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Combination therapy with local radiofrequency ablation and YC-1 inhibits the proliferation and metastasis of hepatocellular carcinoma through activating β-catenin signaling

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Aim: This study was aimed to investigate the possible role of combination therapy of 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) and radiofrequency ablation (RFA) in the treatment of hepatocellular carcinoma (HCC). *Materials and Methods:* The two kinds of HCC HCCLM3 and HepG2 cells were intervened by the RFA and YC-1 respectively to construct the model cell. Then cell proliferation, apoptosis, migration and invasion were analyzed using the MTT, flow cytometry and transwell assay respectively. Moreover, the cell signal-related protein expression was also analyzed using western blotting. *Results:* Our results showed that the combination therapy of RFA and YC-1 significantly inhibited the proliferation and induced the apoptosis of the two kinds of cells (P<0.05). Besides, cell migration and invasion were inhibited by the combination of RFA and YC-1, followed with the EMT symbols of E-cadherin, N-cadherin and vimentin expression in cells (P<0.05). Moreover, the β-catenin signal was activated by the combination treatment. *Conclusion:* Taken together, the data presented in this study reveals that the novel method about combination of RFA and YC-1 may opposite an improve effect on the treatment of HCC than the RFA single treatment. Also, the combination therapy of RFA and YC-1 may well inhibit the development and metastasis of HCC *via* preventing the EMT through activating the β-catenin signal.

1. Introduction

Hepatocellular carcinoma (HCC) remains to be one of the most common malignancies in the world with high morbidity and mortality (Cabibbo et al. 2011). Despite the most effective methods for HCC treatment including surgery and liver transplantation have made certain success, however, the percentage for HCC patients who are suit for the surgery is approximately 5-20 % (Tang 2005; Motoyama et al. 2014; Yong et al. 2014). Therefore, to explore other useful treatment methods for HCC will be of great significance.

It has been reported that radiofrequency ablation (RFA), a thermal ablation technology, which is characterized by advantages like less side effects, rapid recovery, and reuse of this method after recurrence, has been widely used in HCC treatment (Ikeda et al. 2001; Hayakawa et al. 2012). However, studies have shown that incomplete tumor ablation often results in a failure of HCC treatment (Goldreyer 2001; Omaygenc et al. 2015).

3-(5'-Hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) is an antitumor drug acting through a complicated molecular mechanism including cell cycle arrest, apoptosis, anti-angiogenesis, anti-inflammatory, and inhibiting MMPs (Mclaughlin et al. 2000; Yeo et al. 2003). For example, Wang et al, (2005) reported that YC-1 exhibited an antiproliferative effect on HCC by arresting the cell cycle at the G0-G1 stage, and Pan et al. (2009) reported the anti-angiogenesis roles of YC-1 in inhibiting endothelial cell function. In addition, previous studies revealed that YC-1 was sensitive to the HCC cells through cell cycle arresting at the G1 stage, apoptosis inducing, and preventing metastases (Yi-Nan et al. 2006). Although the pivotal roles of RFA or YC-1 on the cure of HCC are known, few have reported a combination therapy of the two methods on HCC treatment.

Thus, the purpose of this study was to investigate the possible effects of combination therapy of YC-1 and RFA in the treatment of HCC using two HCC cell lines by the gene-silencing method. Two kinds of HCC cells were treated with RFA or YC-1, then cell viability, apoptosis, and migration and invasion were detected, followed with the molecular mechanism of cell signal, using various kinds of experimental methods. This study may a provide theoretical basis for the exploration of new therapeutic method for HCC.

2. Investigations and results

2.1. Combination of YC-1 and RFA inhibited the HCC cell proliferation

To analyze the effects of the combination therapy of YC-1 and RFA on the cell proliferation, we used MTT to detect cell viability (Fig. 1). The results showed that both the HCCLM3 and HepG2 cell viability was significantly decreased by the combination of YC-1 and RFA treatment compared to the two controls (P<0.05). This effect gradually declined with time after the cells were treated with YC-1 and RFA.

