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Original Article

Ultrasound-targeted microbubble destruction promotes lipofectamine-mediated shRNA-survivin transfection efficiency thereby inhibiting survivin expression and inducing apoptosis of HepG2 cells

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Abstract: Hepatocellular carcinoma (HCC) is a common cause of cancer death worldwide, but there is currently no satisfactory therapy for HCC. Recent studies have shown that shRNA targeting survivin-induced apoptosis is an efficient, novel strategy for cancer gene therapy. Also, ultrasound-targeted microbubble destruction (UTMD), a new non-invasive method, has also been shown to be an efficient approach for tumor gene therapy. However, relative studies are rare. Therefore, the aim of the present study was to investigate the effect of combining UTMD with lipofectamine (Lipo) on transfection efficiency in human HepG2 cells. The results showed that the efficiency of short hairpin RNA (shRNA) transfer of the UTMD + Lipo + shRNA-survivin group was significantly higher than that of the other groups, while there was no significant difference in cell viability between the UTMD + Lipo + shRNA-survivin group and the single-UTMD + shRNA-survivin or the single-Lipo + shRNA-survivin group. Furthermore, expression levels of survivin mRNA and protein were much lower in the UTMD + Lipo group than those in the other groups. In addition, transfection of shRNA-survivin in the combination of UTMD with Lipo group markedly inhibited proliferation and induced apoptosis of HepG2 cells. In conclusion, UTMD promoted Lipo-mediated shRNA-survivin transfection efficiency, thereby inhibiting survivin expression and inducing apoptosis of HepG2 cells. These results present an improved strategy for gene therapy for HCC.

Keywords: UTMD, lipofectamine, survivin, cell death, HCC

Introduction

Hepatocellular carcinoma (HCC) is a common cause of cancer death worldwide and has become a leading health care expense [1]. In spite of the abundance of research, there is currently no satisfactory therapy for HCC other than surgical resection, conventional chemotherapy, radiotherapy, or liver transplantation for end-stage liver disease. However, the recurrence rate of HCC remains high after surgery and most patients are resistant to chemotherapy and radiotherapy [2, 3]. Therefore, it is urgent to develop more effective treatment strategies for HCC with fewer adverse effects.

With the rapid development of molecular biological techniques, gene therapy has been shown to be an efficient approach for the treatment of diverse diseases, including cancer [4]. RNA interference (RNAi) technology is a new tool for gene therapy with low toxicity, the main mechanism of which is transporting effective nucleic acid fragment into tumor cell, thereby inducing target homologous mRNA degradation [5, 6]. Compared to short interfering RNA (siRNA), which is chemically synthesized, short hairpin RNA (shRNA) expression vectors, which can establish stable expression of shRNA, are considered to be a more effective tool for anticancer therapy. However, technologies to safely and efficiently transport exogenous genes into target cells need to be improved. Furthermore, it also has been demonstrated that the gene delivery system plays a key role in the success of gene therapy. Viral and non-viral vectors have been widely tested in cancer gene therapy, but caveats are associated with both [7, 8]. More recently, ultrasound-targeted microbubble destruction (UTMD), a new non-invasive method, has been advocated as a means to improve the efficiency of non-viral vector sys-

tems. UTMD has been found to increase cell membrane permeability to facilitate more efficient gene delivery than the use of viral vectors [9, 10]. The efficiency of gene delivery is remarkably increased by UTMD in combination with other transfection strategies, such as the use of polyethylenimine and cationic polymers [11, 12]. Additionally, the transfection reagent lipofectamine (Lipo) has been widely used for gene delivery. However, little information is available regarding the effects of UTMD on Lipo-mediated gene delivery.

Moreover, previous studies have indicated that survivin is an inhibitor of apoptosis that is overexpressed in the majority of tumors, especially HCC, which justifies its role as a rational target for HCC therapy [13]. In addition, shRNA targeting survivin-induced apoptosis has also been considered as an efficient, novel strategy for cancer gene therapy [14-16]. shRNA expression vectors could be delivered by UTMD especially when combined with Lipo systems, but related studies are rare [17]. Thus, the aim of the present study was to assess the efficiency of shRNA-survivin transfection and the effect of survivin inhibition mediated by UTMD combined with Lipo as well as subsequent cell death induced by survivin downregulation in the human HCC cell line HepG2. The results of this study suggest that the Lipo-mediated shRNA-survivin transfection efficiency can be significantly enhanced by UTMD to inhibit survivin expression and induce apoptosis of HepG2 cells. These results offer an improved strategy for use of gene therapy for the treatment of HCC.

Materials and methods

Cell culture

HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and a 1% mixture of penicillin G and streptomycin (Gibco). Cells were incubated at 37°C in an atmosphere containing 5% CO_2.

Construction of recombinant plasmid targeting survivin

A 21-mer oligonucleotide sequence (sense, 5'-GGACCACCGCAUCUCUACAdTdT-3') [18, 19] was selected to target the human survivin gene (GenBank accession no. NM_001168). In addition, and a non-homologous, non-coding sequence (sense, 5'-CGUACGCGGAAUACUUCGAdTdT-3') was constructed [20]. All sequences and corresponding recombinant green fluorescent protein (GFP) plasmids were synthesized by Shanghai Invitrogen Biotechnology Co., Ltd. (Shanghai, China). Extracted and purified plasmids (> 1.0 μg/μL) with an optical density (OD) ratio at 260/280 nm of > 1.8 were subjected to restriction enzyme digestion and DNA sequencing.

