

# A new apatinib microcrystal formulation enhances the effect of radiofrequency ablation treatment on hepatocellular carcinoma

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**Introduction:** Radiofrequency ablation (RFA) is the foremost treatment option for advanced hepatocellular carcinoma (HCC), however, rapid and aggressive recurrence of HCC often occurs after RFA due to epithelial–mesenchymal transition process. Although combination of RFA with sorafenib, a molecular targeted agent, could attenuate the recurrence of HCC, application of this molecular targeted agent poses a heavy medical burden and oral administration of sorafenib also brings severe side effects.

**Materials and methods:** In this study, we prepared an apatinib microcrystal formulation (Apa-MS) that sustainably releases apatinib, a novel molecular targeted agent, for advanced HCC treatment. We injected apatinib solution or Apa-MS into subcutaneous HCC tumors.

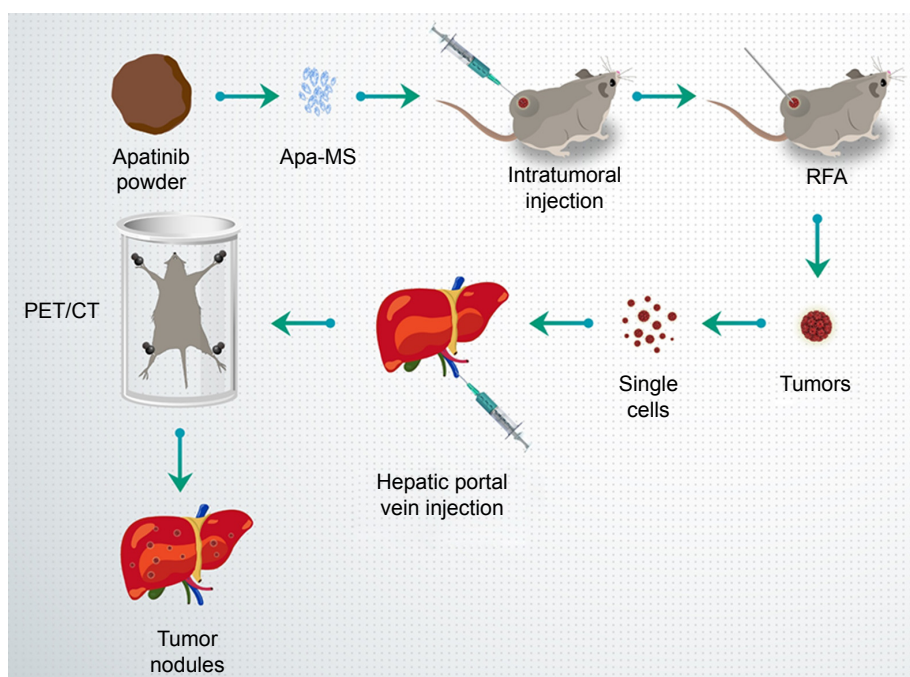
**Results:** It was found that Apa-MS exhibited slow apatinib release in vivo and in turn inhibited the epithelial–mesenchymal transition of HCC cells for extended time. Moreover, in rodent HCC model, Apa-MS enhanced the antitumor effect of RFA treatment.

**Conclusion:** Based on these results, we conclude that Apa-MS, a slow releasing system of apatinib, allows apatinib to remain effective in tumor tissues for a long time and could enhance the antitumor effect of RFA on HCC.

**Keywords:** apatinib microcrystals, radiofrequency ablation, sustained releasing behavior, long-acting efficiency, epithelial–mesenchymal transition, interventional therapy

## Introduction

HCC poses a heavy medical burden worldwide, especially in the People's Republic of China, due to the high infection rate of hepatitis viruses (hepatitis B or C virus).<sup>1,2</sup> Patients often suffer from advanced HCC due to lack of early diagnostic techniques.<sup>3,4</sup> RFA can selectively destroy HCC tissues, and hence, it is believed to be a suitable treatment option for advanced HCC patients with cirrhosis and compromised liver function.<sup>5–7</sup> However, rapid/aggressive recurrence of HCC after RFA treatment is a major drawback of this approach.<sup>8,9</sup> It has been reported that the EMT induced by RFA would be a major reason of HCC recurrence post-RFA, and combination of RFA and molecular targeted agent sorafenib could help to inhibit this process.<sup>10,11</sup> However, there are some obstacles for sorafenib application: 1) advanced HCC patients often have compromised gastrointestinal digestive function that attenuates the absorption of orally administered sorafenib;<sup>12–14</sup> 2) oral administration of sorafenib will result in systemic distribution, making local drug concentration insufficient;<sup>15</sup> and 3) the high daily dose (over 800 mg) imposes a heavy financial burden.<sup>15</sup> Therefore, it is valuable to develop novel and more effective strategies to enhance the antitumor effect of RFA on HCC.



