph node or distant metastasis			0.043
Absent	37	45	
Present	29	69	
Recurrent HCC			0.038
Absent	43	47	
Present	23	67	
HBV			0.056
Negative	47	64	
Positive	19	50	
HCV			0.477
Negative	52	84	
Positive	14	30	

TNM stage for HCC was based on The American Joint Committee on Cancer/International Union Against Cancer staging system.

TAZ down-regulation significantly promoted apoptosis compared to Control and shScr-MHCC-97H cells (Fig. 4C). We then evaluated the effect of shTAZ suppression on the migration and invasion of HCC cells. After 12 h, the area of the wound had been recovered at very low levels in parental and shScramble-infected MHCC-97H cells (Fig. 4D). MHCC-97H cells in which TAZ was knocked down displayed considerably reduced invasiveness as compared to MHCC-97H cells infected with lentivirus expressing shScramble (Fig. 4E). These results indicated that down-regulation of TAZ expression may play an important role in HCC development.

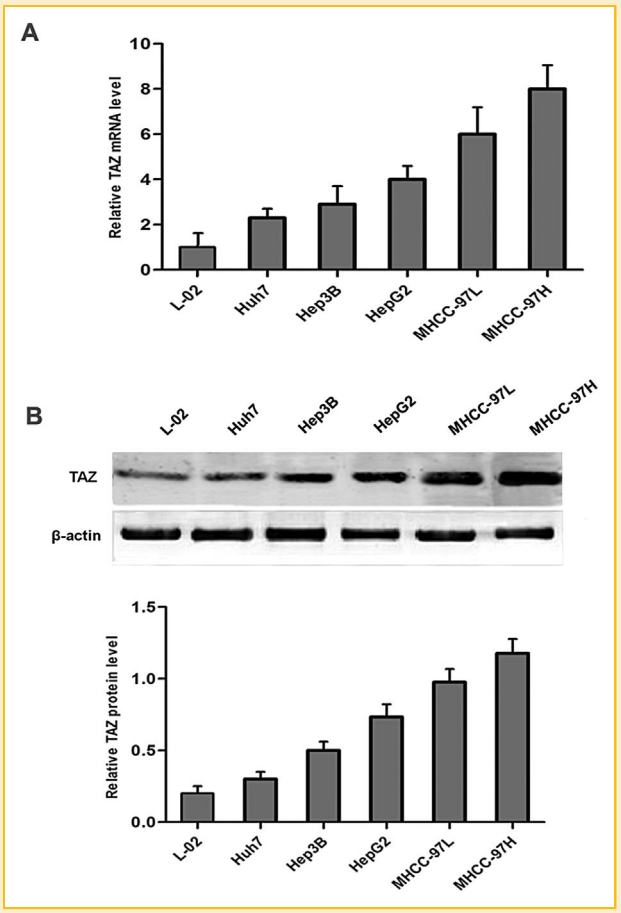

Fig. 2. TAZ expression in HCC cell lines. (A) The mRNA expression level of TAZ was examined in L-02, Huh7, Hep3B, HepG2, MHCC-97L, and MHCC-97H cell lines byReal-timePCR analysis. (B) The protein expressionlevelof TAZ in L-02, Huh7, Hep3B, HepG2, MHCC-97L, and MHCC-97H cell lines by Western blot analysis. β-actin was used as an internal control. The data are representative of triplicate experiments and were calculated as the means ± SD. $^{*}P < 0.05$ indicates the degree of statistical significance.

TAZ DOWN-REGULATION INHIBITS TUMOR FORMATION BYHCC CELLS IN VIVO

To confirm the role of TAZ in the tumorigenesis of HCC cells, we further examined the tumor formation potential in TAZ knockdown HCC-implanted mice. shTAZ-MHCC-97H and shScr-MHCC-97H cells were separately injected into the fat pads of nude mice, and the growth of the tumors was monitored using Vernier calipers. The tumor volume was determined as described in the Materials and Methods section at defined regular intervals (3 days) for 21 days. Compared to shTAZ-MHCC-97H cells, shScr-MHCC-97H cells were compromised in terms of forming tumors at injection sites. Tumors injected with MHCC-97H-shTAZ cells were significantly smaller than those injected with control cells and cells carrying shRNA-Scramble (Fig. 5A and B). Four weeks later, the mice were sacrificed, and the tumors were removed for analysis of tumorigenesis. The immunolabeling of TAZ was weaker in tumors excised from mice in the shTAZ group. There was no significant difference between the control and shScr groups. Moreover, examination of the expression level of the proliferation marker Ki-67 in the tumors showed that the number of Ki-67 positive cells in the TAZ knockdown group was dramatically decreased compared to the control groups. MMP-9, a key molecule in tumor migration and invasion, was also inhibited in the TAZ knockdown group (Fig. 5C). Thus, in vivo assays suggested that down-regulation of TAZ might inhibit the growth and tumorigenicity of HCC cells.