2.2. Combination of YC-1 and RFA induced HCC cell apoptosis

To analyze the effects of the combination therapy of YC-1 and RFA on cell apoptosis, we used flow cytometry (Fig. 2). The treatment of YC-1 and RFA significantly increased the apoptotic percentage of HCCLM3 and HepG2 cells (P<0.05, Fig. 2A and 2B) compared to the control. Besides, the relative mRNA and protein expression of Bcl-2 was decreased while Bax was increased by the treatment of YC-1 and RFA in the two kinds of HCC cells (Fig. 2C and 2D).

2.3. Combination of YC-1 and RFA suppressed cell migration and invasion

We further analyzed the effects of the combination therapy of YC-1 and RFA on cell migration and invasion (Fig. 3). The data showed that the number of migrated cells was significantly decreased by the treatment of YC-1 and RFA compared to the other two groups, control

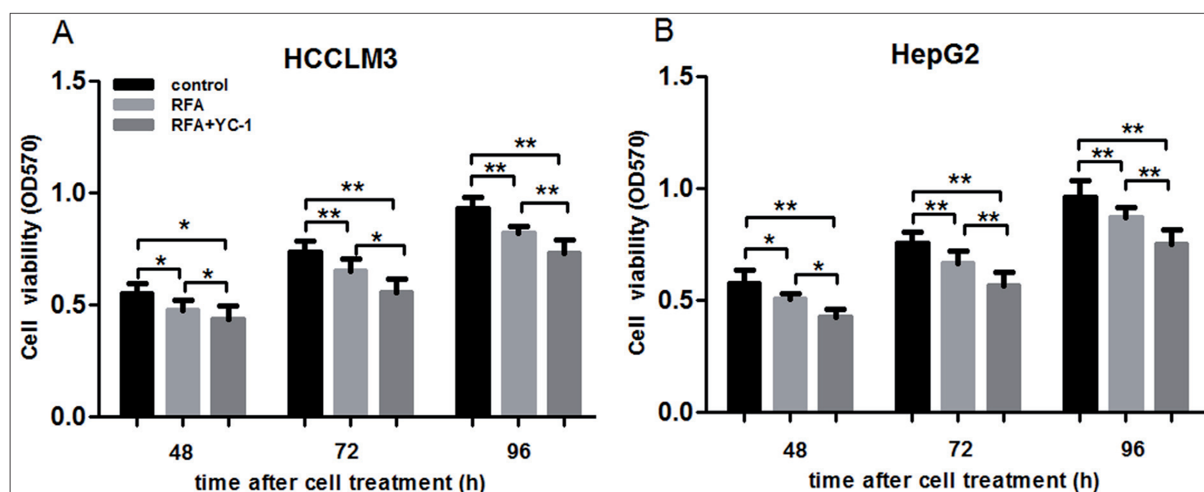

Fig. 1: Combination therapy of YC-1 and RFA inhibits hepatocellular cell viability. A: the combination of RFA and YC-1 significantly decreased the HCCLM3 cell viability with time increasing compared to the control or RFA group; B: the combination of RFA and YC-1 significantly decreased the HepG2 cell viability with time increasing compared to the control or RFA group. *: P<0.05 and **: P<0.01.