**Figure 1** Workflow of the study. Apatinib powder was formulated to Apa-MS and injected into HCC tumors, and then tumors were treated by RFA. After 2–4 weeks, tumors were harvested, and single cells were separated and injected into liver via hepatic portal vein. The nodules in liver formed by HCC cells were measured by PET/CT screening.

**Abbreviations:** Apa-MS, apatinib microcrystal formulation; HCC, hepatocellular carcinoma; RFA, radiofrequency ablation; PET/CT, positron emission tomography/computed tomography.

Apatinib is a newly approved molecular targeting drug for the treatment of advanced HCC.<sup>16,17</sup> However, apatinib is insoluble in water, and the daily dose of apatinib-mesylate tablets is very high (over 850 mg).<sup>18,19</sup> Microcrystallization is a pharmaceutical process that converts drug powder into microcrystals with a diameter of 30–50  $\mu\text{m}$ .<sup>20</sup> Microcrystals have been used to improve the absorption of orally administered drugs due to the fact that microcrystals can fully contact and mix with digestive fluid.<sup>20</sup> Here, for the first time, we report the preparation of a microcrystal formulation that extends the release time of apatinib in vivo. By preparing Apa-MS and studying its antitumor effect in tumor models, we found apatinib was slowly released from Apa-MS in vivo. This approach could overcome the limitation of solubility of apatinib and condense drug concentration in tumor without affecting the surrounding tissue. We also examined whether single-dose administration of Apa-MS could achieve accurate apatinib delivery and enhance the antitumor effect of RFA on HCC cells. Figure 1 presents the workflow of the study.

## Materials and methods

### Cell line and agents

MHCC97-H cells (a highly aggressive HCC cell line) was purchased from the Type Culture Collection of the Chinese

Academy of Sciences and maintained under conditions described in our previous work.<sup>21,22</sup> Apatinib was a gift from Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, People's Republic of China. Sodium dodecyl sulfate, dimethyl sulfoxide, polyethylene glycol 400 and Tween 80 were purchased from Sigma-Aldrich Corporation, St Louis, MO, USA.

### Preparation of apatinib formulations

For preparing Apa-Sol, apatinib was dissolved in DMSO, PEG400 and Tween 80. Then, the solution was carefully and slowly diluted with physiological saline, accompanied by ultrasonic treatment or churning. The ultimate concentrations of DMSO, PEG400 and Tween 80 in solution were 1%, 4% and 4%, respectively, and the concentration of apatinib was almost 1 mg/mL. For preparing apatinib microcrystals,<sup>23</sup> 1.5 g apatinib was dispersed into 50 mL aqueous solution with 6.25% Tween 80 and mixed by magnetic stirring to obtain a relatively uniform coarse suspension. The microcrystals were prepared using a MiniZeta (NETZSCH Machinery and Instruments Co., Ltd., Burlington, MA, USA) machine equipped with the grinding media of yttrium-stabilized zirconium oxide beads (0.6 mm in diameter). The coarse suspension was transferred to the milling bowl, and the agitator speed was set at 3,000 rpm. The apatinib concentration in Apa-MS was almost 30 mg/mL.

## Subcutaneous tumor

All the animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the 302nd Hospital of People's Liberation Army, Beijing, People's Republic of China. Nude SCID mice aged 4–6 weeks were purchased from Si-Bei-Fu Biotechnology Corporation, Beijing, People's Republic of China. All animal studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines. To produce a subcutaneous tumor model,<sup>24</sup> MHCC97-H cells were injected into nude SCID mice ( $1 \times 10^6$  cells per animal). After 2–3 weeks, the tumoral volume reached almost 1,200 mm<sup>3</sup>.

## Pharmacokinetic experiments of apatinib formulations

For in vitro release experiments, the release behavior of Apa-MS was identified by vortex shock method. Apa-MS was added to 10 mL physiological saline with 0.1% Tween 80 and 0.9% NaCl. At different time points, 1 mL solution was sucked out for analysis, and the volume was made up to 10 mL. The release of apatinib into physiological saline at each time point was identified. For in vivo release experiments, 50  $\mu$ L Apa-Sol or Apa-MS was intratumorally injected into subcutaneous tumor formed by MHCC97-H cells. At indicated time points, tumor tissues were harvested, and apatinib was extracted using acetonitrile. The amount of apatinib was identified by liquid chromatography–tandem mass spectrometry following the methods described by Allard et al<sup>25</sup> and He et al.<sup>26</sup>

## RFA treatment

MHCC97-H cells were injected into nude mice ( $1 \times 10^6$  cells per animal). After 4–6 weeks, the tumoral volume reached almost 1,200 mm<sup>3</sup>. RFA of the subcutaneous tumors was performed using a thyroid ablation needle (cat. no UniBlate 700-103587 17G; RITA Company, Crystal Lake, IL, USA). The ablation time was 3–5 minutes, and the temperature was 65°C–70°C. After 2–3 weeks of RFA treatment, the tumoral volume was calculated using the following formula: width<sup>2</sup>  $\times$  length/2. Tumors were harvested 16 days after RFA treatment, and their weights were measured.

