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ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

Knockdown Survivin Expression Reduces the Efficacy of Curcumin Treatment in Hepatocellular Carcinoma Cells

Chin-Sheng Hung, MD[1,2,3,4], Hui-Hsiung Liu, MD, PhD[5], Ming-Te Huang, MD[1,2,3,6], Chao-Wen Cheng, PhD[1], Li-Jen Kuo, MD[1,2,3,4], Yuan-Soon Ho, PhD[7,8], Chih-Hsiung Wu, MD, PhD[1,2,3,6], Chien-Ming Chen, MS[8], Po-Li Wei, MD, PhD[1,2,3,4], and Yu-Jia Chang, PhD[1,2,3,4,7]

[1]Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan; [2]Department of Surgery, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan; [3]Division of General Surgery, Department of Surgery, Taipei Medical University Hospital, Taipei Medical University, Taipei, Taiwan; [4]Cancer Research Center, Taipei Medical University Hospital, Taipei Medical University, Taipei, Taiwan; [5]Department of Public Health, School of Public Health, Taipei Medical University, Taiwan, Taipei, Taiwan; [6]Division of General Surgery, Department of Surgery, Shuang Ho Hospital, Taipei Medical University, Taipei, Taiwan; [7]Center of Excellence for Cancer Research, Taipei Medical University, Taipei, Taiwan; [8]School of Medical Laboratory Science and Biotechnology, College of Medical Science and Technology, Taipei Medical University, Taipei, Taiwan

ABSTRACT

Background. Survivin is a potential therapeutic target for cancer. Increased survivin expression promotes cell survival and therapeutic resistance. However, there is little information regarding whether the expression level of survivin affects curcumin treatment in hepatocellular carcinoma (HCC).

Methods. Survivin expression was suppressed in HCC cells using a short interfering RNA (siRNA) technique. The anticancer effects of curcumin were examined using a biosensor system, MTT assay, TUNEL assay, and cell cycle analysis.

Results. Curcumin resistance developed in cells with suppressed survivin, in contrast to the parental cells, as determined by survival assays. Cell cycle analysis and TUNEL assays revealed that the apoptotic cell population was increased in the scrambled-siRNA cells treated with curcumin compared with the survivin-siRNA cells. Suppression of survivin expression resulted in curcumin resistance via the modulation of Bcl-2 and Bax expression.

Conclusions. We conclude that the expression levels of survivin may mediate the therapeutic efficacy of curcumin in HCC cells.

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Po-LiWei, MD, PhD
e-mail: poliwei@tmu.edu.tw

Y.-J. Chang, PhD
e-mail: r5424012@tmu.edu.tw

Hepatocellular carcinoma (HCC) is the fifth most common type of neoplasm and the third most common cause of cancer-related deaths worldwide. HCC is considered to be a major health problem in Asia and Africa. The incidence of HCC is steadily increasing in Europe and America because of the escalating prevalence of cirrhosis-related risk factors, including viral infection, diabetes, obesity, and excessive alcohol intake.[1,2] Conventional therapies at the early stages of the disease include surgical resection, liver transplantation, radiofrequency ablation, and ethanol injection. With such treatments, the 5-year survival rate is 50–70 %. Unfortunately, the majority of patients (70–85 %) present with an advanced or unresectable disease, and the current therapeutic options at this stage are limited.[3,4] The use of molecularly targeted therapies, such as the multikinase inhibitor sorafenib, in treating advanced HCC highlights the need to identify additional clinically relevant molecular targets for therapeutic intervention.

Survivin, a member of the family of inhibitor of apoptosis proteins, is undetectable in terminally differentiated adult tissues, but is strongly expressed in most human tumors and fetal tissues.[5] Survivin inactivates caspase-9 and the adapter protein Apaf-1 to suppress the cell death

that occurs through extrinsic or intrinsic pathways. Survivin also counteracts the G2/M apoptotic checkpoint of the cell cycle.[6–8] Thus, survivin overexpression may inhibit apoptosis and accelerate mitotic activity in tumor cells. Survivin protein expression is associated with poor clinicopathological parameters, local recurrence, and disease-free survival in HCC patients.[9–11] Stable survivin knockdown via short hairpin RNA abrogates the growth of human HCC in vitro and in vivo.[12] Several studies have shown that survivin expression is regulated by the activation of the signal transducer and activator of transcription 3 (STAT3).[13,14] Constitutive activation of STAT3 has been reported in approximately 50 % of HCC patients.[15] Novel approaches to downregulate survivin expression in HCC may serve as potential targeted therapies in the future.