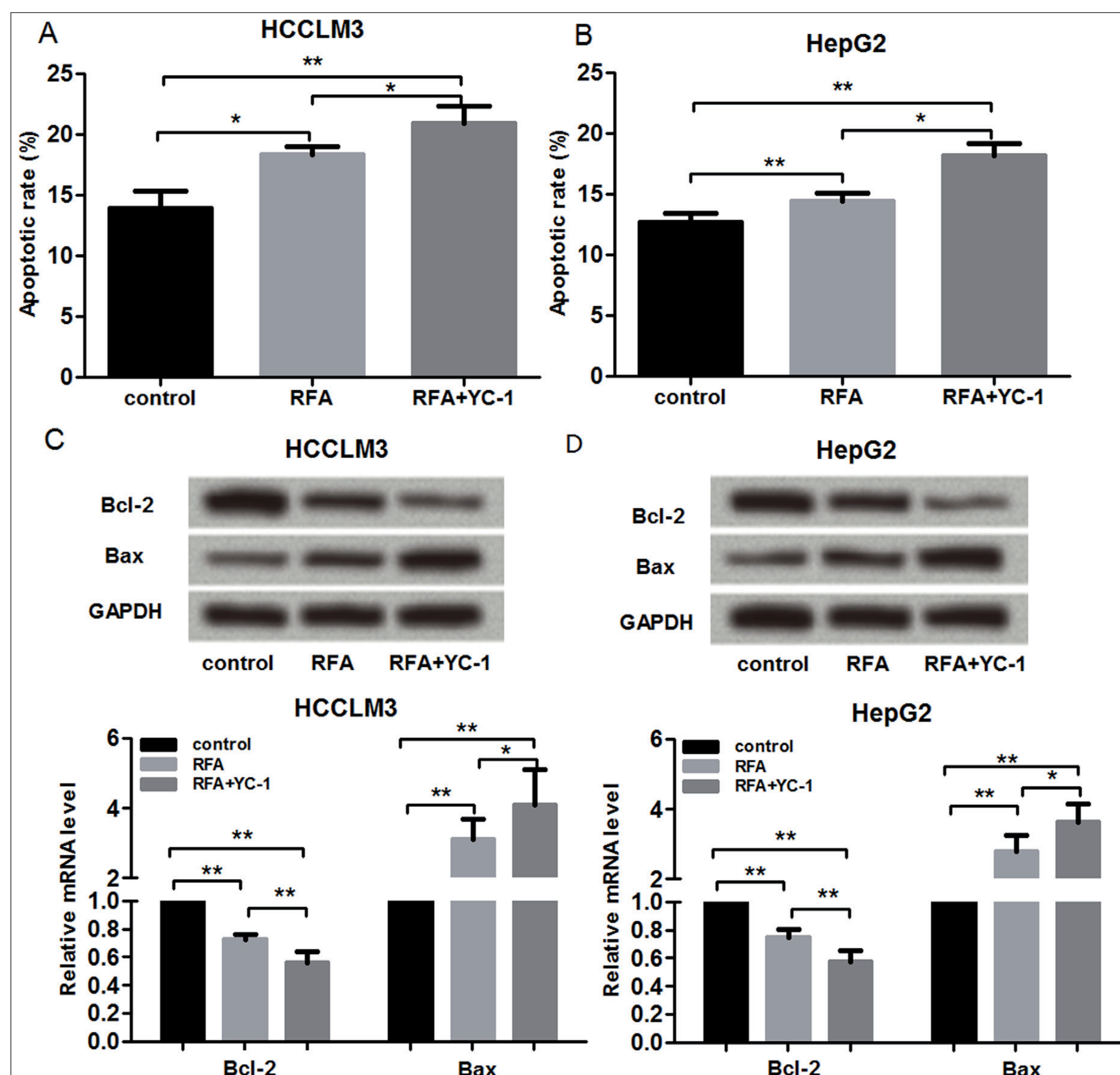

Fig. 2: Combination therapy of YC-1 and RFA induces hepatocellular cell apoptosis. A: the combination of RFA and YC-1 significantly increased the percentage of apoptotic HCCLM3 cells compared to the control or RFA group; B: the combination of RFA and YC-1 significantly increased the percentage of apoptotic HepG2 cells compared to the control or RFA group; C: the relative mRNA and protein expression of cell apoptosis-related protein Bcl-2 was decreased while Bax was increased by the combination of RFA and YC-1 in HCCLM3 cells; D: the relative mRNA and protein expression of cell apoptosis-related protein Bcl-2 was decreased while Bax was increased by the combination of RFA and YC-1 in HepG2 cells. *: P<0.05 and **: P<0.01.