Preparation of lipid microbubbles (LMs)

LM ampoules were newly manufactured by the Department of Ultrasound, Xinqiao Hospital, the Third Military Medical University, Chongqing, China, and mechanically shocked for 2-3 min at a rate of 5000 ± 100 oscillations/min to obtain an LM suspension. The oscillation amplitude was 20 ± 5 mm using reciprocating motion.

Optimization of UTMD parameters

Plasmids at a final concentration of 20 μg/mL were added to each group of cells before exposure. Cells were treated at ultrasonic intensities of 0.4, 0.8, 1.2 and 1.6 W/cm^2 with a duty cycle (DC) of 10%, 20%, or 50% at each intensity. Cell groups with the highest luciferase activity were exposed for various durations (30, 90, and 180 s) to LM at a series of concentrations (50, 100, 150, and 250 μL/mL). Then, cell viability and GFP expression were detected.

Experimental grouping and gene delivery

After the cells were washed three times with phosphate-buffered saline (PBS) solution, 1 mL of antibiotic-free DMEM medium was added to each well for following groups of cells: blank control group (Group A), shRNA-suvivin group (Group B), UTMD + shRNA-survivin group (Group C), Lipo + shRNA-survivin group (Group D), and UTMD + Lipo + shRNA-survivin group (Group E). Positive recombinant plasmids (0.8 μg/well) were added to groups B, C, D and E, while Lipo 2000 was added to groups D and E at a dose of 2 μL/well. After incubating at room temperature for 5 min, the cells were mixed and then incubated for an additional 20 min. Then, LMs (200 μL/well) were added to groups C and E. The ultrasound exposure parameters for groups

C and E were optimized in this study. Three wells of each group were sequentially sonicated using a probe close to the bottom of the culture plate using Anshin ultra as a coupling agent. After irradiation, the plate bottom was wiped and the plate was placed back into the incubator. After incubation for 5-6 h, each well was rinsed with PBS solution to remove nonviable cells and cell debris. Then, the medium was replaced with 0.5 mL of fresh DMEM solution without plasmids. The data were obtained from at least four independent experiments.

Cell viability measurement

A Cell Counting Kit-8 (CCK-8) detection kit (Tongren Chemical Institute, Japan) was used to measure cell viability according to the manufacturer's protocols. HepG2 cells were treated as indicated in section 2.5. Briefly, 1000 cells were plated in each well of a 96-well plate and CCK-8 solution (20 μL/well) was added to the wells at various time-points and the plate was incubated at 37°C for another 2 h. Subsequently, viable cells were counted by absorbance measurements with a monochromator microplate reader (Safire II; Tecan Schweiz AG, Männedorf, Switzerland) at a wavelength of 450 nm. The OD value at 450 nm was reported as the percentage of viable cells in relation to the control group (set as 100%).

Detection of the transfection efficiency

At 24 h after transfection, GFP expression was observed under a confocal microscope. Then, the cells were digested, suspended in PBS buffer, and centrifuged at 1000 rpm for 5 min to discard the supernatant. After repeating this process, the cell suspension was adjusted to the proper concentration and transfection efficiency was quantified by flow cytometry.

Quantitative real-time PCR (qPCR) assay

After transfection for 48 h, total RNA was extracted from cultured cells for reverse transcription qPCR using the SYBR Premix Ex Taq TMII (Perfect Real Time) kit (Takara Bio, Inc., Shiga, Japan). A pair of specific primer sequences (forward: 5'-ACCACCGCATCTCTACATTC-3', reverse: 5'-AGTCTGGCTCGTTCTCAGTG-3') was used to amplify human survivin cDNA. The predicted size of the product was 113 bp. A 25 μL reaction containing 4 μg of cDNA, 12.5 μL of SYBR Master mix, and 1 μL of each primer was amplified with an initial denaturation step at 95°C for 10 min followed by 45 cycles of amplification at 95°C for 15 s and 60°C for 1 min. All amplification reactions were performed in triplicate and the averages of the threshold cycles (Ct) were used to interpolate curves using 7300 System SDS RQ Study Software (Thermo Fisher Scientific Inc., Waltham, MA, USA). Results are presented as the ratio of each mRNA compared with the mRNA expression level of glyceraldehyde 3-phosphate dehydrogenase.

Western blot analysis

Total cell lysate was analyzed by western blot analysis as previously described [21]. Briefly, 30-50 μg of protein were resolved by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electroblotted onto polyvinylidene difluoride membranes for western blot analysis. Blots were probed with 1:1,000-diluted primary antibodies overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies. After the proteins were visualized by enhanced chemiluminescence exposure to X-ray film, the blots were scanned and densitometric analysis was performed on the scanned images using Scion Image-Release Beta 4.02 software (http://scion-corporation.software.informer.com/).