Curcumin, a polyphenol derived from the turmeric plant (*Curcuma longa*), has been widely studied in the areas of chemoprevention and chemotherapy.[16] Curcumin modulates cancer cell growth by regulating the activity of several key transcription factors, including NF-κB, AP-1, Egr-1, STATs, PPAR-γ, β-catenin, Nrf2, EpRE, p53, CBP, the androgen receptor (AR), and AR-related cofactors.[17–19] Additionally, curcumin downregulates survivin expression in pancreatic cancer cell lines by preventing STAT3 phosphorylation.[20] Work from our laboratory has shown that curcumin inhibits HCC proliferation by inducing endoplasmic reticulum stress and mitochondrial dysfunction, and suppressing GRP78 causes HCC to become resistant to curcumin.[21,22]

Although the survivin expression level correlates with the tumor grade, it is unclear whether the cytotoxic effect of curcumin on HCC is altered by the level of survivin expression. The purpose of this study is to examine the role of survivin in curcumin-induced growth inhibition in HCC. We found that decreased survivin expression induces resistance to curcumin treatment.

MATERIALS AND METHODS

Chemicals, Reagents, and Cell Culture

Curcumin, propidium iodide (PI), Triton X-100, Tris–HCl, neomycin, trypan blue, EDTA, MTT, ribonuclease A, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). The survivin, Bcl2, Bax, Bad, Bim, and β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The HCC cell lines were grown in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 10 % fetal bovine serum (Gibco BRL, Grand Island, NY), and 2 % penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) in a humidified incubator with 5 % CO_2 at 37 °C, as previously described.[22,23]

Generation of Survivin-Knockdown HepJ5 and Mahlavu Cells

Survivin expression was suppressed in HepJ5 and Mahlavu cells using small interfering RNA (siRNA), as previously described.[22–24] The target sequence for the human survivin mRNA was 5′-TGGGAGCCAGATGACG ACC-3′, and the scrambled siRNA sequence was 5′-AAG GTGGTTGTTTTGTTCACT-3′. The survivin-targeted and scrambled siRNAs were inserted into the pSUPERIOR vector, and the inserts were confirmed by sequencing. The survivin siRNA and scrambled siRNA plasmids were transfected into HCC cells, and the stably transfected cells were neomycin-selected, as previously described.[25,26] Survivin expression levels were examined using real-time RT-PCR and western blotting.

MTT Assay Cells (2×10^4) were seeded onto 24-well plates and incubated overnight. Various concentrations of curcumin (0–50 μM) or its vehicle (70 % ethanol) were added to the cells. The medium was aspirated at particular time intervals, and the cells were incubated in 0.25 mg/ml MTT for 1 h. The formazan was extracted with DMSO, and the optical density was measured at 550 nm using a spectrophotometer (GE Healthcare, Piscataway, NJ).

DAPI Staining

Cells (approximately 2×10^5) were plated onto 4-well chamber slides and incubated with curcumin (20 μM) for 48 h. The cells were fixed, stained with DAPI, and photographed under a fluorescence microscope, as previously described.[21]

Flow Cytometry for Cell Cycle Analysis Cells (3×10^5) were plated onto 6-well plates overnight and then incubated with curcumin (20 μM) or its vehicle (70 % ethanol) for 48 h. The cells were harvested and washed with PBS for various time intervals. The cells were fixed in pure methanol, treated with RNase A (final concentration, 40 μg/ml), and stained with PI (40 μg/ml) for 30 min at room temperature. The stained cells were analyzed using a flow cytometer (BD Biosciences, San Jose, CA), and DNA content was quantified using Modfit software (Verity Software House, Inc., Topsham, ME). The percentage of hypodiploid cells (sub-G1) was used to quantify the dead cells.

TUNEL Assay Cells (3×10^5) were plated onto 6-well plates overnight and then treated with curcumin (20 μM) or its vehicle (70 % ethanol) for 48 h. The cells were harvested and washed with PBS. The morphology associated with DNA fragmentation was detected via terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labeling (TUNEL) using an Apo-BrdU In Situ DNA Fragmentation Assay Kit (Bio Vision, Mountain View, CA), according to the manufacturer's instructions. The TUNEL-positive cells were analyzed using flow cytometry.