DISCUSSION

HCC is the third leading cause of cancer-related death in the world. Elucidating the molecular mechanisms underlying the development and progression of HCC is critical for its prevention, diagnosis, and treatment. Recently, the Hippo signaling pathway was shown to play an important role in the development and differentiation of biosystems. TAZ, as a downstream target of the Hippo pathway, is known to modulate mesenchymal stem cell differentiation toward osteoblasts and adipocytes. Knockout of the TAZ gene in mice

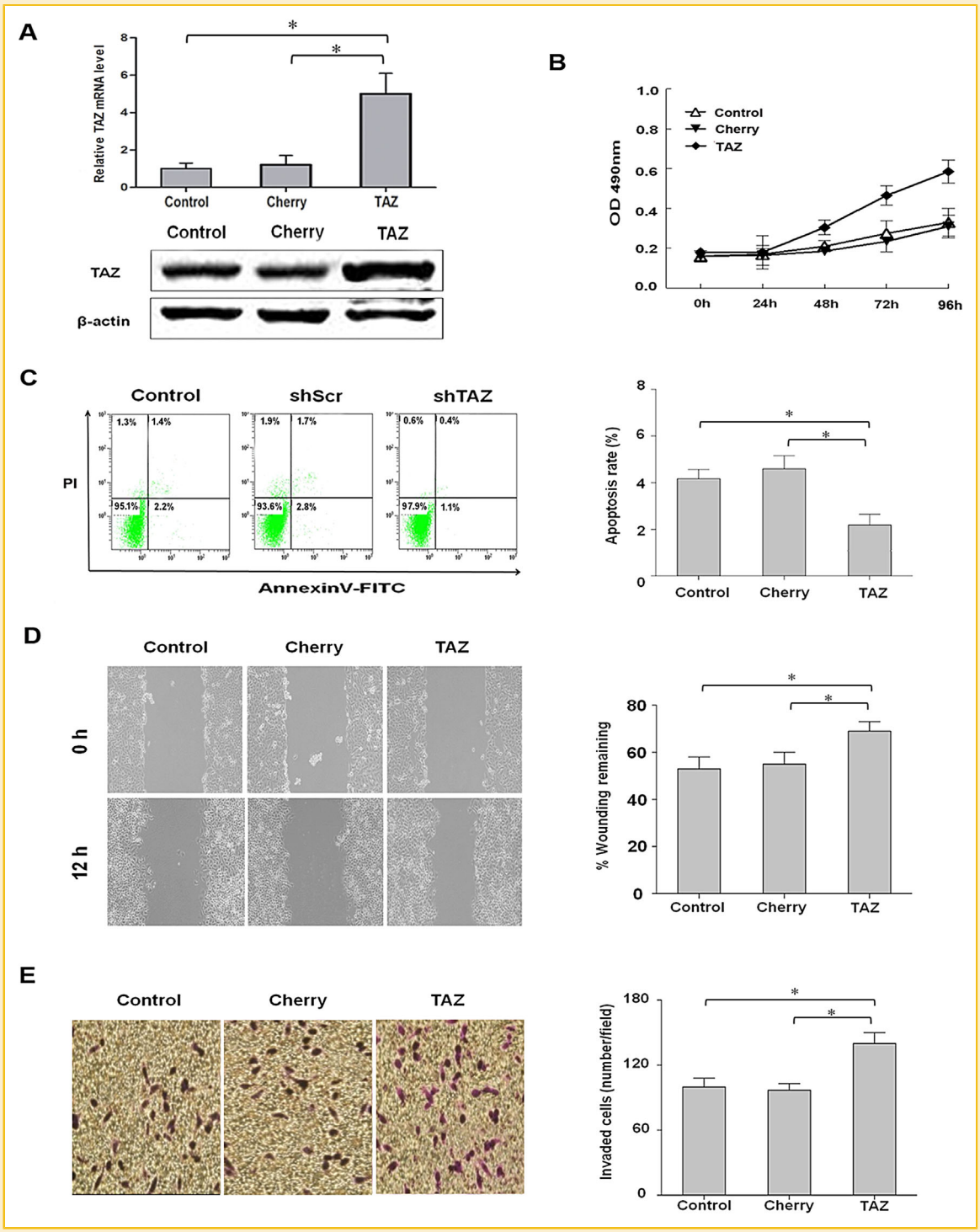

Fig. 3. Overexpression of TAZ in Huh7 cells promotes cell growth, inhibits cell apoptosis, and enhances cell migration, and invasion. (A) mRNA and protein levels of TAZ in Control (Huh7 wild type cells), Cherry (Huh7 cells infected with the overexpression control plasmid), and TAZ (Huh7 cells infected with the TAZ overexpression plasmid) cells, which were examined via Real-time PCR and Western blot, respectively. (B) Cell growth curves. The viability of cells was detected in MTT assays at 24, 48, 72, and 96 h. (C) Apoptosis rate. Apoptotic cells were stained with Annexin V/PI and analyzed via flow cytometry. The data are shown in histograms. (D) A wound-healing migration assay was performed in the cells. Wound healing by migrating cells was imaged at 0–12 h. (E) Cell invasion is depicted in phase contrast images and through graphical representation. The invasion rate of controlcells was defined as 100%.The data are representative of triplicate experiments and were calculated as the means ± SD. $^{*}P < 0.05$ indicates the degree of statistical significance.