Flow cytometry assay for cell apoptosis

Cells were harvested, washed three times with ice-cold PBS, and assessed for apoptosis using an annexin-V-fluorescein isothiocyanate and propidium iodide double staining kit (Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's instructions. Cell apoptosis was analyzed on a FACScan flow cytometer (Becton Dickinson & Co., Franklin Lakes, NJ, USA).

Statistical analysis

Data are presented as the mean ± standard deviation (SD). The statistical analysis was conducted with the t-test and one-way analysis of variance using SPSS ver. 18.0 software (IBM-SPSS, Inc., Chicago, IL, USA). A probability (*P*) value of < 0.05 was considered statistically significant.

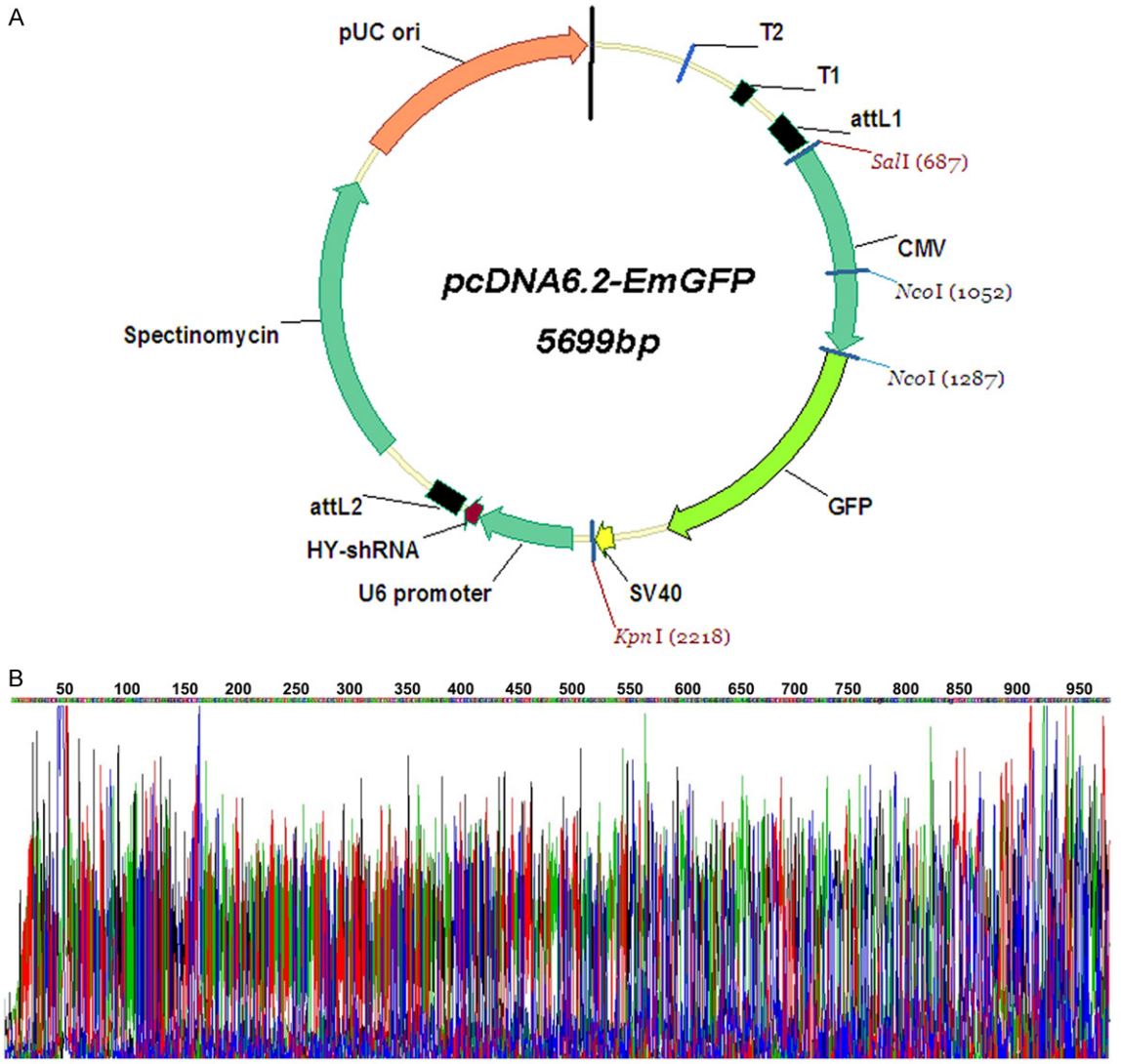

Figure 1. Plasmid identification. A. Schematic diagram of shRNA-survivin recombinant plasmids. B. Sequence analysis revealed that shRNA template fragment targeting survivin gene was successfully inserted into the plasmid.

Results

Plasmid identification results

Restriction enzyme digestion of recombinant plasmids yielded a target band of approximately 5.7 kb, consistent with the expected fragment size. Sequence analysis revealed that the shRNA template fragment targeting the survivin gene was successfully inserted to RNAi-specific plasmids, which confirmed the reliability of the recombinant plasmid (**Figure 1A** and **1B**).