Protein Extraction and Western Blot Analysis

Cells were treated with curcumin (20 μM) for 48 hours. Proteins were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, as previously described.[22,23] Briefly, the cells were washed with cold PBS and lysed with lysis buffer (Thermo Scientific, Rockford, IL) containing protease inhibitors (Complete Protease Inhibitor Tablets, Boehringer Mannheim, Indianapolis, IN). Proteins (25 μg) were separated using 10 % SDS-PAGE under reducing conditions and electrotransferred onto PVDF membranes (GE Healthcare Piscataway, NJ). The membranes were incubated overnight with survivin, Bcl-2, Bax, or *β*-actin antibodies at 4 °C and were then probed with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000). The products were visualized with an enhanced chemiluminescence reagent (GE Healthcare Piscataway, NJ) and were detected using a VersaDoc 5000 (Bio-Rad Laboratories), as previously described.

RNA Preparation and Real-Time RT-PCR

Total RNA was extracted from the cells using TRIZOL reagent, as previously described.[11,22,23] The extracted RNA was purified using a genomic DNA elimination mixture and was subsequently reversed-transcribed into cDNA. The cDNA was amplified from 2 μg of total RNA in a final volume of 20 μl using MMLV (Moloney murine leukemia virus) reverse transcriptase at 37 °C for 90 min. For validation, real-time RT-PCR was performed using the Power SYBR-Green real-time RT-PCR system and an ABI 7500 Fast detection system (Applied Biosystems, Foster City, CA). The reaction conditions were: 95 °C for 3 min, 95 °C for 15 sec, and 60 °C for 1 min (40 cycles). The primer sequences for target genes have been previously described and are: *β*-actin (forward, 5′-AGCGCGGCTACAGCTTCA-3′; reverse, 5′-GGCCATCTCTTGCTCGAAGT-3′); and survivin (forward, 5′-AGGTCATCTCGGCTGTTCCTG-3′; reverse, 5′-TCATCCTCACTGCGGCTGTC-3′).

Statistical Analysis

All of the experiments were repeated a minimum of 3 times. All of the data are expressed as the means ± SD. The data presented in certain figures are from a representative experiment that was quantitatively similar to the replicate experiments. The statistical significance was determined using the *t* test (2-tailed) to compare 2 data sets.

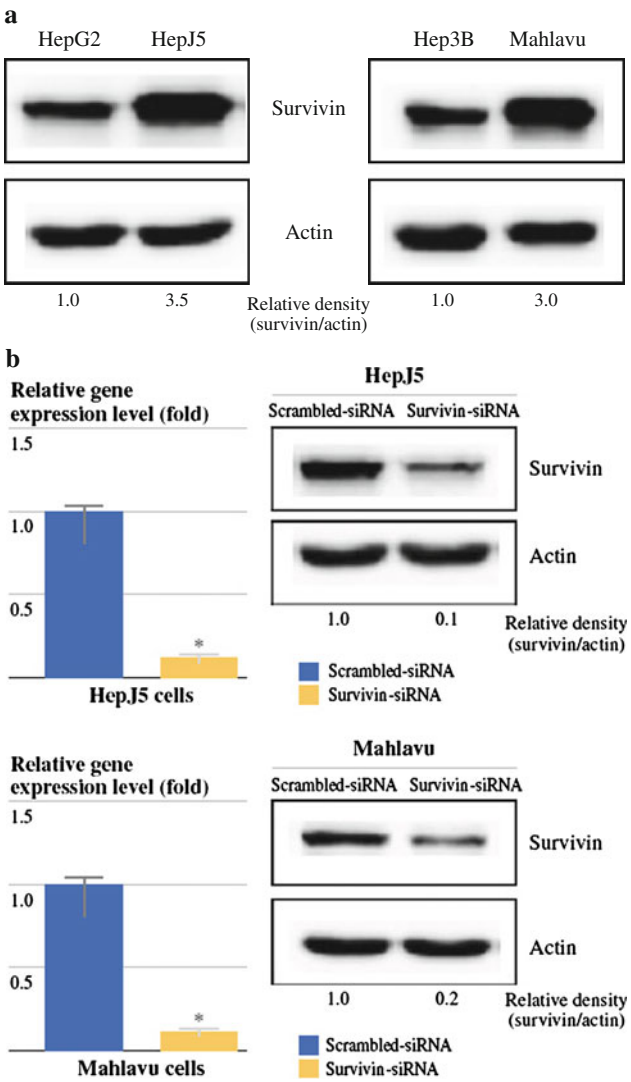

FIG. 1 Survivin expression in HCC cells. **a** Survivin expression in HCC cells (HepG2, Hep3B, HepJ5, and Mahlavu) was determined using western blotting. **b** Suppression of survivin expression in HepJ5 and Mahlavu cells using siRNA technique. Survivin expression was determined using real-time RT-PCR and western blotting. All of the experiments were independently repeated at least 3 times. $^*P < 0.05$