## qPCR experiments

Total RNA of cells or clinical specimens was extracted using a PARIS™ kit (Applied Biosystems; Thermo Fisher Scientific Corporation, Waltham, MA, USA) and reverse transcribed by MultiScribe™ Reverse Transcriptase (Applied

**Table 1** Primers used in this work

Genes	Primer sequence (5'–3')
E-cadherin	Forward: 5'-AAGGCACGCCTGTCTGAAGCA-3' Reverse: 5'-ACGTTGTCCCGGGTGTCTATCCT-3'
N-cadherin	Forward: 5'-TGCGCGTGAAGGTTTGCCAGT-3' Reverse: 5'-TGGCGTTCTTTATCCCGGCGT-3'
Vimentin	Forward: 5'-ACCGCACACAGCAAGGCGAT-3' Reverse: 5'-CGATTGAGGGCTCTAGCGGTT-3'

Biosystems; Thermo Fisher Scientific Corporation). The qPCR experiments were performed as described previously.<sup>27</sup> The level of  $\beta$ -actin mRNA was measured as an internal control. Primers used in qPCR are listed in Table 1.

## Intrahepatic tumor model

To produce an in situ liver tumor model,<sup>28,29</sup> HCC cells were separated from subcutaneous tumors formed by MHCC97-H cells and directly inoculated into the right lobe of the liver of mice ( $1 \times 10^5$  cells per animal). After 4–8 weeks, nude mice were injected intravenously with 100  $\mu$ Ci of <sup>18</sup>F-FDG, and the animals were examined using a PET/CT scanner (Philips Corp., Amsterdam, the Netherlands). Two-minute CT and 10-minute PET scans were performed 45 minutes after the <sup>18</sup>F-FDG injection. A NaI (TI) well counter (China Atom Corp., Beijing, People's Republic of China) was used to measure the radioactivity of organs (liver) and blood.<sup>29,30</sup> The liver organ photographs were analyzed using ImageJ Software (version 1.51j8; National Institutes of Health, Bethesda, MD, USA). The percentage of red areas indicated the relative HCC amount.<sup>29,30</sup>

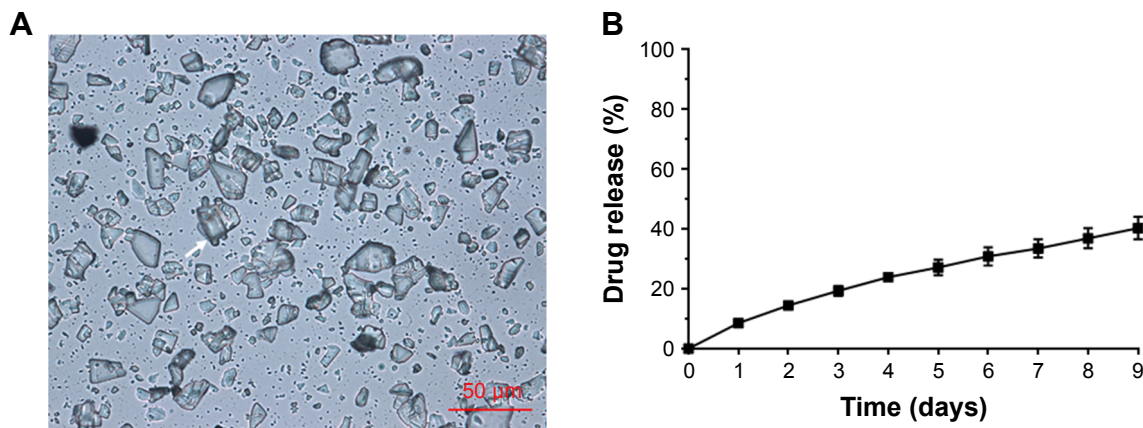
## Statistical analysis

All statistical analyses were performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Statistical significance was analyzed by two-way ANOVA with Bonferroni correction. Differences between groups were examined by paired-sample *t*-test.

