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Original Research

Liposomal clodronate combined with Cisplatin or Sorafenib inhibits hepatocellular carcinoma cell proliferation, migration and invasion by suppressing FOXQ1 expression

Yilong Zhou[1, 2#], Yuan Chen[2#], Chunyang Ma[2], Bingfeng Shao[2], Feng Zhang[1*]

[1] Department of liver Surgery/Liver Transplantation Center, the First Affiliated Hospital of Nanjing Medical University, Nanjing, 210029, China
[2] Department of Surgery, Nantong Tumor Hospital, Nantong, 226300, China

[*]**Correspondence to:** igrd59@163.com

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[#] Yilong Zhou and Yuan Chen contributed equally to this paper.

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Abstract: The purpose of this study was to investigate the effect of liposomal clodronate combined with Cisplatin or Sorafenib on the FOXQ1 expression and biological function of hepatocellular carcinoma cell lines. The expression of FOXQ1 was determined in normal hepatic cell line and hepatocellular carcinoma cell lines using quantitative real-time polymerase chain reaction (qRT-PCR). HepG2 and MHCC97H cells were administered low, medium and high concentrations of cisplatin (3, 5 and 7 μg/ml) or Sorafenib (2, 7 and 20 μg/ml) in combination with liposomal clodronate (LC, 20μg/ml), and the expression of FOXQ1 in each group was determined. Cell migration, MTT and transwell assays were used to determine the effects of the treatments on biological functions of HepG2 and MHCC97H cells. qRT-PCR showed that the expression of FOXQ1 mRNA was higher in the four hepatocellular carcinoma cell lines than in normal cells, and the expression of FOXQ1 mRNA in HepG2 and MHCC97H cells were more dominant. All the tested doses of Cisplatin, but only high dose Sorafenib down-regulated the expression of FOXQ1. However, Sorafenib at low and medium concentrations had no significant effect on the expression of FOXQ1. When Cisplatin or Sorafenib was administered in combination with LC, the expression level of FOXQ1 was significantly reduced. Cell migration, MTT and transwell assays showed that proliferation, migration and invasion were inhibited when each drug was administered alone, but was stronger when the drugs were combined with liposomal clodronate. Liposomal clodronate combined with Cisplatin or Sorafenib down-regulates the expression of FOXQ1 in HepG2 and MHCC97H hepatoma cells, and inhibits their proliferation, migration and invasion.

Key words: FOXQ1; Cisplatin; Sorafenib; Liposomal clodronate.

Introduction

Hepatocellular carcinoma (HCC) is a frequently-occurring malignant tumor affecting human health due to its poor prognosis and poor clinical response to traditional chemotherapy and radiotherapy (1). Its mortality ranks third in the world, and second in cancer mortality in Asia (2). Factors involved in the etiology of HCC are yet to be unraveled (3). However, hepatitis B and hepatitis C may be major predisposing factors for HCC (4). Some strategies used for improving the lives of HCC subjects include liver transplant surgery and chemotherapy. However, the prognosis of HCC still remains unimpressive due to drug resistance and high metastatic rates of HCC (5). Therefore, there is need for development of more effective treatment strategies for HCC.

The transcription factor, foxhead box protein Q1 (FOXQ1) is intimately linked to the pathogenesis of tumors, and it is abnormally highly expressed in colon cancer, ovarian cancer, breast cancer, non-small cell lung cancer, bladder cancer, and glioma, and pancreatic ductal carcinoma (6). Studies have shown that FOXQ1 is highly expressed in hepatocellular carcinoma tissues, indicating that FOXQ1 may contribute to the occurrence and metastasis of hepatocellular carcinoma (7). Systemic chemotherapeutic effect is poor in hepatocellular carcinoma. Thus, a new therapeutic regimen for HCC will be of great clinical significance. Surgery is the main method for the treatment of hepatocellular carcinoma (HCC), but a lot of patients miss the best surgical opportunity due to the late diagnosis. Hence, non-surgical targeted drug therapy and chemotherapy have become the main treatment choice for these patients. Cisplatin (DDP) is a widely used anti-tumor chemotherapeutic drug in clinical practice, but it has a lot of side effects, and drug resistance easily develops when it is applied in high doses (8). Therefore, many researchers have tried to combine different compounds with traditional chemotherapy drugs to enhance sensitivity, reduce side effects and minimize drug resistance. Sorafenib is the first oral multi-kinase inhibitor, a multi-target molecular target drug that inhibits Raf-1 kinase, VEGFR2/3, FLT3, Ret, C-kit, PDGFR and other targets (9). It is a multi-target anti-tumor drug that targets serine/threonine kinases and receptor tyrosine kinases on tumor cells and tumor blood vessels (10). A number of studies have shown that Sorafenib has clear and definite curative effects on advanced primary hepatocellular carcinoma (HCC), and results in significant prolongation of the survival time of advanced hepatocellular carcinoma patients (11).