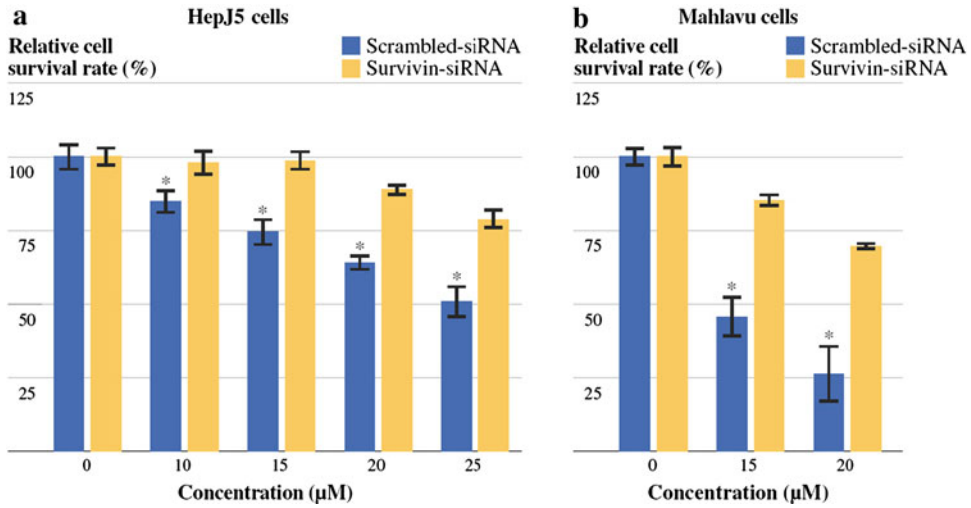

FIG. 2 Percentage of viable HepJ5 and Mahlavu cells following treatment with curcumin. **a** Survivin-siRNA and scrambled-siRNA cells (2×10^4 cells/well; 24-well plates) were plated in Dulbecco's modified Eagle's medium with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate and 10 % fetal bovine serum in the presence of various doses of curcumin for 48 h. Cell survival was determined using the MTT assay. **b** The survivin-siRNA and scrambled-siRNA Mahlavu cells were treated with various doses of curcumin for 48 h. Cell survival was determined using the MTT assay. All of the experiments were independently repeated at least 3 times. $*P < 0.05$

RESULTS

Knockdown of Survivin Increases the Resistance of HCC to Curcumin Treatment

Survivin expression was examined in various cell lines (HepG2, Hep3B, HepJ5, and Mahlavu). The HepJ5 and Mahlavu cells had the highest ectopic expression of survivin compared with the HepG2 and Hep3B cells (Fig. 1a). Survivin-knockdown (survivin-siRNA) and scrambled-siRNA cells were generated using a siRNA targeted against survivin in HepJ5 and Mahlavu cells. The stably transfected cells were selected by antibiotic, and the expression levels of survivin were determined by real-time RT-PCR and western blot. As shown in Fig. 1b, survivin expression was reduced by more than 80 % at both the transcriptional and translational levels. Curcumin-induced cytotoxicity was examined in the survivin-siRNA and scrambled-siRNA cells. Curcumin caused a dose-dependent inhibition of cell proliferation in both the survivin-siRNA and scrambled-siRNA cells (Fig. 2). Interestingly, less cytotoxicity was observed in the survivin-siRNA cells.

Knockdown of Survivin Reduces Curcumin-Induced Apoptosis Cell cycle distributions and apoptosis were analyzed via flow cytometry in survivin-siRNA and scrambled-siRNA cells following curcumin treatment. Cells treated in the presence or absence of curcumin (20 μM) for 48 h were harvested and fixed. As shown in Fig. 3a, the percentage of HepJ5 cells in sub-G1 was higher in the cells harboring the scrambled siRNA (39.31 %) compared with the survivin siRNA (9.75 %). Similar cell cycle distributions were observed in Mahlavu cells containing the scrambled siRNA or survivin siRNA following curcumin treatment (Fig. 3b).