## Results

### Characterization of Apa-MS

Apa-MS was prepared as described in the Materials and methods section. As shown in Figure 2A, Apa-MS contained irregularly shaped crystals with a particle diameter of 30–50  $\mu$ m. Apa-MS released apatinib at a slow speed, and almost 60% (40.24% $\pm$ 3.52%) of apatinib remained after 9 days in vitro (Figure 2B). Next, the in vivo release of apatinib was examined. We prepared Apa-Sol as a control. Apa-Sol or Apa-MS was intratumorally injected into



**Figure 2** Particle size and in vitro release rate of Apa-MS. **(A)** Apa-MS has an average particle size of 30–50 µm. Arrow indicates Apa-MS. Scale bar = 50 µm. **(B)** The in vitro release curve of apatinib was plotted with drug release (%) at indicated time points. **Abbreviation:** Apa-MS, apatinib microcrystal formulation.

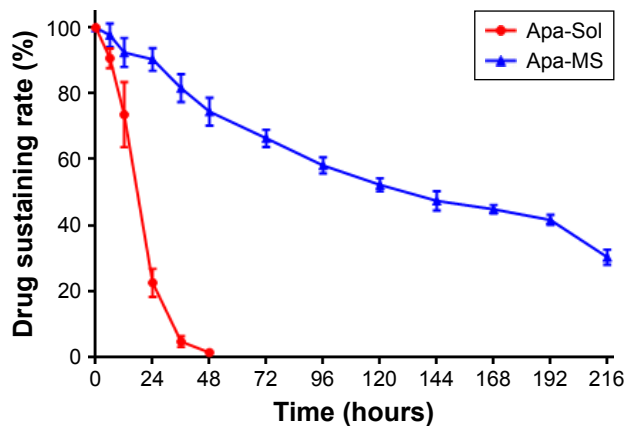
subcutaneous MHCC97-H tumors. Tumor tissues were harvested at indicated time points, and apatinib concentration was tested. As shown in Figure 3, following single-dose administration of Apa-Sol, apatinib was completely cleared from tumor tissues at 48 hours, whereas clearance of Apa-MS was much slower: almost 30% (30.2%±2.00%) of Apa-MS remained in tumors at 216 hours (9 days).

### Apa-MS enhanced RFA-induced inhibition of the growth of HCC cells in vivo

Apa-Sol or Apa-MS was injected intratumorally into RFA-treated subcutaneous HCC tumor model. As shown in Figure 4, Apa-MS administration reduced the tumor growth and enhanced the effect of RFA treatment. It has been previously reported that RFA induced the EMT of HCC cells by decreasing the expression of E-cadherin and increasing

the expression of N-cadherin and vimentin.<sup>31</sup> Therefore, we examined the transcriptional level of these proteins in tumor tissues after the subcutaneous HCC tumor growth experiments. As shown in Figure 5, an increase in the mRNA expression of E-cadherin and decrease in the mRNA expression of N-cadherin and vimentin were observed. Apa-MS also attenuated the EMT process of HCC cells induced by RFA at mRNA level. Moreover, the protein levels of E-cadherin, N-cadherin and vimentin were examined (Figure 6). Single-dose administration of Apa-MS was found to inhibit the RFA-induced EMT at protein level (Figure 6). Thus, we found that single-dose administration of Apa-MS inhibited the RFA-induced EMT in HCC.

We also tested the effect of Apa-MS on intrahepatic HCC model, using PET/CT imaging of whole animals and liver organ photographs. As shown in Figure 7A, PET/CT images of whole animals indicated that RFA treatment decreased the intrahepatic growth of HCC cells and Apa-MS enhanced the effect of RFA. Moreover, HCC cells formed multifocal nodules in liver. RFA treatment decreased the nodules formation of HCC cells, and Apa-MS enhanced the antitumor effect of RFA (Figure 7B). Quantitative results including radioactivity ratio of liver to blood (Figure 7C) and relative nodule number (percentage of nodules in liver) (Figure 7D) confirmed the findings of PET images of whole animal (Figure 7A) and liver organ images (Figure 7B). These in vivo data support our hypothesis that Apa-MS enhances the effect of RFA treatment on HCC.