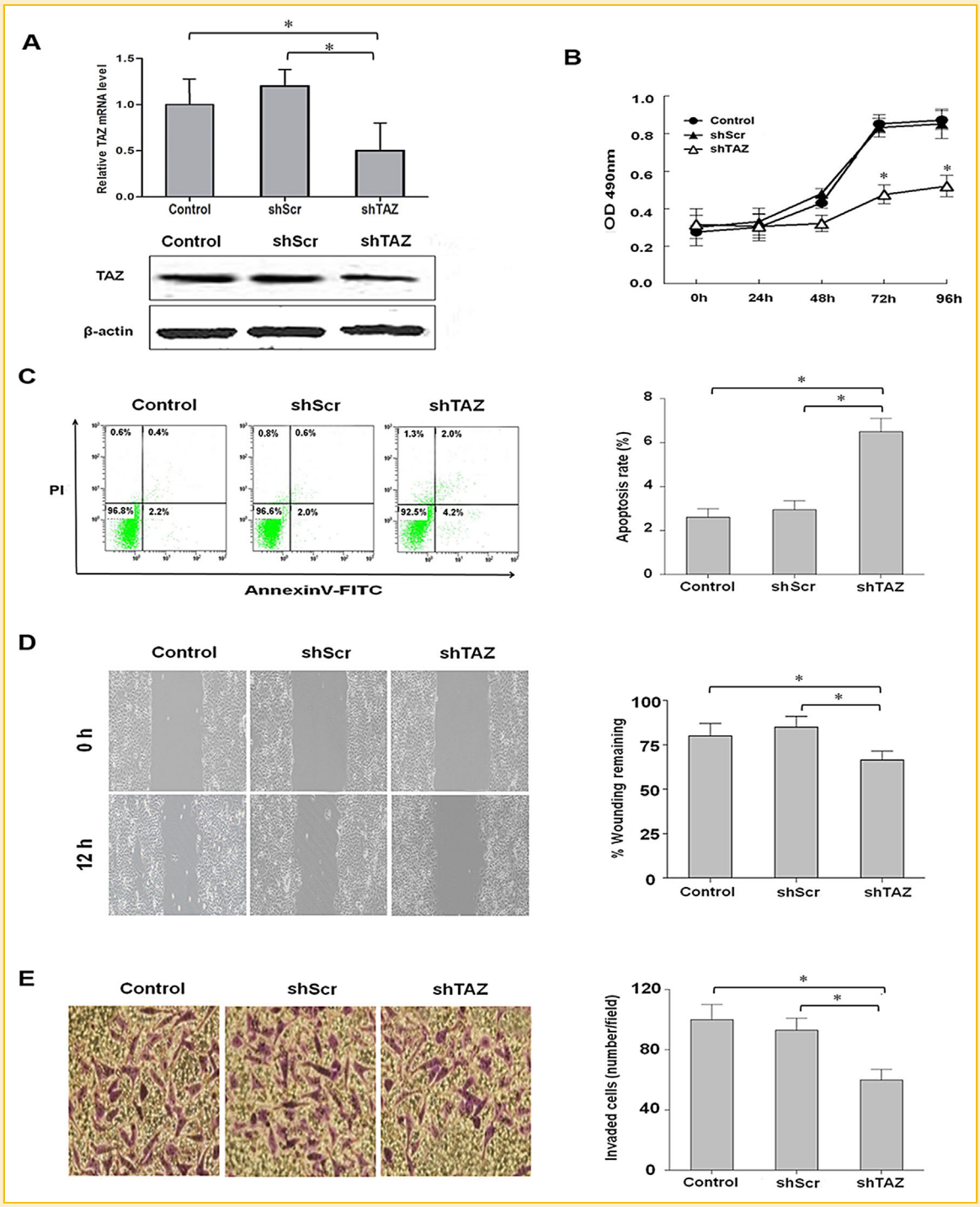

Fig. 4. shRNA-mediated knockdown of TAZ in MHCC97H cells inhibits cell growth, induces cell apoptosis and suppresses cell migration and invasion. (A) mRNA and protein levels of TAZ in Control (MHCC-97H wild type cells), shScr (MHCC-97H cells infected with lentivirus containing the control Scramble shRNA) and shTAZ (MHCC-97H cells infected with lentivirus containing shRNAtargeting TAZ) cells were examined via Real-time PCR and Western blot, respectively. (B) Cell growth curves.The viability of the cells was detected in MTT assays at 24, 48, 72, and 96 h. (C) Apoptosis rate. Apoptotic cells were stained with Annexin V/PI and analyzed via flow cytometry. The data are shown in histograms. (D) Wound-healing migration assayswere performed for the cells. Wound healing by migratingcells at was imaged at 0–12 h. (E) Cell invasion is depicted in phase contrast images and through graphical representation. The invasion rate of control cells was defined as 100%. The data are representative of triplicate experiments and were calculated as the means ± SD. $^{*}P < 0.05$ indicates the degree of statistical significance.