Detection of Apoptosis by DAPI Staining and TUNEL Assays Curcumin-induced apoptosis was further examined using DAPI staining. Apoptosis was observed in the survivin-siRNA and scrambled-siRNA cells following curcumin (20 μM) treatment (Fig. 4). Apoptotic cells were identified by small and round nuclei, in contrast to condensed, larger, and deep white nuclei. These data suggest that the apoptotic effects of curcumin were more prominent in the scrambled-siRNA cells. A TUNEL assay was used to further visualize the apoptotic effects of curcumin. Positive TUNEL staining was increased in survivin-siRNA and scrambled-siRNA HepJ5 cells following curcumin treatment (Fig. 5). The number of curcumin-induced TUNEL-positive HepJ5 cells was increased in cells harboring the scrambled-siRNA (22.98 %) compared with cells with the survivin-siRNA (1.08 %) after treating with curcumin. These results were confirmed in Mahlavu cells. As shown in Fig. 5b, the survivin-siRNA Mahlavu cells exhibited lower apoptosis (1.07 %) following curcumin treatment compared with the scrambled-siRNA cells (60.79 %). These data indicate that curcumin promotes apoptosis in both survivin-siRNA and scrambled-siRNA cells, but the scrambled-siRNA cells are more sensitive to curcumin.

Curcumin Influences Bcl-2and Bax Expression in Mahlavu Cells To characterize the molecular mechanism by which survivin alters curcumin-induced apoptosis in Mahlavu