Studies have shown that activated macrophages cause systemic inflammatory responses, induce lipid peroxidation and membrane structure damage, cause damage to liver and other extra-hepatic organs, and ultimately lead to multiple organ dysfunction syndrome by promoting excessive secretion of cytokines (12). Liver damage can further aggravate systemic inflammatory responses and increase mortality by affecting the metabolism of toxins and the release of excess inflammatory mediators (13). Liposomal clodronate (LC) is currently recognized as a macrophage scavenger (14). Clodronate is encapsulated in liposomes and can be engulfed by macrophages. It causes macrophage apoptosis; it has no significant effect on the proliferation of smooth muscle cells and endothelial cells without phagocytosis, but it selectively eliminates systemic mononuclear macrophages (15). In this study, the core hypothesis is that LC plays an auxiliary role in liver cancer treatment. Thus, the study was aimed at investigating the effects of LC combined with Cisplatin or Sorafenib on the expression of FOXQ1, and on the biological functions of HCC cell lines.

Materials and Methods

Cell culture and administration

The cell lines LO2, HepG2, MHCC97L, MHCC97H and SMMC-7721 were purchased from ATCC (USA). All cells were cultured in DMEM complete medium containing 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin in an incubator at 37 °C, 95% humidity and 5% CO_2. The experiment used 28 groups. The grouping details and treatments are shown in Table 1.

Quantitative real-time polymerase chain reaction

The expression of FOXQ1 was assayed using was RT-PCR. Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer′s instructions. Then, the RNA (1μg) was converted to cDNA in two cycles, one for 15 min at 37°C, and the other for 5 sec at 85°C. The resultant cDNA was subjected to RT-PCR using the primers shown in Table 2. The PCR conditions were: 95°C for 5 min for one cycle, 40 cycles for 15 sec at 95 °C, and 60°C for 60 sec; 1 cycle at 95°C for 15 sec, 60°C for 1 min and 95°C for 15sec. Thermal cycling and real-time detection were conducted on StepOnePlus RT-PCR Systems (ABI). The resultant mRNA products were quantified with the $2^{-\Delta\Delta Ct}$ procedure.

MTT assay

Cells in the various treatment groups were subjected to MTT assay in line with standard procedures. The resultant formazan crystals from each assay were solubilized in DMSO, and the absorbance of the solution was read at 490 nm. Each assay was done in triplicate.

Table 1. Grouping details.

Group	Description/treatment
1	HepG2 (Control)
2	Liposomal clodronate (20μg/mL)
3	Sorafenib (2μg/mL)
4	Sorafenib (7μg/mL)
5	Sorafenib (20μg/mL)
6	Cisplatin (3μg/mL)
7	Cisplatin (5μg/mL)
8	Cisplatin (7μg/mL)
9	Liposomal clodronate+Sorafenib (2μg/mL)
10	Liposomal clodronate+Sorafenib (7μg/mL)
11	Liposomal clodronate+Sorafenib (20μg/mL)
12	Liposomal clodronate+Cisplatin (3μg/mL)
13	Liposomal clodronate+Cisplatin (5μg/mL)
14	Liposomal clodronate+Cisplatin (7μg/mL)
15	MHCC97H (Control)
16	Liposomal clodronate (20μg/mL)
17	Sorafenib (2μg/mL)
18	Sorafenib (7μg/mL)
19	Sorafenib (20μg/mL)
20	Cisplatin (3μg/mL)
21	Cisplatin (5μg/mL)
22	Cisplatin (7μg/mL)
23	Liposomal clodronate+Sorafenib (2μg/mL)
24	Liposomal clodronate+Sorafenib (7μg/mL)
25	Liposomal clodronate+Sorafenib (20μg/mL)
26	Liposomal clodronate+Cisplatin (3μg/mL)
27	Liposomal clodronate+Cisplatin (5μg/mL)
28	Liposomal clodronate+Cisplatin (7μg/mL)