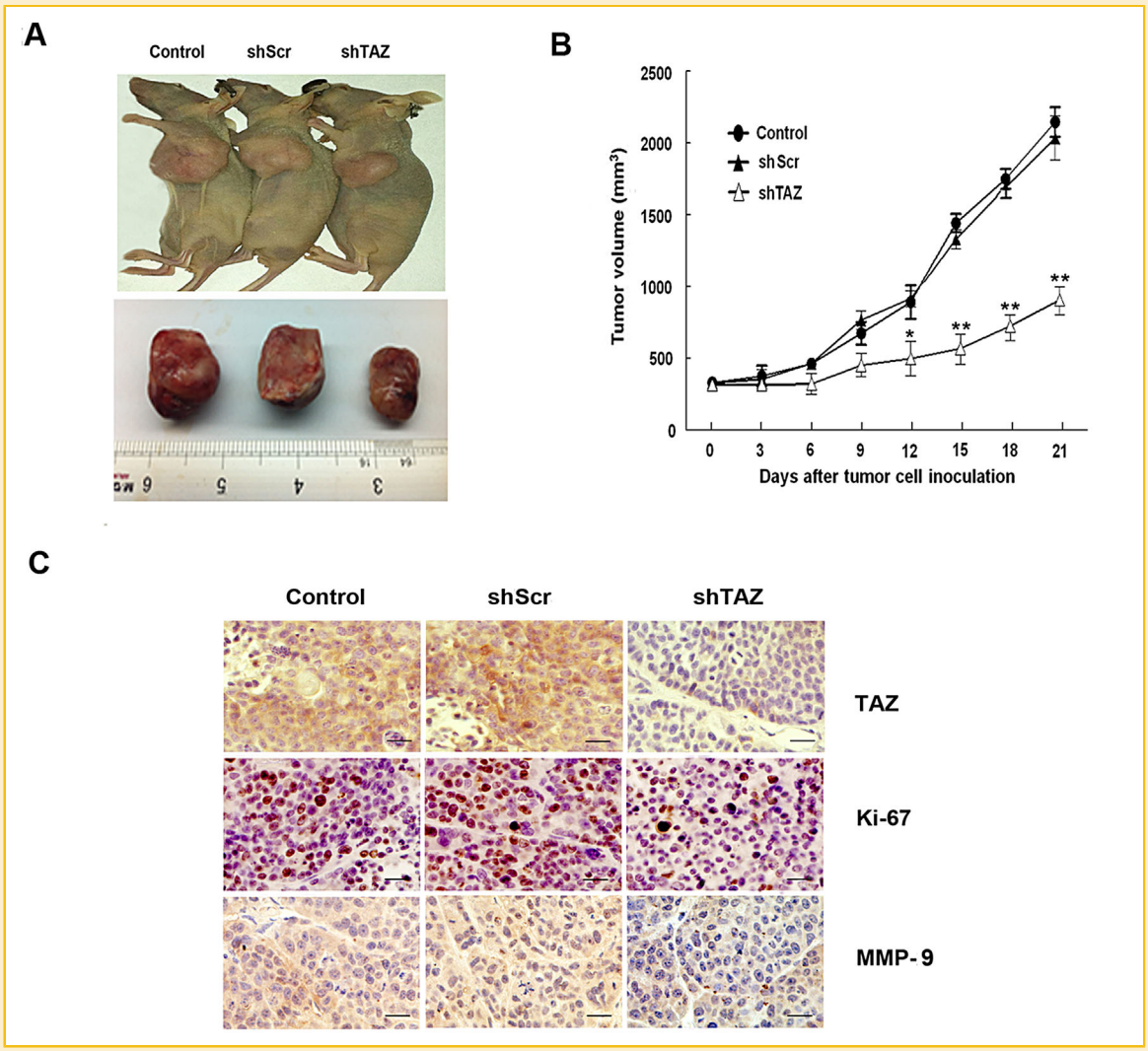

Fig. 5. Stable silencing of TAZ suppresses tumorigenicity in nude mice. MHCC-97H cells were infected with lentivirus containing shRNAtargeting TAZ (shTAZ) or control scramble shRNA (shScramble) and then individually injected into the left flanks or the thoracic mammary fat pads of the mice ($n = 5$ in each group). (A) Four weeks later, the mice were sacrificed, and representative mice and excised tumors are shown. (B) Tumor growth curve. Tumor growth was assessed every 3 days until day 21 by measuring two perpendicular diameters and calculating the volume in mm^3. Statistical analysis was performed using the means ± SD. $^{*}P < 0.05$ or $^{**}P < 0.01$indicates the degree of statistical significance. (C) Primary tumors were removed for histological examination. Representative immunohistochemistry staining showing the expression levels of TAZ, Ki-67, and MMP-9 in the tumor tissues of each group (Magnification 400×).

resulted in only minimal developmental abnormalities in surviving TAZ-deficient mice, although the number of pups born was only approximately half the expected Mendelian ratio in some mouse strains. In addition, the TAZ-deficient mice were reported to show distorted lung and kidney architectures [Hossain et al., 2007; Makita et al., 2008]. Several signaling components of the Hippo pathway have been implicated as tumor suppressors, while the downstream effector TAZ, which is negatively regulated by the Hippo signaling cascade, is known to be overexpressed in various cancers and plays an important role in tumor initiation and progression [Chan et al., 2009; de Cristofaro et al., 2011; Zhou et al., 2011].