Optimization of UTMD parameters

As shown in **Figure 2A**, an ultrasonic intensity of up to 1.2 W/cm^2 was tolerated by HepG2 cells, which yielded a transfection efficiency of about 19.47 ± 1.91% (**Figure 2B** and **2C**). When the ultrasonic intensity was fixed at 1.2 W/cm^2, a DC of up to 20% was tolerated by HepG2 cells (**Figure 2D**) and the transfection efficiency was about 21.4 ± 2.52% (**Figure 2E** and **2F**). Furthermore, exposure time also affected cell viability within a certain range. Exposure of no more than 90 s was tolerated by HepG2 cells when ultrasonic intensity was fixed at 1.2 W/cm^2 with a DC of 20% (**Figure 2G**). The transfection efficiency was about 22.10 ± 2.16% after ultrasound exposure for 90 s (**Figure 2H** and **2I**). The addition of LMs at concentrations of up to 150 μL/mL was tolerated by HepG2 cells (**Figure 2J**). The transfection efficiency

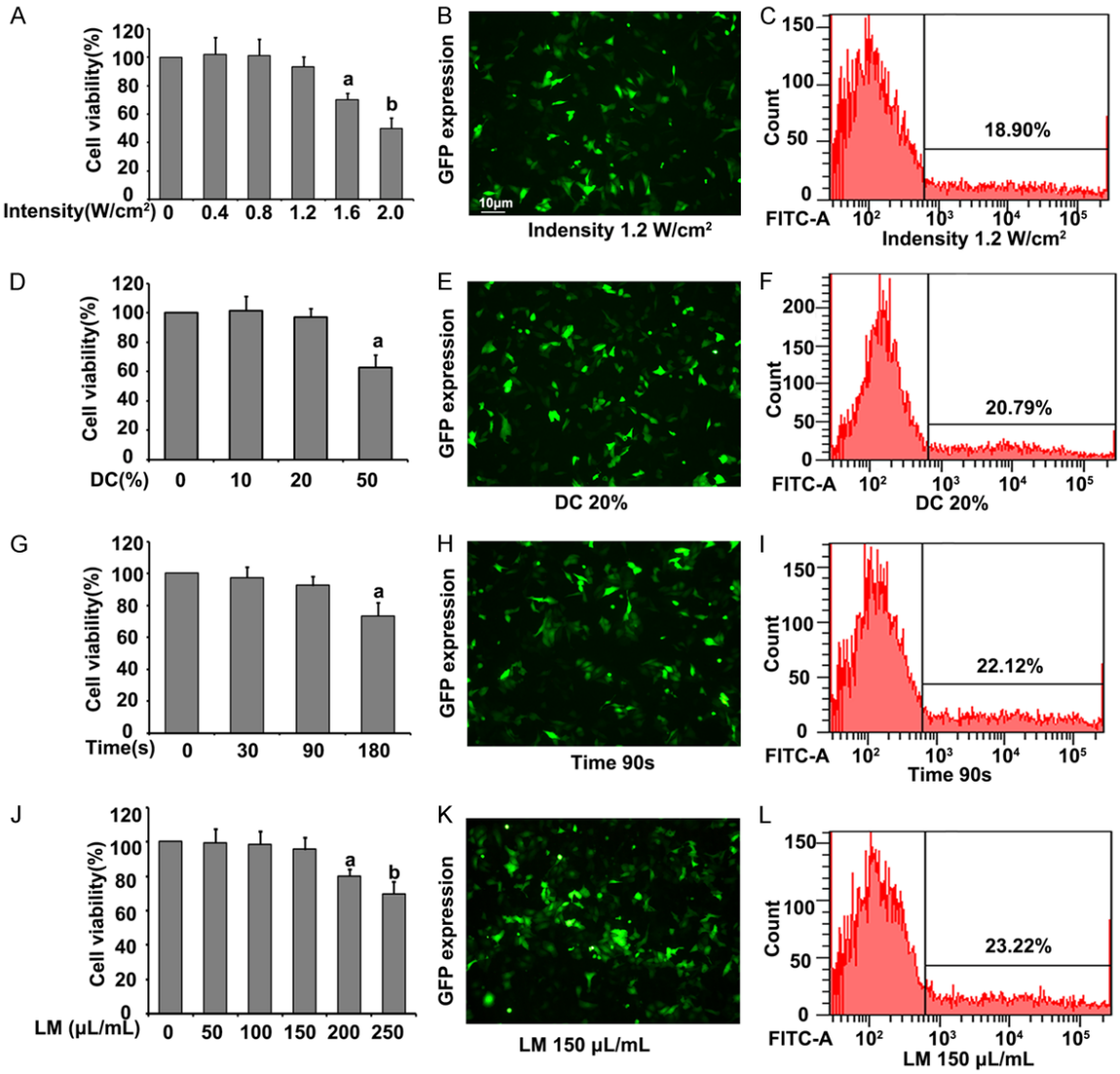

Figure 2. Optimization of UTMD parameters. Optimization of ultrasonic intensity. A. Cell viability of different experimental groups was detected using the CCK-8 kit. B. Transfection efficiency of HepG2 cells exposed to ultrasound at an intensity of 1.2 W/cm^2 was observed by confocal microscopy. C. Transfection efficiency of HepG2 cells exposed to ultrasound at an intensity of 1.2 W/cm^2 was observed by flow cytometry. Optimization of DC. D. Cell viability of different experimental groups was detected using the CCK-8 kit; E. Transfection efficiency of HepG2 cells exposed to ultrasound at an intensity of 1.2 W/cm^2 and DC of 20%, as observed by confocal microscopy. F. Transfection efficiency of HepG2 cells exposed to ultrasound at anintensity of 1.2 W/cm^2 and DC of 20%, as observed by flow cytometry. Optimization of exposure time. G. Cell viability of different experimental groups was detected using the CCK-8 kit. H. Transfection efficiency of HepG2 cells exposed to ultrasound at an intensity of 1.2 W/cm^2 and DC of 20% for 90 s, as observed by confocal microscopy. I. Transfection efficiency of HepG2 cells exposed to ultrasound at an intensity of 1.2 W/cm^2 and DC of 20% for 90 s, as observed by flow cytometry. Optimization of LM concentration. J. Cell viability of different experimental groups was detected using the CCK-8 kit. K. Transfection efficiency of HepG2 cells exposed to ultrasound at an intensity of 1.2 W/cm^2 and DC of 20% with LM (150 μL/mL) for 90 s was observed by confocal microscopy. L. Transfection efficiency of HepG2 cells exposed to ultrasound at anintensity of 1.2 W/cm^2 and DC of 20% with LM (150 μL/mL) for 90 s, as observed by flow cytometry. Values are presented as means ± SD (n = 4); $^aP < 0.05$, $^bP < 0.01$ versus the vehicle-treated control group.

was about 23.67 ± 2.07% at an LM concentration of 150 μL/mL (**Figure 2K** and **2L**). Thus, ultrasound at 1.2 W/cm^2 with DC of 20% combined with LM (150 μL/mL) for 90 s was set as the optimal parameters to treat cells in the following experiments.