Cell migration assay

The cells were incubated in air-dried 24-well Matrigel plates at 37°C in an atmosphere with 5% CO_2. A line of uniform thickness was made on each well with a sterile tip when the base of plate was entirely occupied by cells. The cells were photographed (0 h) prior to incubation for 24 h. Following 24 h incubation, the medium was siphoned off and the plate was rinsed thrice with phosphate-buffered saline to eliminate remnants of scratch-derived cell debris. Subsequently, pictures of the cells were taken in a serum-free solution. The distance migrated after 24 h was estimated using ImageTool software.

Transwell chamber assay

A thin Matrigel gel membrane was formed in each transwell chamber at 37°C, transferred to a 24-well plate, and subjected to hydration with medium for 2h. The medium was drained off from both chambers, fol-

Table 2. PCR primers sequences.

Primer	Sequence (5'-3')
FOXQ1(Human)-RT-F	CGACTGCTTCGTCAAGGTGCTG
FOXQ1(Human)-RT-R	CCCCGTCGGCGAAGGTGTA
GAPDH(Human)-RT-F	AGAAGGCTGGGGCTCATTTG
GAPDH(Human)-RT-R	AGGGGCCATCCACAGTCTTC

lowed by addition of 0.5ml of cell suspension (5×10^4 cells/ml) and 0.5ml of medium. After incubating for 24 h at 37°C, the medium was removed, and the cells were rinsed in phosphate-buffered saline and subjected to fixation in cold methanol. This was followed by staining for 10 min using 0.1% crystal violet. Excess stain was washed away under tap water, followed by air-drying of the cells and photographing using Olympus Inverted Microscope at six fields of vision at a determined site. A hit counter was used in enumerating cells that were transferred to the lower compartment.

Statistical analysis

Data analysis was done with GraphPad Prism 6.0 software. Data are presented as mean ± SD. Two-group comparison was done using *t*-test, while ANOVA was used for multi-group comparisons. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

Results

Expression levels of FOXQ1 in different cell lines

Results from qRT-PCR showed that the expression of FOXQ1 in LO2 cell line was significantly lower than those in hepatocellular carcinoma cells (HepG2, MHCC97L, SMMC-7721 and MHCC97H) ($p<0.01$; Figure 1). It is worth noting that the expression levels of FOXQ1 in HepG2 and MHCC97H cell lines were significantly higher than those in MHCC97L and SMMC-7721 cell lines ($p<0.05$). Therefore, HepG2 and MHCC97H cell lines were used in subsequent studies.

Combination therapy significantly reduced the expression of FOXQ1

The results showed that LC monotherapy resulted in significant down-regulation of FOXQ1 expression in hepatocellular carcinoma cell lines ($p<0.05$; Figure 2). Cisplatin monotherapy also significantly down-regulated the expression of FOXQ1 in a concentration-dependent manner in HCC cells ($p<0.05$). However, the low and middle concentrations (2 and 7 μg/ml) of Sorafenib had no obvious effects on the expression level of FOXQ1 ($p>0.05$), while the high concentration of Sorafenib (20 μg/ml) significantly reduced the expression of FOXQ1 ($p<0.05$). Combination therapy with LC and different concentrations of Cisplatin and Sorafenib significantly reduced the expression of FOXQ1 ($p<0.01$).

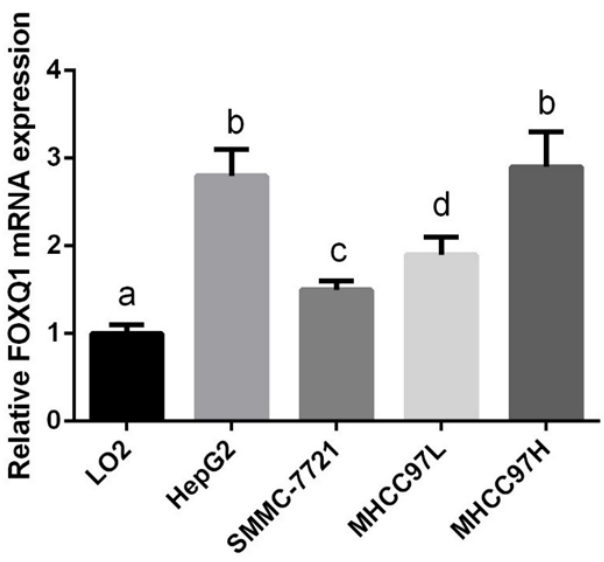

Figure 1. Expressions of FOXQ1 mRNA in normal liver cell line and hepatocellular carcinoma cell lines, as assayed using qRT-PCR. Bars with the same letters are comparable, while bars with different letters differ significantly.