In the present study, we used 180 clinical tissue samples to explore the role of TAZ in HCC for the first time. We examined TAZ protein expression in paired primary HCC samples through immunohistochemical analysis. We found that TAZ expression occurred at a very low level in paired tumor-adjacent normal liver tissues and was markedly increased in most primary HCC tumor tissues. Consistent with these observations, Western blot analyses also showed that TAZ expression was increased in most HCC tumor tissues compared to the corresponding non-tumorous liver tissues. In the immunohistochemical analysis, increased TAZ expression in HCC was observed to be significantly associated with tumor size, lymph node or distant metastasis, TNM stage, and recurrence. Additionally, most of the poorly differentiated HCC samples were positive for TAZ expression, but TAZ expression was much weaker in the moderately and well-differentiated tumor samples. Thus, increased TAZ expression is

correlated with poor differentiation in HCC cells and may further promote HCC progression. Kaplan–Meier survival analysis showed that HCC patients who displayed positive TAZ expression generally exhibited a worse prognosis than those who showed negative expression. These results indicated that up-regulation of TAZ expression may play a role in the development of HCC. We detected the expression of TAZ at both the transcriptional and translational levels in current HCC cell lines and found that the highly invasive HCC cells expressed high levels of TAZ, whereas the weakly invasive cells expressed low levels of TAZ.