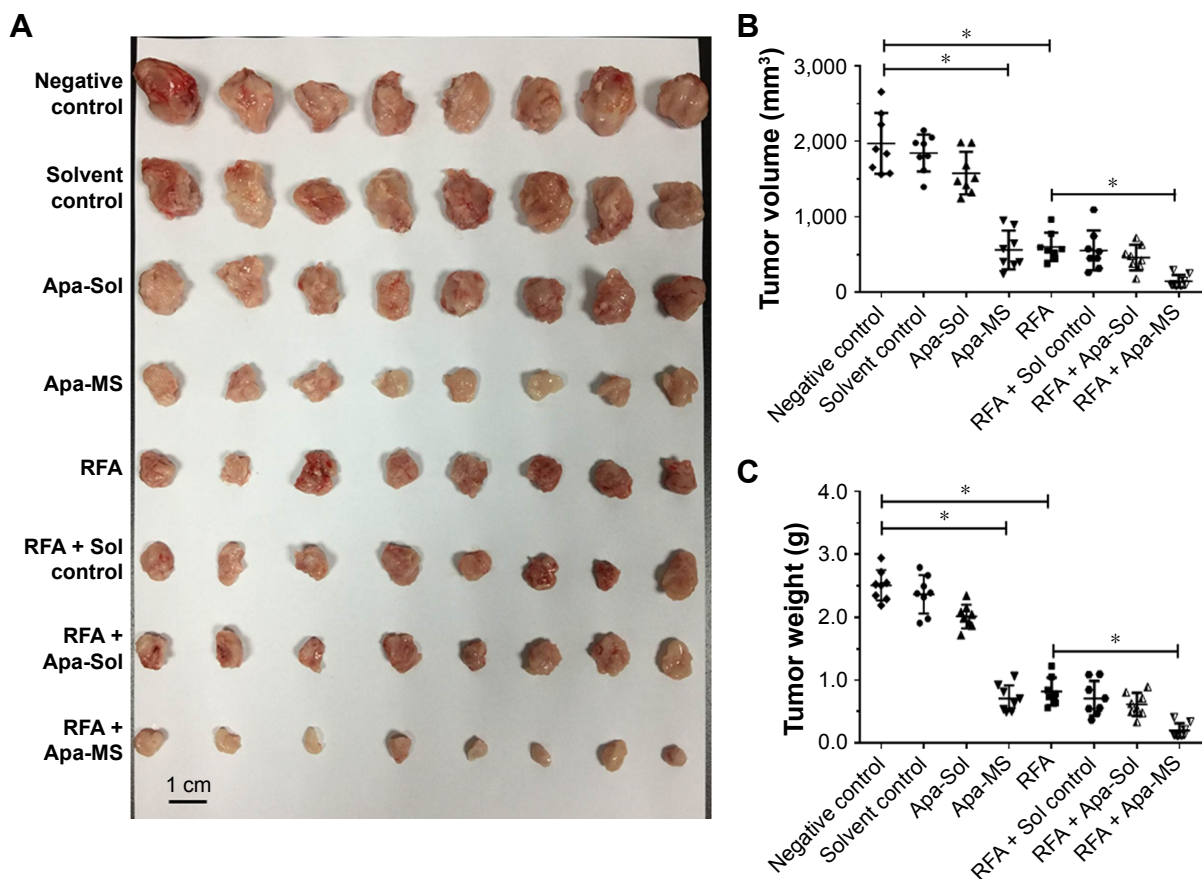


**Figure 3** The in vivo release curve of apatinib formulations. Apa-Sol or Apa-MS was injected in tumor tissues. The release rates of apatinib in tumors from Apa-Sol or Apa-MS at the indicated time points are shown (mean ± SD, n = 10). **Abbreviations:** Apa-Sol, apatinib solution; Apa-MS, apatinib microcrystal formulation.

### Discussion

Currently, RFA is the prime treatment option for late-stage HCC, particularly for patients with cirrhosis or for those