Combination therapy significantly inhibited the proliferation of HepG2 and MHCC97H cells

Results from MTT assay showed that the proliferation of untreated HCC cells was obvious with increase in time, but there was no obvious proliferation or decrease in proliferation in the drug-administered groups. Cell proliferation was significantly reduced at 24 h and 48 h in LC-DDP combination therapy and LC-Sorafenib combination therapy, respectively ($p<0.05$; Figure 3). These results suggest that drug administration significantly reduced cell proliferation, when compared to the untreated HepG2 and MHCC97H cells. The inhibitory effect of combination therapy on cell proliferation was better than that of monotherapy.

Combination therapy significantly inhibited the migration of HepG2 and MHCC97H cells

The scratch test was performed to measure cell migration. The results showed that at 0 h, the untreated HepG2 and MHCC97H cells in the control group and the administered cells in the treated groups adhered well: there was no cell adherence at the scratch point. However, after 24 h, the cells started to migrate to the middle of the scratch. The scratch in the control group was covered by cells, but the migrated distances of all drug-administered groups were significantly shorter than that of the control group ($p<0.05$). As expected, the distances migrated in the combination therapy were

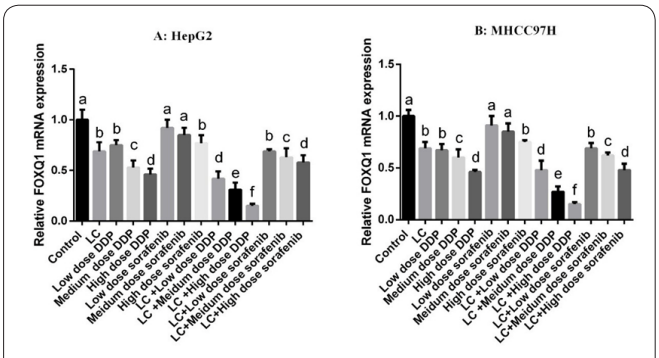

Figure 2. FOXQ1 mRNA expressions after drug administrations. A: FOXQ1 mRNA expressions in HepG2 cell; B: FOXQ1 mRNA expressions in MHCC97H cell. Bars with the same letters are comparable, while bars with different letters differ significantly.

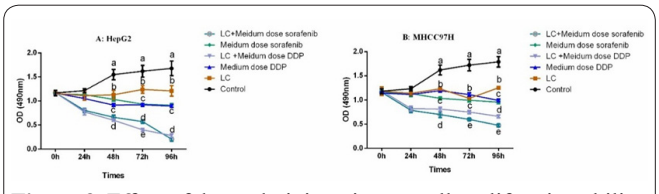

Figure 3. Effect of drug administration on cell proliferation ability, as determined using MTT assay. A: OD values in HepG2 cells; B: OD values in MHCC97H cells. Points with the same letter are comparable; points with different letters have significant differences between them.

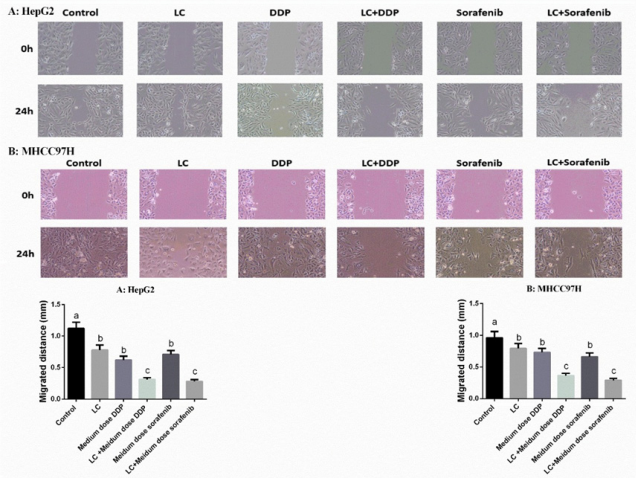

Figure 4. Scratch assay showed that cell migration ability was significantly reduced after administration of drugs. A: distances migrated by HepG2 cells; B: distances migrated by MHCC97H cells. Bars with the same letters are comparable, while bars with different letters differ significantly.