Based on the differences in TAZ expression observed in the HCC cells lines, we modulated TAZ expression to further examine its critical role in HCC progression. Both gain-of-function (via over-expression) and loss-of-function (via shRNA-mediated knockdown) approaches were used, and the effect of TAZ in regulating HCC cell growth and motility was directly demonstrated through a series of in vitro assays. Our experimental data showed that the down-regulation of TAZ expression could significantly inhibit cell proliferation, promote apoptosis, and suppress the migratory and invasive properties of HCC cells. In parallel experiments, we also found that the down-regulation of TAZ expression suppressed tumor growth in injectable mouse models. Consistent with our results, previous research has shown that decreased TAZ expression in breast cancer cells inhibits cellular transformation and xenograft tumor growth [Cordenonsi et al., 2011; Zhou et al., 2011].

YAP, a protein that is highly homologous to TAZ, was recently identified as a candidate oncogene in HCC. In clinical studies, YAP overexpression in HCC patients was found to be significantly associated with poor tumor differentiation and high serum levels of alpha-fetoprotein (AFP), which is a widely used serum biomarker for liver malignancy. More importantly, YAP was shown to be an independent predictor of HCC-specific disease-free survival and overall survival [Xu et al., 2009]. The expression of cyclin E was found to be decreased in hepatoma cells which transfected with siRNA targeting YAP. In addition, the tumorigenicity of these hepatoma cells was reduced compared to controls after injection into recipient mice [Zender et al., 2006]. Recently, miR-375 was reported to negatively regulate the expression of YAP and its primary target CTGF. In addition, ectopic expression of miR-375 in HCC cells inhibits cell proliferation and invasion [Liu et al., 2010a]. Liu et al. also demonstrated that overexpression of YAP inhibits the apoptosis of HCC cells via activation of the AKT and ERK1/2 signaling pathway [Liu et al., 2010b]. Therefore, the TAZ homolog YAP plays an important role in the development of HCC.

Based on our current knowledge of TAZ as a coactivator of gene transcription, one possible mechanism for the action of TAZ is interaction with other transcriptional activators to enhance the transcription of genes that are involved in tumor cell proliferation, EMT, migration, and invasion. The TEAD family is a key group of downstream transcription factors that mediate the function of TAZ. Zhang et al. showed that disruption of TEAD-TAZ binding or silencing of TEAD expression blocks the function of TAZ to promote cell proliferation and to induce EMT. Moreover, CTGF has been identified as a direct target gene of the TAZ-TEAD complex [Zhang et al., 2009] and has been implicated in the development and progression of multiple human cancers [Sala-Torra et al., 2007; Chu et al., 2008]. A recent study demonstrated that KLF5 may be the key transcription factor that interacts with the TAZ to promote breast cell proliferation and tumor growth. TAZ through WW domain and PY motif interacts with KLF5 and protects KLF5 from WWP1-mediated ubiquitination and degradation [Zhao et al., 2012]. The KLF5 protein is rapidly degraded through the proteasome following ubiquitination by E3 ubiquitin ligases [Chen et al., 2005]. KLF5 has been shown to act as an oncogenic transcription factor in several cancer types including breast cancer [Zheng et al., 2009] and intestinal cancer [McConnell et al., 2009]. Additionally, KLF5 is involved in multiple cancer signaling pathways, such as the Ras-mitogen-activated protein kinase [Usui et al., 2004], Wnt-β-catenin [McConnell et al., 2009], transforming growth factor β-Smad [Guo et al., 2009], and Hippo pathways. Accordingly, further studies are required to delineatethe biological functions and precise mechanisms of TAZ.

Taken together, our results revealed that TAZ is a differentially expressed molecule in HCC, and decreasing the expression of TAZ may inhibit the malignant phenotype of HCC. As an important regulator, TAZ might be as a new diagnostic and therapeutic biomarker in hepatocellular carcinoma.

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