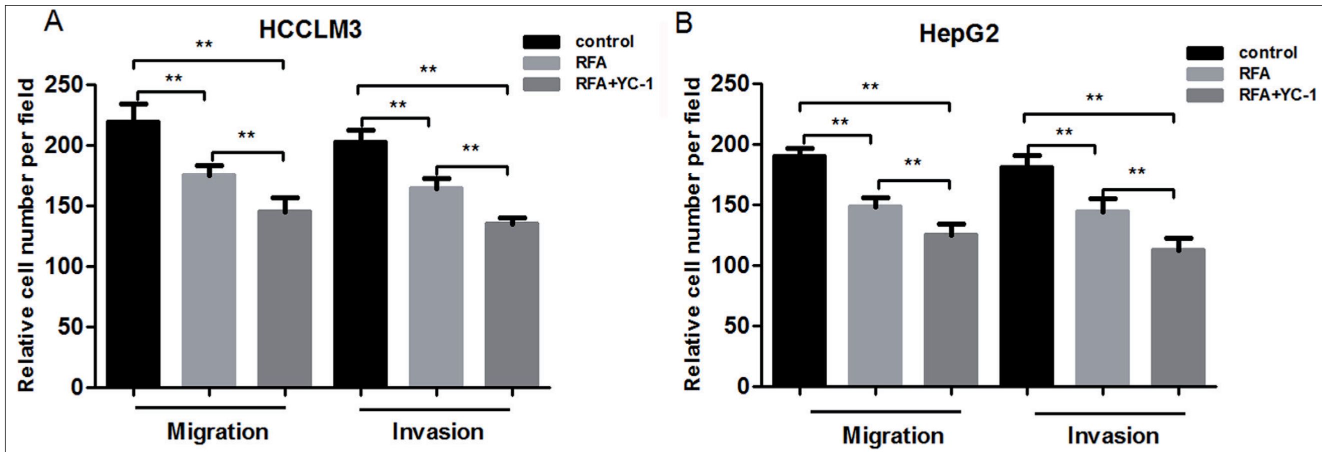

Fig. 3: Combination therapy of YC-1 and RFA suppresses hepatocellular cell migration and invasion. A: the combination of RFA and YC-1 significantly decreased the number of migrated and invaded HCCLM3 cells compared to the control or RFA group; B: the combination of RFA and YC-1 significantly decreased the number of migrated and invaded HepG2 cells compared to the control or RFA group. *: P<0.05 and **: P<0.01.

Fig. 4: Combination therapy of YC-1 and RFA on expression of epithelial-mesenchymal transition (EMT)-related protein expression. A: the relative mRNA and protein expression of E-cadherin was increased, while N-cadherin and vimentin were decreased by the treatment of RFA and YC-1 compared to the other two groups in HCCLM3 cells; B: the relative mRNA and protein expression of E-cadherin was increased, while N-cadherin and vimentin were decreased by the treatment of RFA and YC-1 compared to the other two groups in HepG2 cells. *: P<0.05 and **: P<0.01; C: cell morphology assay revealed that the HCCLM3 cells were alike as the EMT cells in the RFA and YC-1 group.