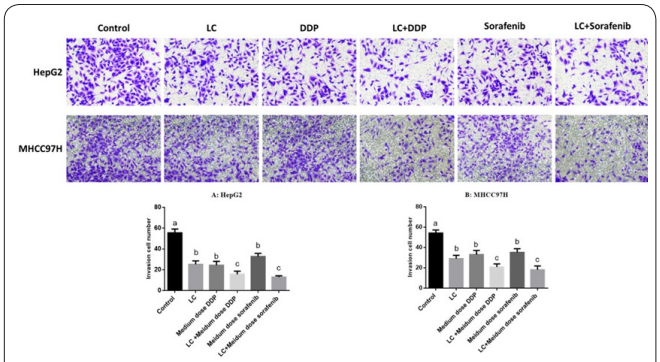

Figure 5. Transwell chamber assay showing that cell invasion ability was significantly reduced after drug administrations. A: cell numbers in HepG2 cells; B: cell numbers in MHCC97H cells. Bars with the same letters are comparable, while bars with different letters differ significantly.

markedly smaller, relative to the other groups (p<0.05; Figure 4). This suggests that Cisplatin and Sorafenib can reduce the migration of HepG2 and MHCC97H cells. Moreover, the inhibitory effect of combination treatment with LC was much better than that of monotherapy.

Combination therapy significantly inhibited the invasion of HepG2 and MHCC97H cells

Transwell chamber assay was performed to determine cell invasion. The results showed that the number of invaded cells in the control group was higher than that in the combination therapy and monotherapy groups (Figure 5), while the number of invaded cells was significantly lower in the combination therapy groups than in the monotherapy groups (p<0.05).

Discussion

Due to rapid development and metastatic potential of primary hepatocellular carcinoma, patients with surgery usually experience tumor recurrence, poor prognosis, and a low 5-year survival. Therefore, early diagnosis of patients with metastasis and recurrence, and timely and effective treatment are key to extending the survival of patients with HCC. At present, it is the focus of research to find new and more effective targeted therapy for HCC so as to improve the diagnosis and therapy of this disease.

As a traditional chemotherapeutic drug, Cisplatin (DDP) has remarkable anti-tumor effect and wide anti-tumor spectrum, and is widely used in the treatment of various solid tumors (16). The main target site of Cisplatin is DNA. Cisplatin leads to DNA cross-linking, inter-strand cross-linking, DNA-protein cross-linking, and DNA replication. Thus, one of the key points of Cisplatin chemotherapy concerns improved sensitivity of cancer cells to the drug (17). Although Cisplatin has become the standard chemotherapy drug recommended by the International Cancer Organization for first-line HCC, serious side effects and drug resistance have affected its use (18). Therefore, new models such as combination therapy for treating tumors have been considered for better results. The rapid development of molecularly targeted drugs provides a new choice for the treatment of HCC, and new molecular targeted drugs have also achieved exciting results in clinical practice. Sorafenib is a novel multi-target signal transduction inhibitor (19). Previous studies have shown that the mechanism involved in the anti-tumor effect of Sorafenib is mainly the inhibition of HCC cell proliferation through suppression of Raf and down-regulation of the expressions of pMEK and pERK. Sorafenib also induces apoptosis of HCC cells and inhibits the expressions of VEGFR and PDGFR, thereby preventing angiogenesis in tumor tissues (20, 21). It is currently the world's first multi-target targeted therapeutic drug approved for clinical use (22). However, tumor cell signaling is a complex system which cannot be effectively suppressed by curbing the progression of the tumor. Thus, newer and more effective treatment options need to be explored.