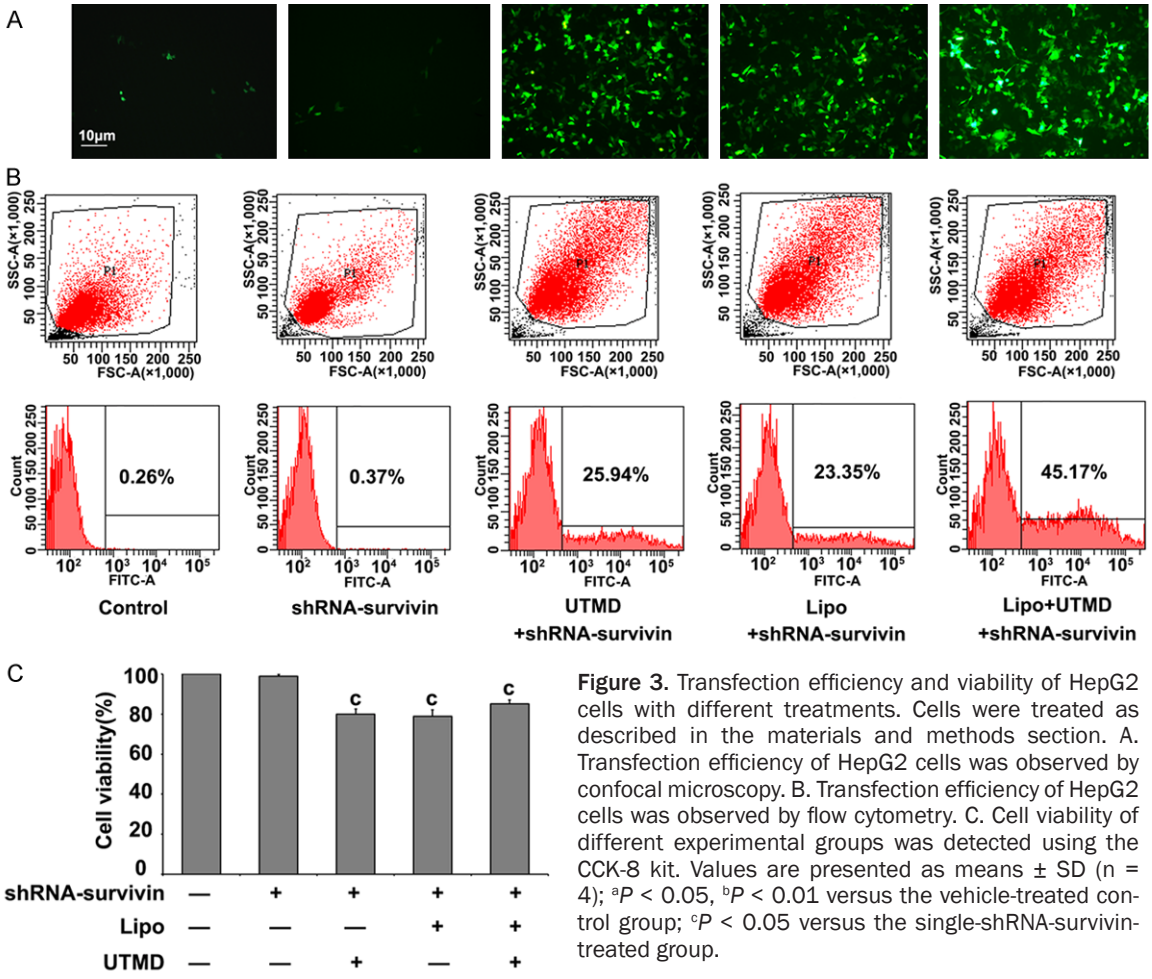

Figure 3. Transfection efficiency and viability of HepG2 cells with different treatments. Cells were treated as described in the materials and methods section. A. Transfection efficiency of HepG2 cells was observed by confocal microscopy. B. Transfection efficiency of HepG2 cells was observed by flow cytometry. C. Cell viability of different experimental groups was detected using the CCK-8 kit. Values are presented as means ± SD (n = 4); $^{a}P < 0.05$, $^{b}P < 0.01$ versus the vehicle-treated control group; $^{c}P < 0.05$ versus the single-shRNA-survivin-treated group.

Transfection of shRNA-survivin with lipo in combination with UTMD had the highest transfection efficiency in HepG2 cells

To determine the effect of combining UTMD with Lipo transfection on transfection efficiency in HepG2, the cells were divided into five groups: untreated, shRNA-survivin, shRNA-survivin with Lipo, shRNA-survivin with UTMD, and shRNA-survivin with both Lipo and UTMD. Transfection efficiency was first determined by observing fluorescent cells under a confocal microscope, which showed that the Lipo combined with UTMD group had the highest transfection efficiency, which was also significantly higher than that of single-Lipo group (**Figure 3A**). Furthermore, fluorescence intensity and the proportion of transfected cells were quantified by flow cytometry. No fluorescence was detected in the untreated group. The percentages of transfected (fluorescent) cells were 0.69 ± 0.18% in the