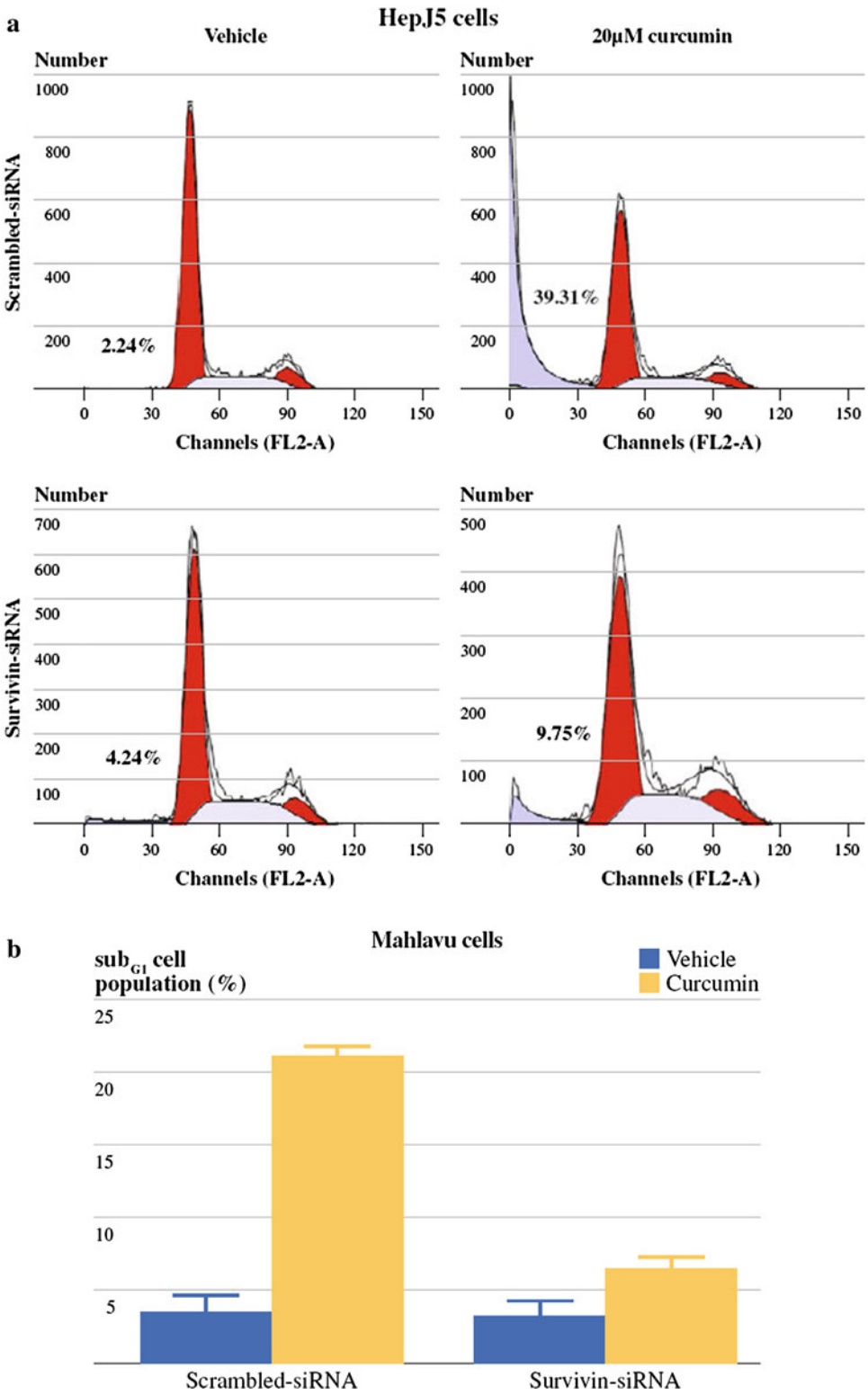

FIG. 3 Cell cycle population and percentage of survivin-siRNA and scrambled-siRNA HCC cells in sub-G1 after curcumin treatment. Scrambled-siRNA and survivin-siRNA cells were exposed to 20 μM curcumin for 48 h. The cells were harvested, and their cell cycle distributions were analyzed. The data represent means ± SD of 3 experiments. $^{*}P < 0.05$

cells, we examined the expression of apoptosis-associated proteins in response to curcumin. The survivin-siRNA and scrambled-siRNA cells were treated with curcumin for 48 h, and the expression levels of Bcl-2, Bax, Bad, and Bim were detected using Western blotting. Decrease of Bcl-2 level and increase of Bax level were observed in the scrambled-siRNA cells, but no change in Bad and Bim expression levels in scrambled-siRNA and survivin-siRNA

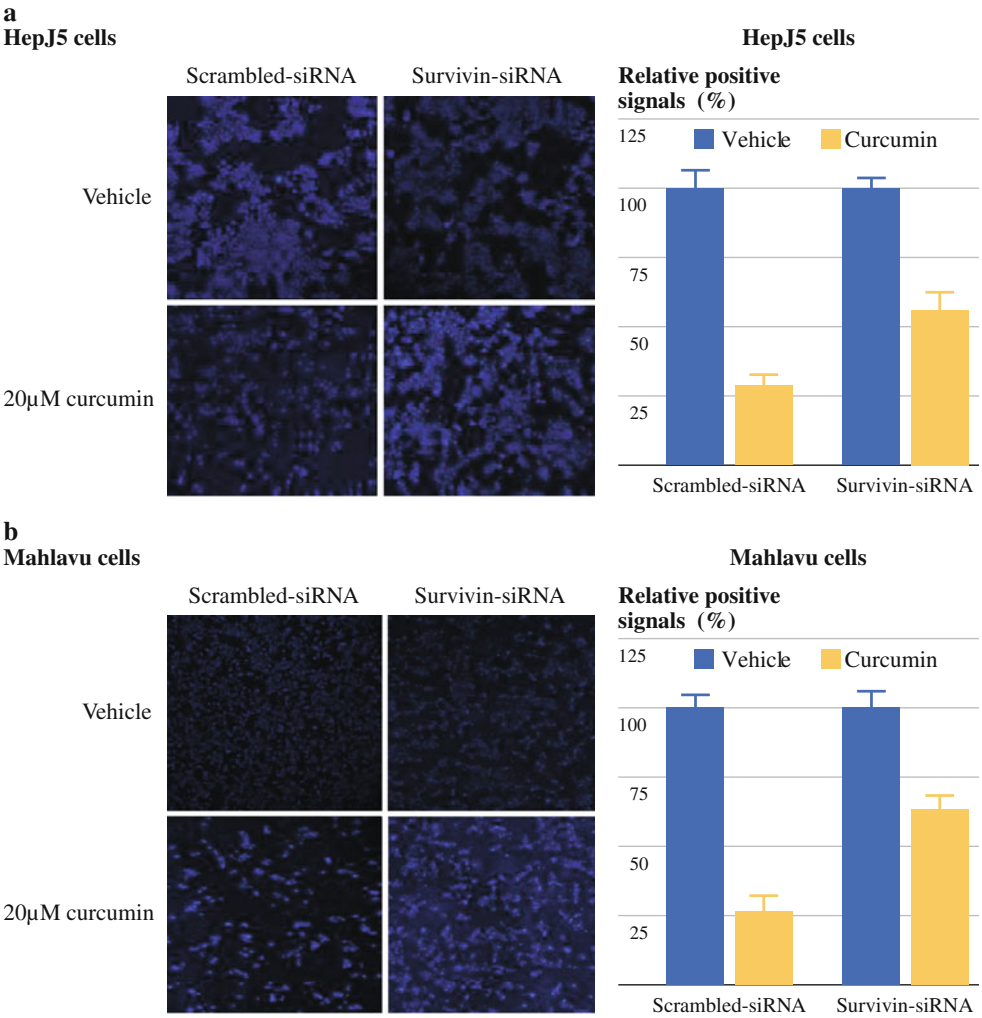

FIG. 4 Apoptosis in curcumin-treated HCC cells was examined using DAPI staining. Scrambled-siRNA and survivin-siRNA cells (2×10^5) were plated in 4-well chamber slides and incubated with curcumin (20 μM) for 48 h. The cells were fixed, stained with DAPI, and photographed under a fluorescence microscope