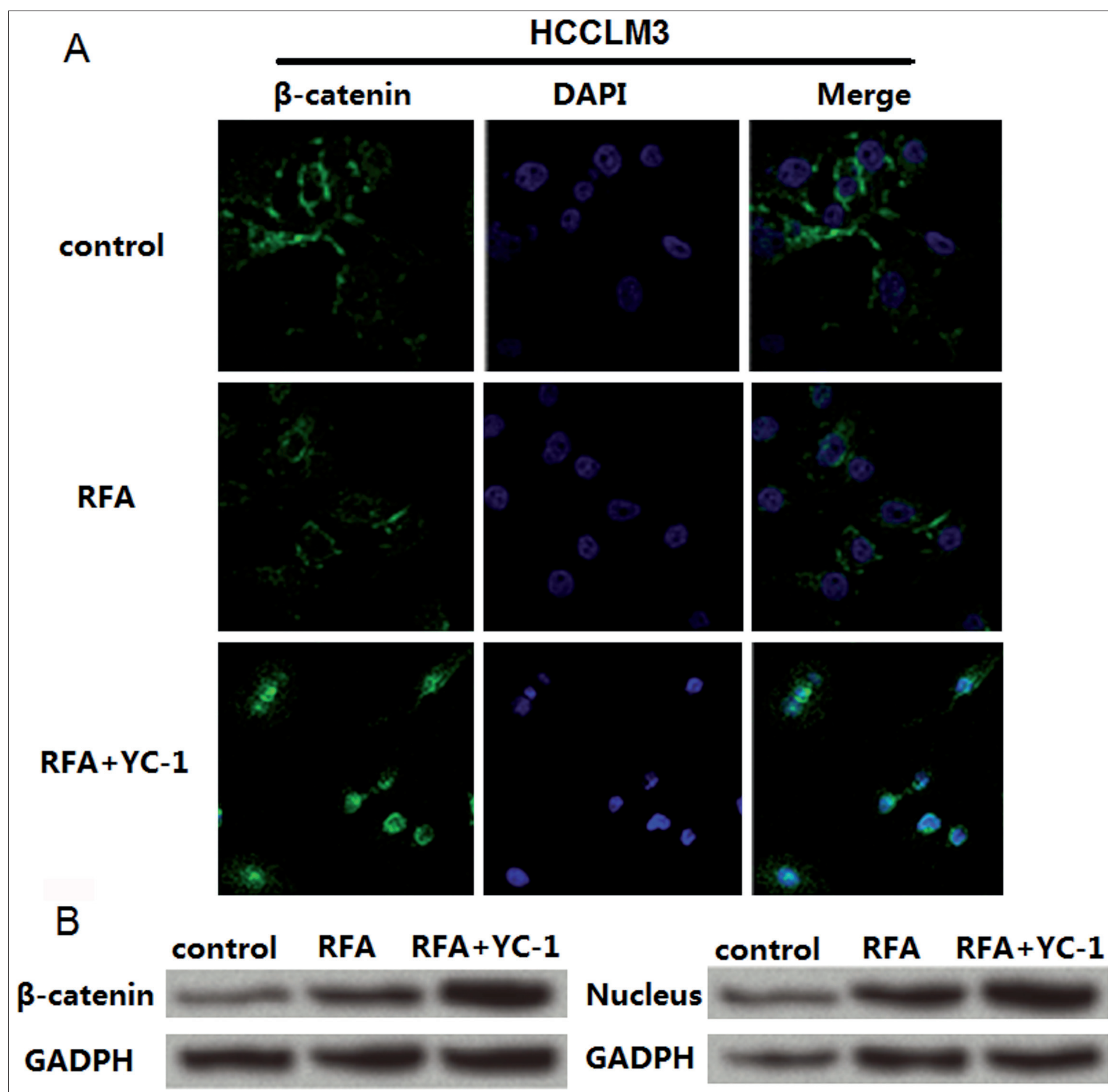

Fig. 5: Combination therapy of YC-1 and RFA on the β-catenin signal expression in HCCLM3 cells. A: immunofluorencent assay revealed that β-catenin was mainly existing in the nucleus instead of in the cytoplasm in the YC-1 and RFA treated group; B: western blot showed that the total amount of β-catenin protein was higher in the YC-1 and RFA treated group. Also, the expression of β-catenin in HCCLM3 nucleus was also more than the other two control group.

and the RFA group (Fig. 3A and 3B). Additionally, the invasive cells were also significantly decreased by the combination treatment. These results indicated that the combination treatment of YC-1 and RFA may suppress the migration and invasion of HCC cells.