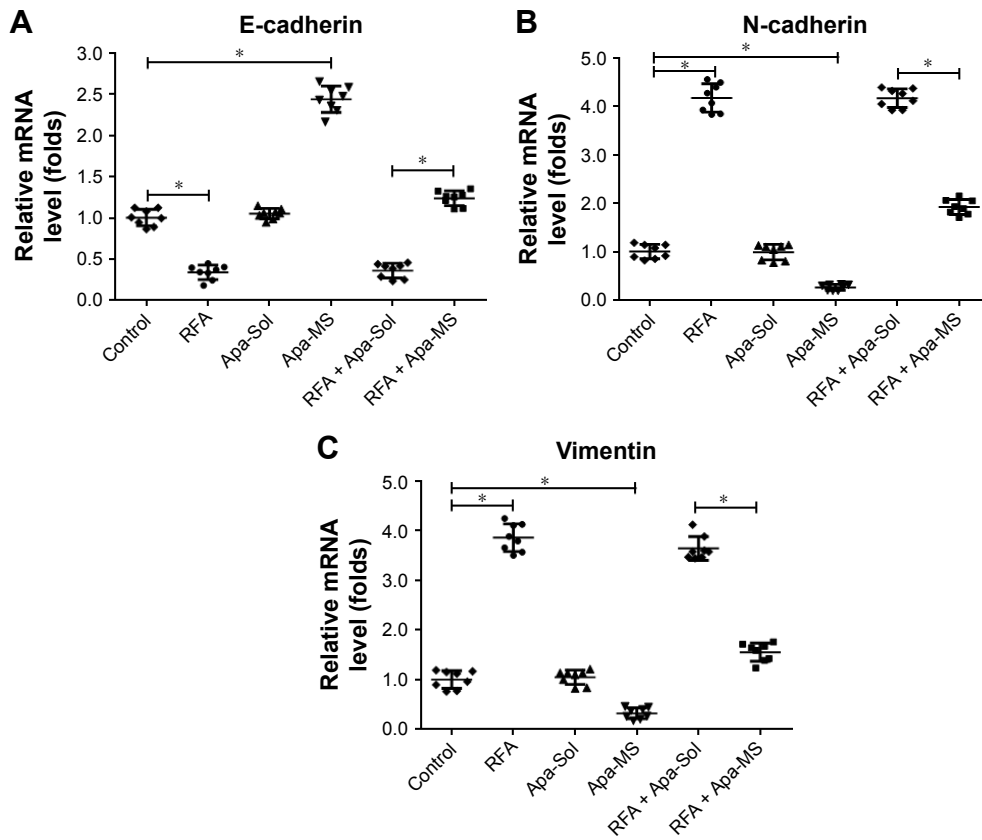


**Figure 4** Single dose of Apa-MS but not Apa-Sol inhibited the in vivo growth of HCC cells and enhanced the effect of RFA on subcutaneous HCC model. The experimental animals were divided into the following groups: no-treatment group (negative control), solvent control group, Apa-Sol group, Apa-MS group, RFA group, RFA + solvent control group, RFA + Apa-Sol group and RFA + Apa-MS group. Results are shown as (A) photographs, (B) volume (mean  $\pm$  SD,  $n = 8$ ) and (C) weight of tumors (mean  $\pm$  SD,  $n = 8$ ). \* $P < 0.05$ .

**Abbreviations:** Apa-MS, apatinib microcrystal formulation; Apa-Sol, apatinib solution; HCC, hepatocellular carcinoma; RFA, radiofrequency ablation.

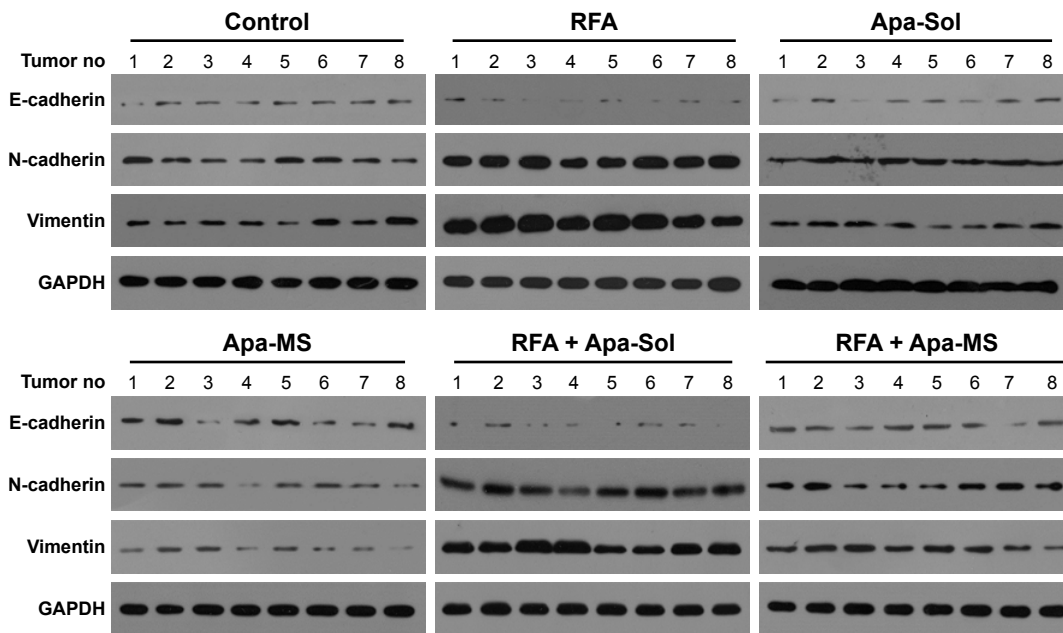
whose liver functional reserve precludes radiotherapy.<sup>32</sup> However, HCC can aggressively and rapidly recur after RFA.<sup>32</sup> To protect nontumor liver tissue, we cannot indefinitely increase the ablation temperature or power during RFA treatment. On the other hand, RFA may induce cellular stress and pathological changes in HCC cells.<sup>33,34</sup> Several studies suggested that the elevated EMT may account for post-RFA recurrence.<sup>33–35</sup> Therefore, inhibiting the EMT process during RFA treatment may be a useful strategy to overcome the recurrence of HCC after RFA treatment. Apatinib is a novel and effective molecular targeted agent for advanced HCC treatment; it may enhance the effect of RFA treatment and suppress EMT process.<sup>36</sup> However, apatinib is insoluble in water, making treatment combination difficult to be applied. To overcome this obstacle, this work aimed to prepare a novel formulation of apatinib that is easily administrated and has elongated effect. This work, for the first time, established a slow releasing system for apatinib based on its insoluble features. The average size of the apatinib microcrystals in