shRNA-survivin alone group, which was not statistically different from that of the control. In contrast, the percentage of positive cells in the UTMD + Lipo + shRNA-survivin group was significantly higher than that of the Lipo + shRNA-survivin group and the UTMD + shRNA-survivin group (43.22 ± 5.23% vs. 25.55 ± 1.39% and 23. 78 ± 2.14%, respectively; **Figure 3B**). As indicated in **Figure 3C**, the cell viabilities of the Lipo + shRNA-survivin group, UTMD + shRNA-survivin group, and the UTMD + Lipo + shRNA-survivin group were significantly decreased, as compared to the untreated group. However, these differences in cell viability among the three groups were not significant. These results suggested that UTMD effectively induced Lipo-mediated shRNA transfection.

Transfection of shRNA-survivin in combination with UTMD and lipo resulted in the highest degree of survivin downregulation

To further investigate the effects of combining UTMD with Lipo transfection on survivin expres-

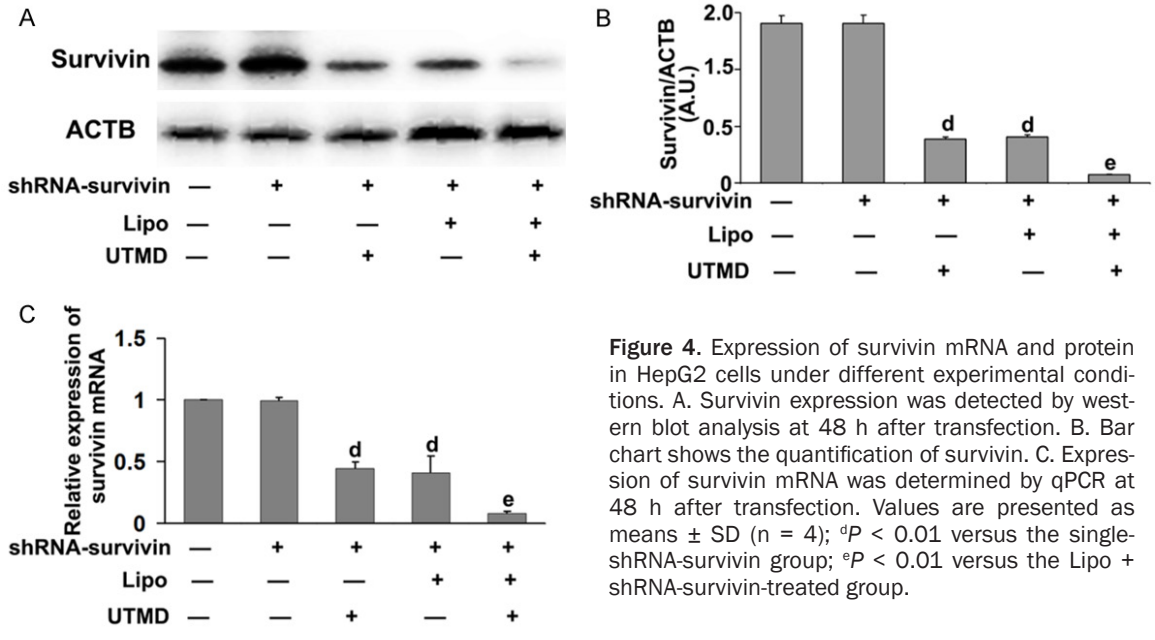

Figure 4. Expression of survivin mRNA and protein in HepG2 cells under different experimental conditions. A. Survivin expression was detected by western blot analysis at 48 h after transfection. B. Bar chart shows the quantification of survivin. C. Expression of survivin mRNA was determined by qPCR at 48 h after transfection. Values are presented as means ± SD (n = 4); $^{d}P < 0.01$ versus the single-shRNA-survivin group; $^{e}P < 0.01$ versus the Lipo + shRNA-survivin-treated group.