2.4. *Combination of YC-1 and RFA inhibited the EMT*

Since we found that the combination of YC-1 and RFA may correlate to cell migration and invasion, we investigated the expression of EMT-related protein by the qRT-PCR and western blotting (Fig. 4). The data displayed that the expression of E-cadherin was increased while N-cadherin and vimentin were decreased by the treatment of YC-1 and RFA in the two kinds of HCC cells (Fig. 4A and 4B). In addition, we observed the HCCLM3 cell morphology as previously described (Zhang et al. 2014), and found that the cells in the YC-1 and RFA group were almost the same as the epithelial-mesenchymal transition cells (Fig. 4C). These results indicated that the combination treatment of YC-1 and RFA may suppress EMT in HCC cells.

2.5. *Combination of YC-1 and RFA activated the β-catenin signal*

Based on the former findings, we further analyzed the effects of combination of YC-1 and RFA on the cell signal protein expression to investigate the possible molecular mechanism (Fig. 5). The immunofluorescence assay revealed that β-catenin exists in the cytoplasm, while it was transferred into the nucleus in the YC-1 and RFA group (Fig. 5A). Accordingly, we analyzed the protein expression of β-catenin in the cytoplasm and nucleus from cells in each group, and the results showed that β-catenin was highly expressed in the nucleus from the YC-1 and RFA treated cells (Fig. 5B). These results implied that the β-catenin signal can be activated by the combination of YC-1 and RFA.

3. Discussion

RFA has been firstly used in the treatment of HCC in 1996, and has become the most common thermal ablation technique for clinical of HCC treatment (Shiina et al. 2009). Although RFA offers great advantages for HCC patients, relapses are frequently occurring due to incomplicate ablation (Goldreyer 2001; Omaygenc et al. 2015). In this study, we developed a novel method, which is the combination therapy of RFA and YC-1 for HCC treatment. First, we analyzed the effects of combination therapy of RFA and YC-1 on the HCC cells biological processes including cell viability and apoptosis. Previous evidence revealed that the application of or RFA inhibited HCC cell proliferation but induced cell apoptosis (Cioni et al. 2001; Livraghi et al. 2001). In agreement with previous results, our study revealed that compared to the RFA single treated cells, the tumor cell viability was suppressed (Fig. 1), whereas apoptosis was induced (Fig. 2) by the combination therapy of YC-1 and RFA, implying the improvement effects of this combination therapy on HCC.

Meanwhile, we further analyzed the effects of combination therapy of YC-1 and RFA on cell migration and invasion. Our results showed that the HCC cell migration and invasion were better inhibited by the combination of YC-1 and RFA than by RFA single treatment (Fig. 3), suggesting a suppressive effect of the combination on HCC metastasis. Besides, EMT is a kind of basic physiological change, which plays certain roles in the embryonic development, chronic inflammation, fibrosis and tumor migration and invasion (Yang and Weinberg 2008; Nieto, 2009). It has been demonstrated that EMT contributes to metastasis and invasion of HCC (Park et al. 2007). E-Cadherin, N-cadherin and vimentin are the EMT symbols that play certain roles in the metastasis of HCC, such as the N-cadherin overexpression or N-cadherin or vimentin downregulation results in the suppression of HCC metastasis (Luo et al. 2010; Stella et al, 2010; Hee Jeong et al. 2014). Our results revealed that EMT was well inhibited by the application of RFA and YC-1, and N-cadherin was overexpressed while N-cadherin and vimentin were downregulated (Fig. 4), implying that the combination therapy may suppress HCC metastasis through inhibiting EMT.