the formulation was 30–50  $\mu\text{m}$ . Apatinib microcrystals could not be identical; however, in our work, the diameter of the particles was relatively uniform. Due to restrictions in the current pharmaceutical techniques, there were many smaller particles. The size of the particle would affect its release, and hence, it is necessary to prepare apatinib microcrystals with different diameters to reveal the relationship between diameter and release behavior. Moreover, our results showed that Apa-MS extended the releasing duration of apatinib as compared with the control formulation. Single-dose administration of Apa-MS enhanced the effect of RFA and had a long-acting effect of inhibiting the EMT process of HCC cells induced by RFA. Apa-MS had several advantages compared with other formulations: 1) Apa-Sol was cleared from tumor tissues within 48 hours, but Apa-MS remained in the tissues for a longer period; 2) only drugs that are soluble could be loaded into microspheres, and Apa-MS exhibited excellent solubility; 3) microcrystals were more tolerant to high temperatures during RFA compared to gelatin sponge



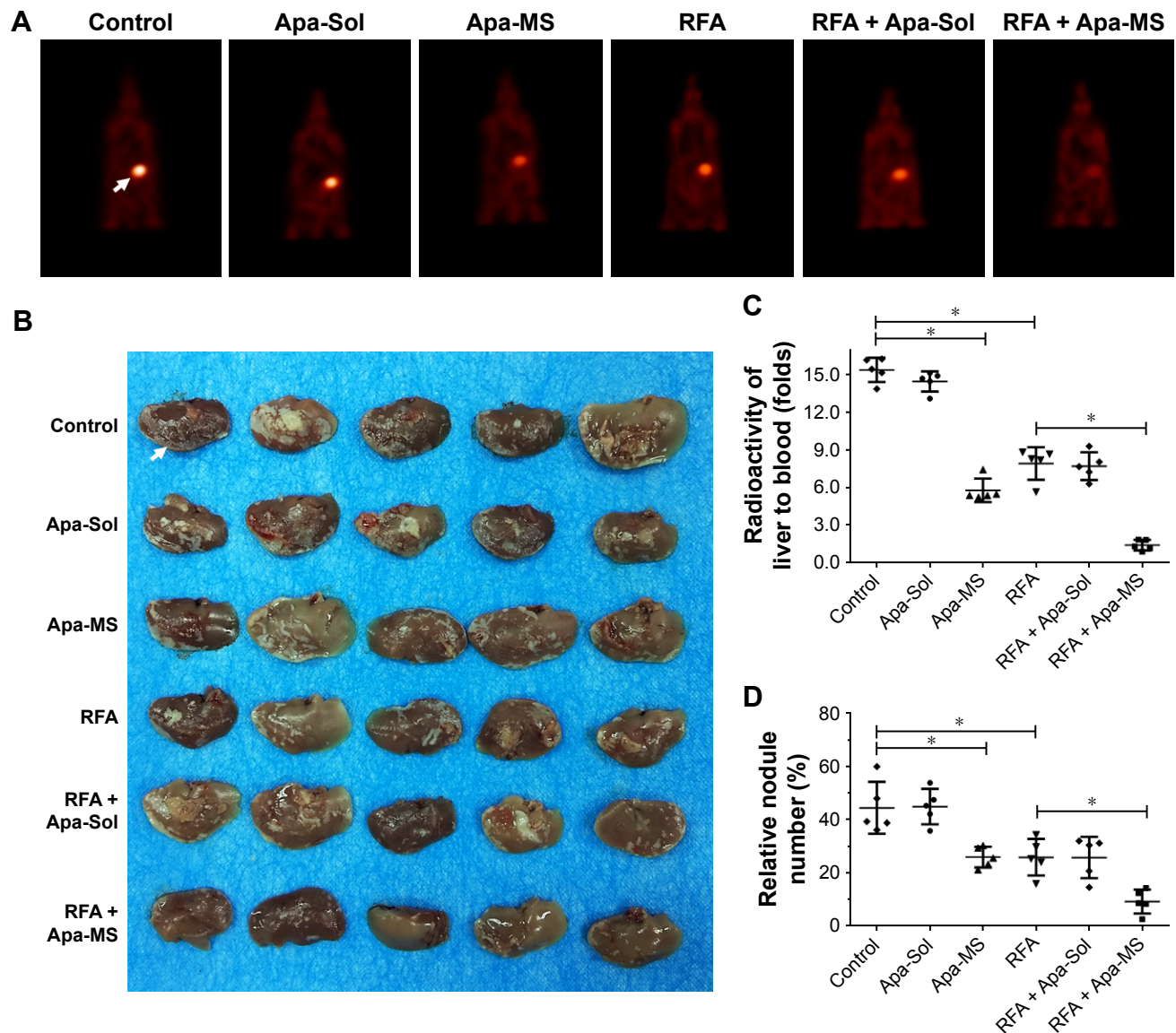
**Figure 5** Apa-MS but Apa-Sol inhibited the EMT of HCC cells induced by RFA at mRNA level. Tumors from solvent control group, RFA group, Apa-Sol group, Apa-MS group, RFA + Apa-Sol group and RFA + Apa-MS group were harvested, and total mRNA was extracted. The relative mRNA level of (A) E-cadherin, (B) N-cadherin and (C) vimentin is shown as mean  $\pm$  SD. \* $P$ <0.05.