cells (Fig. 6). These data are consistent with the apoptosis data and suggest survivin suppression induces curcumin resistance by modulating Bcl-2 and Bax expression.

DISCUSSION

Survivin is an anti-apoptotic protein, and its expression is frequently observed in various cancers, such as lung, breast, pancreatic, and colon cancers.[27–29] Additionally, survivin is expressed in hepatocellular carcinoma, and upregulation of the survivin proto-oncogene correlates with HCC progression.[11,15] In this study, we also demonstrate that the expression level of survivin is higher in the poorly differentiated Mahlavu cell. Recently, survivin is regarded as a new cancer therapeutic target, and we found that suppression of survivin in HCC by siRNA resulted in decreased HCC growth.[30]

Curcumin and its analogs provide anticancer effects by suppressing various survival proteins, such as survivin.[31] In the era of tailored cancer treatment such as the cetuximab for colon cancer with wide type K-ras and the trastuzumab for breast cancer with HER-2 expression, there is little information regarding whether the expression level of survivin may alter the therapeutic efficacy of curcumin.[32,33] In this study, we investigated the effect of different survivin expression on curcumin-induced growth inhibition in HCC. Although suppression of survivin resulted in decreased HCC growth, it also made HCC cells more resistant to curcumin-induced growth inhibition (Fig. 2). These data demonstrate that survivin expression is an important factor involved in mediating curcumin-induced HCC cell growth inhibition.

It is still unclear why survivin suppression promotes curcumin resistance. Recent studies have shown that decreased survivin levels may contribute to the downregulation of GRP78.[30] Furthermore, GRP78 suppression reduces the therapeutic efficacy of curcumin in HCC.[21] Taken together, decreased survivin levels may reduce the efficacy of curcumin via GRP78.

Many anticancer compounds target survivin, but it is impossible to cure a complex disease such as cancer using