Furthermore, we analyzed the molecular mechanism for the combination therapy of RFA and YC-1 on HCC at the intracellular signal level. β-Catenin signal transduction plays pivotal roles in the development of cell transformation and apoptosis, which is also correlated to the abnormal activation, development and progression of tumors (Gumbiner 1995; Behrens 2000). It has been said that the β-catenin signal activation was a way for EMT in tumors (Li and Zhou 2011), and the β-catenin-independent activation of TGF-β indicates a promotion effect on tumor metastasis (Pang et al. 2015; Kretzschmar et al. 2016). Our data revealed that the β-catenin protein was highly expressed in the nucleus from cells treated by the combination of RFA and YC-1 (Fig. 5), suggesting an activation of β-catenin signal by RFA and YC-1.

In conclusion, the data presented in this study reveal that a combination of RFA and YC-1 may lead to better treatment of HCC than RFA alone. Also, combination therapy may well inhibit the development and metastases of HCC *via* preventing EMT through activating the β-catenin signal. This study may also provide the theoretical basis for novel strategies in the clinical treatment of HCC.

4. Experimental

4.1. Cell culture

Human hepatocellular HCCLM3 (high metastatic potential) and HepG2 (low metastatic potential) cells were used for the *in vitro* assay in this study (established at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China). The cells were cultured in the Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Rockville, MD, USA) with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin at 37 °C in a humidified atmosphere containing 5% CO_2.

4.2. RFA treatment and drug intervention

A multipole RF ablation instrument and bipolar ablation needle (purchased from Beijing Blade Opto-Electronic Technology Development Co., Ltd) was used on the nude mice. In nude mice, a bipolar electrode (outer diameter 1.0 mm, active length 10 mm) was used for RFA at 2 W for 45 s with complete puncture of the right lung upper lobe, which corresponded to a total energy output of 90 J. RF was applied for 5 min with the generator output titrated to maintain a designated tip temperature (70±2 °C, 90±20 mA).

YC-1 (Sigma-Aldrich) possesses antiplatelet activity and decreases hypoxia-induced HIF-1α accumulation and stability. Therefore, YC-1 was dissolved in saline and administered intraperitoneally at a preoperative dose of 100 mg/kg body weight, followed by postoperative doses of 30 mg/kg body weight.

HCCLM3 cells and HepG2 cells were seeded into 6-well plates at a density of 5×10^4 cells per well. After 24 h of incubation, the plates were sealed with parafilm and submerged in a water bath set to the target temperature for 10 min. Cells were separated into 3 groups, control (cells without any treatment), RFA group (cells treated with RFA only), and RFA+YC-1 group (cells treated with the two conditions).

4.3. Cell proliferation assay

Cell proliferation ability was assessed using MTT assay as previously described (van Meerloo et al. 2011). Briefly, cells cultured in DMEM medium containing 20% FBS at logarithmic stage (5×10^3) were transfected into the 96-well plates. After for 24 h cultivation, supernatant was abandoned and followed with addition of 20 µL MTT every 24 h and incubation for 4 h. After that, 150 µL dimethylsulfoxide (DMSO) was used to mix with the cells for 10 min. Absorbance of cells in each well was observed at 570 nm under an absorption spectrophotometer (Olympus, Japan).

4.4. Cell migration and invasion assay

Cell migration and invasion were assessed by Transwell assay (Corning). Briefly, 8×10^4 cells in serum-free DMEM were seeded into the upper chamber of each well of 24-well plates containing 8.0-mm pore size membranes. DMEM containing 10% FBS was added to the lower chamber of each well. After 48 h of incubation, cells that had reached the underside of the membrane were stained with Giemsa (Sigma), counted at 6200 magnification in five randomly selected areas per well. The cell invasion assay was carried out similarly, except that 80 mL Matrigel (0.8 mg/mL, BD Biosciences) was added to each well 6 h before cells were seeded on the membrane.