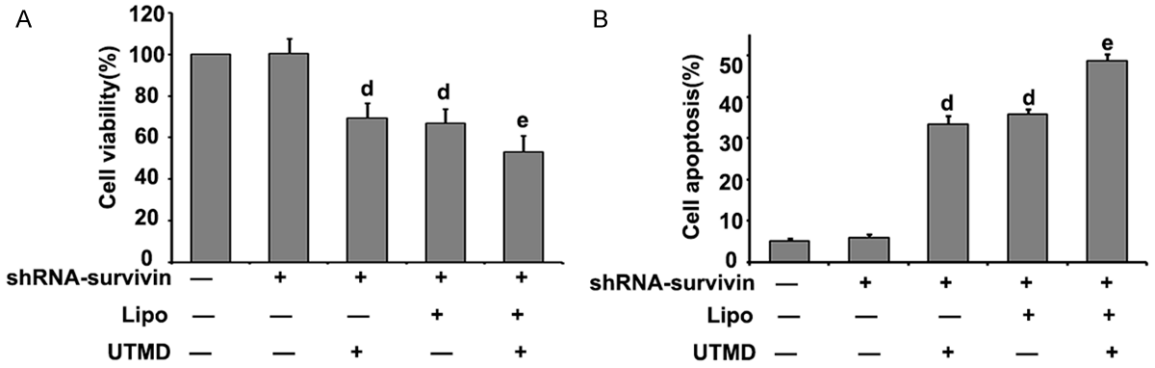

Figure 5. Apoptosis and viability of HepG2 cells at 48 h after different treatments. A. Viability of transfected-HepG2 cells was detected using the CCK-8 assay. B. Apoptosis of transfected-HepG2 cells was detected by flow cytometry. Values are presented as means ± SD (n = 4); $^{d}P < 0.01$ versus the single-shRNA-survivin group; $^{e}P < 0.01$ versus the Lipo + shRNA-survivin-treated group.

sion in HepG2 cells, expression levels of survivin mRNA and protein were detected by qPCR and western blotting, respectively. The results showed that survivin expression was evidently lower in the Lipo + shRNA-survivin group than that in the single-shRNA-survivin group (**Figure 4A** and **4B**). There was no difference in survivin expression between the Lipo + shRNA-survivin group and UTMD + shRNA-survivin group (**Figure 4A** and **4B**). However, the UTMD + Lipo + shRNA-survivin group had the most significant inhibitory effects on survivin expression in HepG2 cells, with survivin levels markedly lower than those of the UTMD + shRNA-survivin group or the Lipo + shRNA-survivin group (**Figure 4A** and **4B**). In addition, the same results were found for survivin mRNA expression (**Figure 4C**). These results suggested that UTMD induced Lipo-mediated shRNA transfection targeting survivin and efficiently inhibited survivin expression.

UTMD combined with lipo increased shRNA-mediated inhibition of survivin there by promoting apoptosis of HepG2 cells

As survivin expression was markedly inhibited by shRNA-survivin transfection in combination with UTMD and Lipo, cell viability and apoptosis caused by survivin downregulation were then

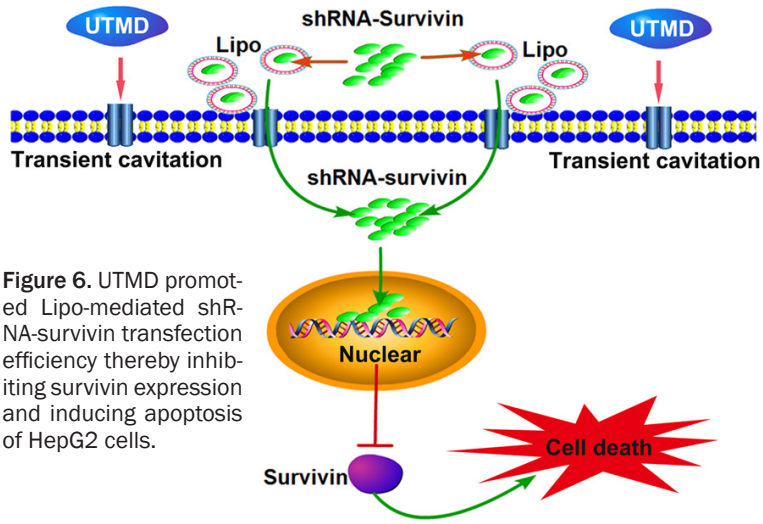

Figure 6. UTMD promoted Lipo-mediated shRNA-survivin transfection efficiency thereby inhibiting survivin expression and inducing apoptosis of HepG2 cells.