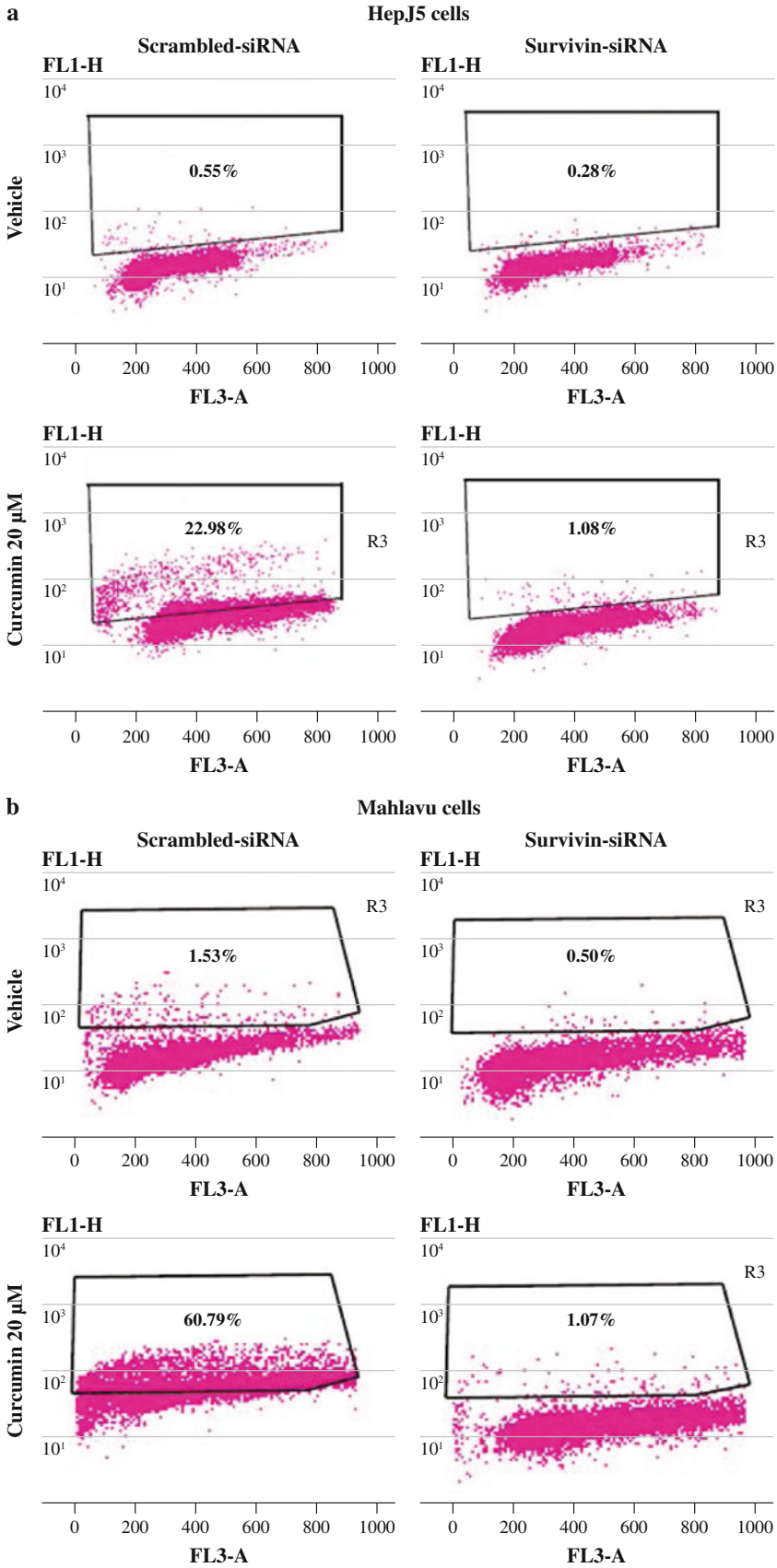

FIG. 5 TUNEL assays. The scrambled-siRNA and survivin-siRNA cells (3×10^5) were plated in 6-well plates and incubated overnight. The cells were treated with curcumin (20 μM) for 48 h. The morphology associated with cellular DNA fragmentation was detected using terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labeling (TUNEL) using an Apo-BrdU In Situ DNA Fragmentation Assay Kit, according to manufacturer's instructions. The TUNEL-positive cells were analyzed using flow cytometry. All of the experiments were independently repeated at least 3 times

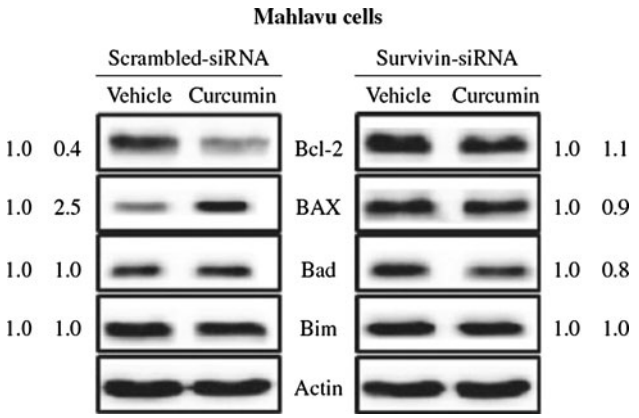

FIG. 6 Curcumin alters apoptosis-related signaling molecules in Mahlavu cells. Proteins were extracted from the scrambled-siRNA and survivin-siRNA cells following a 48-hour treatment with curcumin (20 μM). The expression levels of Bcl-2, Bax, Bad, and Bim were examined using western blotting. The expression of the Bcl-2 anti-apoptotic protein in the scrambled-siRNA cells was decreased following curcumin treatment. Curcumin did not alter Bax expression in the scrambled-siRNA or survivin-siRNA cells

a single drug. Combination therapies may become a trend in clinical cancer therapy. More information concerning the efficacy of particular drugs in combination with the expression status of target proteins, such as survivin, should translate to better treatment options for cancer patients. There is currently no clinical data evaluating the efficacy of curcumin against tumors with low levels of survivin expression. Such information may provide valuable insight into potential combination therapies involving curcumin and survivin-targeting compounds.

In summary, the extent of HCC malignancy is strongly associated with survivin expression. Furthermore, survivin expression alters the sensitivity of HCC cells to curcumin-induced cytotoxicity. Collectively, these data suggest that survivin expression may be a clinically relevant molecular target for HCC therapy. Analyzing survivin expression in HCC tumors prior to treatment should identify those patients who would benefit from survivin-targeting agents.

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