The liver has the ability to induce antigen-specific tolerance which may be destroyed by chronic viral hepatitis due to immune-mediated mechanisms (23). The occurrence and progression of HCC are complex multi-factor processes arising from series of molecular events such as abnormal signal transduction, abnormal secretion of angiogenic factors, imbalance in proto-oncogenes and tumor suppressor genes, and disorders in growth factor receptors (24, 25). The FOXQ1 is a carcinogenic transcription factor (26). A large amount of research evidence has indicated that the expression of FOXQ1 is associated with tumorigenesis, metastasis and prognosis; it is highly expressed in breast cancer, colon cancer, pancreatic cancer, ovarian cancer, glioma and other tumors (27, 28). *In vitro* experiments have shown that high expression of FOXQ1 inhibits tumor cell apoptosis caused by chemotherapeutic drugs, thereby inducing drug resistance (29). Studies have shown that overexpression of FOXQ1 in HCC leads to poor prognosis, and promotes metastasis of cancer cells (30). FOXQ1 is a downstream regulatory gene in the Wnt/β-Catenin signaling pathway, and is extremely important for the metastasis of hepatoma cells which is mediated by the β-Catenin pathway (31). It has been demonstrated that FOXQ1 changes the tumor microenvironment by regulating Versican V1, a direct target for FOXQI which promotes HCC cell metastasis and

enhances macrophage recruitment (32). Overexpression of FOXQ1 in HCC cells can significantly increase macrophage infiltration and expression of inflammatory mediators, while macrophages, which are important factors in tumor microenvironment, regulate angiogenesis in tumor tissues (32, 33). Studies have shown that in invasive human breast cancer, the presence of macrophages is associated with microvessel density, VEGF content, tumor grade, lymph node metastasis, and poor overall survival. In a variety of tumor xenograft models, simultaneous injection of macrophages increases VEGF abundance and neovascularization (34).

In summary, these studies demonstrate the importance of tumor-associated macrophages in regulating tumor angiogenesis and promoting metastasis. Liposomal clodronate (LC) is a recognized cleansing agent for macrophages. It is widely used in scientific research, and its functions and mechanisms of action are relatively well described (35). It eventually leads to cell death by blocking macrophage energy metabolism (36). Previous studies have shown that when SMMC7721 cells were implanted into the left lobe of nude mouse liver, and LC was administered, the expressions of CCL2, TNF-α, IL-6 and IL-8 in the host and tumor tissues were significantly reduced (37). Therefore, it can be hypothesized that the combination therapy of LC and chemotherapy might reduce drug resistance, thereby improving chemotherapy sensibility of tumor cells so as to achieve better anti-tumor effect.

The results presented in this study showed that FOXQ1 was highly expressed in hepatoma cell lines, when compared with normal hepatocytes. Therefore, it can be speculated that the high expression of FOXQ1 in hepatoma cells may be related to its biological function. In this study, after administration of different concentrations of Cisplatin, the expression levels of FOXQ1 in HepG2 and MHCC97H cell lines were significantly downregulated. In HepG2 cells, only the high concentration of Sorafenib down-regulated the expression of FOXQ1 significantly, when compared to the control group. It may be speculated that FOXQ1 is not a direct target of Sorafenib, since low and medium concentrations of Sorafenib had no obvious effect on the expression of FOXQ1, but high concentration of Sorafenib indirectly reduced the expression of FOXQ1 by affecting different targets. When Cisplatin or Sorafenib was combined with LC to treat these two hepatocellular carcinoma cells, the decrease in FOXQ1 expression was more significant than that seen in monotherapy. It is worth noting that the expression level of FOXQ1 was significantly downregulated even by LC in combination with low/medium concentration of Sorafenib. The results on the biological function of hepatocellular carcinoma cells confirmed that LC, Sorafenib or Cisplatin reduced the proliferation, migration and invasion of hepatocellular carcinoma cells, while the combination therapy significantly reduced the proliferation, migration and invasion of HCC cells, when compared to monotherapy.

Except for surgery, the therapeutic strategy for hepatocellular carcinoma is mainly based on chemotherapy and targeted therapy. The present study confirms that the combination of LC and conventional chemotherapeutic drugs or targeted drugs can improve the efficacy of these drugs *in vitro*. Further research is still needed to investigate the effect of combination therapy *in vivo*.

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Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Feng Zhang; Yilong Zhou, Yuan Chen, Chunyang Ma, Bingfeng Shao and Feng Zhang collected and analysed the data; Yilong Zhou and Yuan Chen wrote the text and all authors have read and approved the text prior to publication.
Yilong Zhou and Yuan Chen contributed equally to this work and should be considered as co-first authors.

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