**Abbreviations:** Apa-MS, apatinib microcrystal formulation; Apa-Sol, apatinib solution; EMT, epithelial–mesenchymal transition; HCC, hepatocellular carcinoma; RFA, radiofrequency ablation.



**Figure 6** Apa-MS but Apa-Sol inhibited the EMT of HCC cells induced by RFA at protein level. Tumors from solvent control group, RFA group, Apa-Sol group, Apa-MS group, RFA + Apa-Sol group or RFA + Apa-MS group were harvested, and total protein was extracted. The protein level of E-cadherin, N-cadherin and vimentin was examined by their antibodies. GAPDH was chosen as a loading control.

**Abbreviations:** Apa-MS, apatinib microcrystal formulation; Apa-Sol, apatinib solution; EMT, epithelial–mesenchymal transition; HCC, hepatocellular carcinoma; RFA, radiofrequency ablation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.



**Figure 7** Single dose of Apa-MS but not Apa-Sol inhibited the in vivo growth of HCC cells and enhanced the effect of RFA on intrahepatic HCC model. Single cells from tumors of solvent control group, RFA group, Apa-Sol group, Apa-MS group, RFA + Apa-Sol group and RFA + Apa-MS group were harvested and injected into liver via hepatic portal vein. After 3–4 weeks, animals were examined using a PET/CT scanner. Quantitative results are shown as (A) PET images of whole animals, (B) photographs of liver with multifocal nodules of HCC, (C) radioactivity ratio of liver to blood and (D) relative nodule number. \* $P < 0.05$ .

**Abbreviations:** Apa-MS, apatinib microcrystal formulation; Apa-Sol, apatinib solution; HCC, hepatocellular carcinoma; RFA, radiofrequency ablation; PET/CT, positron emission tomography/computed tomography.

particles or liposomes; and 4) Apa-MS released a very high concentration (30–50 mg/mL) of apatinib and permitted injecting a large amount of drug within a small volume. Thus, this work extended our knowledge on apatinib, and injection of Apa-MS into HCC tissues of patients guided by CT or digital subtraction angiography would be a promising combination strategy to enhance the effect of RFA.<sup>37</sup>

## Conclusion

We conclude that the new apatinib formulation Apa-MS allows single-dose apatinib to be slowly released in tumor

tissues for up to 9 days and enhances the antitumor effect of RFA on HCC.

## Abbreviations

<sup>18</sup>F-FDG, <sup>18</sup>F-radiolabeled fluorodeoxyglucose; Apa-MS, apatinib microcrystal formulation; Apa-Sol, apatinib solution; CT, computed tomography; EMT, epithelial–mesenchymal transition; HCC, hepatocellular carcinoma; PET, positron emission tomography; qRT-PCR, quantitative real-time polymerase chain reaction; RFA, radiofrequency ablation; SCID, severe combined immune deficiency.



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## Author contributions

All authors made substantial contributions to the design and conception of the study, and acquisition, analysis and interpretation of data, and took part in either drafting or revising the manuscript. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

## Disclosure

The authors report no conflicts of interest in this work.

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