investigated. Cells were treated as described in the material and methods section and cell viability and apoptosis were detected at 48 h following treatments. The results showed that cell viability was lowest in the UTMD + Lipo + shRNA-survivin group, which was lower than that of the Lipo + shRNA-survivin group or the UTMD + shRNA-survivin group (**Figure 5A**). Furthermore, the same results were found in the apoptotic rate of HepG2 cells (**Figure 5B**). These results confirmed that UTMD promoted Lipo-mediated shRNA transfection by efficiently targeting survivin, thereby inhibiting survivin expression and inducing apoptosis of HepG2 cells.

Discussion

HCC is one of the most common malignant tumors worldwide and is associated with high morbidity and mortality rates [1]. However, there is currently no satisfactory therapy for HCC. It has been demonstrated that apoptosis is dysregulated in tumor cells, which results in resistance to cell death. Hence, the induction of apoptosis is considered as a major strategy for anti-tumor therapy [22]. Survivin is a member of the mammalian inhibitor of apoptosis protein family, which has become the best studied anti-apoptotic protein [13]. Survivin is overexpressed in the majority of tumors, including HCC, and is closely associated with tumor cell differentiation, proliferation, invasion, and metastasis. Therefore, targeted inhibition of the survivin gene is particularly attractive as an ideal anti-cancer strategy both in vitro and in vivo [23]. Recently, RNAi, which is highly efficient and specific for blocking expression of target genes, has become a novel promising strategy for cancer gene therapy and antivirus and gene drug selection [24, 25]. Moreover, use of shRNA expression vectors is considered to be more effective than siRNA. Also, shRNA targeting survivin-induced apoptosis has also been considered as an efficient, novel strategy for cancer gene therapy. Previous studies have found that shRNA plasmid transfection targeting survivin inhibits gene expression and induces apoptosis in cervical cancer cells both in vitro and in vivo [26, 27]. The results of the present study showed that shRNA-survivin transfection significantly inhibited survivin expression and induced apoptosis of HepG2 cells. A recent study showed that shRNA-mediated SOX9 downregulation effectively inhibited cell proliferation and induced apoptosis in the human HCC cell line Hep3B [28]. These results indicate that shRNA-mediated gene downregulation could be a predominant strategy in HCC therapy.

Sonoporation is the main mechanism of UTMD, which is a non-invasive approach for transfer of DNA or drugs into cells, by producing reversible small holes in the cell membrane, as observed by scanning electron microscopy [29]. A number of studies have shown that the great power released by "transient cavitation" functions as a driving force [30, 31]. The shock waves and jet flow generated by the bursting of LMs destroys the local integrity of the cell membrane, induces reversible permeability of the cell membrane, and promotes DNA penetration, thereby improving genetic transduction efficiency [32]. UTMD has several advantages over other methods, such as low cytotoxicity, low immunogenicity, and repeatable applicability. A previous report [33] indicated that UTMD was a useful tool for gene delivery, while the combination of UTMD with other transfection strategies (such as the use of polyethylenimine or cationic polymers) could obtain higher transfection rates than the use of polyethylenimine or cationic polymers alone for shRNA delivery [34, 35]. Moreover, Lipo has been widely used for gene delivery. Koo et al. [36] used Lipo to transfer microRNA-145 into human glioblasto-

ma cells and Wang et al. [37] reported that this reagent was useful for transfection. However, relevant studies about the effect of UTMD on Lipo-mediated gene delivery are currently rare. Therefore, the aim of the present study was to determine whether the combination of UTMD and Lipo could improve shRNA intracellular delivery. As expected, the average transfection rate was 43.22% ± 5.23% in the UTMD + Lipo + shRNA-survivin group, which was the highest rate of all measured groups. Exposure to Lipo alone or UTMD + Lipo resulted in negligible differences in cell membrane injury and induced no significant difference in cell viability. These findings indicate that the combined use of these two approaches can significantly improve gene transfection efficiency, while not increasing the rate of cell death. Moreover, Zheng et al. [38] used microbubbles to promote Lipo-mediated siRNA transduction to rat retina and Xue et al. [39] transduced siRNA into HepG2 cells, and came to the same conclusion that the combination of Lipo, microbubbles, and ultrasound exposure effectively reduced target gene expression. The results of the present study complemented previous work on combining UTMD with RNAi transfection, which reduced toxicity to normal cells and may open up avenues to new therapeutic strategies for HCC-directed therapy.

These results showed that the use of UTMD with Lipo could be an effective strategy for cancer gene therapy and the use recombinant shRNA as vectors presents a powerful technique for gene analysis, which led to significant suppression of survivin protein expression in vitro (**Figure 6**). Thus, the survivin gene could be an ideal target for cancer genetic therapy in HCC. However, further in vivo research is required to investigate whether this approach is suitable for efficient, specific, and noninvasive gene transfer in animals and humans.

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Disclosure of conflict of interest

None.

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