4.5. Cell apoptosis assay

Cells apoptosis was quantified with a flow cytometry using Annexin V-FITC cell apoptosis kit (Invitrogen) according to manufacturer's protocol. Briefly, cells were transfected with vectors for 36 h, followed by the replacement of cell culture medium with serum-free DMEM medium. Total cells were harvested and then washed using PBS buffer (PH 7.4) three times, and then resuspended in the staining buffer. After that, 5 µL of annexin-V-FITC and 5 µL of propidium iodide (PI) were mixed with the cells. After being cultivated at room temperature for 10 min, mixtures were analyzed using the FACScan flow cytometry. Annexin V-positive and propidium iodide-negative cells were considered to be apoptotic cells.

4.6. qRT-PCR

Total RNA from cells was isolated using Trizol Reagent (Invitrogen, CA, USA) as previously described, and was treated with RNase-free DNase I (Promega Biotech, USA). Consequently, the concentration and purity for the isolated RNA were measured with SMA400 UV-VIS (Merinton, Shanghai, China). Purified RNA at density of 0.5 µg/µL with nuclease-free water was used for cDNA synthesis with the PrimerScript 1st Strand cDNA Synthesis Kit (Invitrogen, USA). Expression of targets were detected in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-qPCR Kit (Takara, China). Phosphoglyceraldehyde dehydrogenase (GAPDH) was chosen as the internal control. Primers used for targets amplification were shown in the Table.

Table: Primers used for targets amplification in this study

Target	primer	Sequence (5'-3')
GAPDH	Sense	GGGTGGAGCCAAACGGGTC
	Antisense	GGAGTTGCTGTTGAAGTCGCA
Bcl-2	Sense	TTGTGGCCTTCTTTGAGTTCGGTG
	Antisense	GGTGCCGGTTCAGGTACTCAGTCA
Bax	Sense	ATGGACGGGTCCGGGGAG
	Antisense	TCAGAAAACATGTCAGCTGCC
β-Catenin	Sense	GGGCGGCACCTTCCTACTTC
	Antisense	AGCTCCCTCGCGGTTCAT
E-Cadherin	Sense	GAACTCAGCCAAGTGTAAAAGCC
	Antisense	GAGTCTGAACTGACTTCCGC
N-Cadherin	Sense	GCGGAGAGGAAGACCAGGA
	Antisense	TAGTTGGGCTCCGAGTGCAT
Vimentin	Sense	CCTTGAACGCAAAGTGGAATC
	Antisense	GACATGCTGTTCCTGAATCTGAG

4.7. Western blot assay

The cells were lapped with RIPA assay (radioimmunoprecipitation, Sangon Biotech, China) lysate containing PMSF (phenylmethanesufonyl fluoride, Sigma, USA), then centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatant was collected for the measurement of protein concentrations using BCA protein assay kit (Pierce, Rochford, IL). For Western blotting, 50 µg protein per cell lysate was subjected to a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferred onto a polyvinylidencefluoride (PVDF) membrane (Millipore). Then the PVDF membranes were blocked in Tris Buffered Saline Tween (TBST) containing 5% non-fat milk for 1 h at room temperature. Consequently, the membrane was incubated with rabbit anti-human antibodies (Bcl-2, Bax, β-catenin, E-cadherin, N-cadherin, vimentin, 1:100 dilution, Invitrogen) and overnight at 4 °C. Then membrane was incubated with horseradish-peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Finally, the PVDF membranes were washed three times with 1× TBST buffer for 10 min each. The signals were detected after incubation with a chromogenic substrate using the enhanced chemiluminescence (ECL) method. Additionally, GAPDH served as the internal control.

4.8. Statistical analysis

All data were expressed as mean±standard error of mean (SEM) in this study. Independent sample t-test was used to calculate the difference between two groups using the graph prism 5.0 software (GraphPad Prism, San Diego, CA). Statistical differences between groups were analyzed by a Student's t-test and ANOVA for parametric data. $p<0.05$ or $p<0.01$ was set as the level of statistical significance.

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