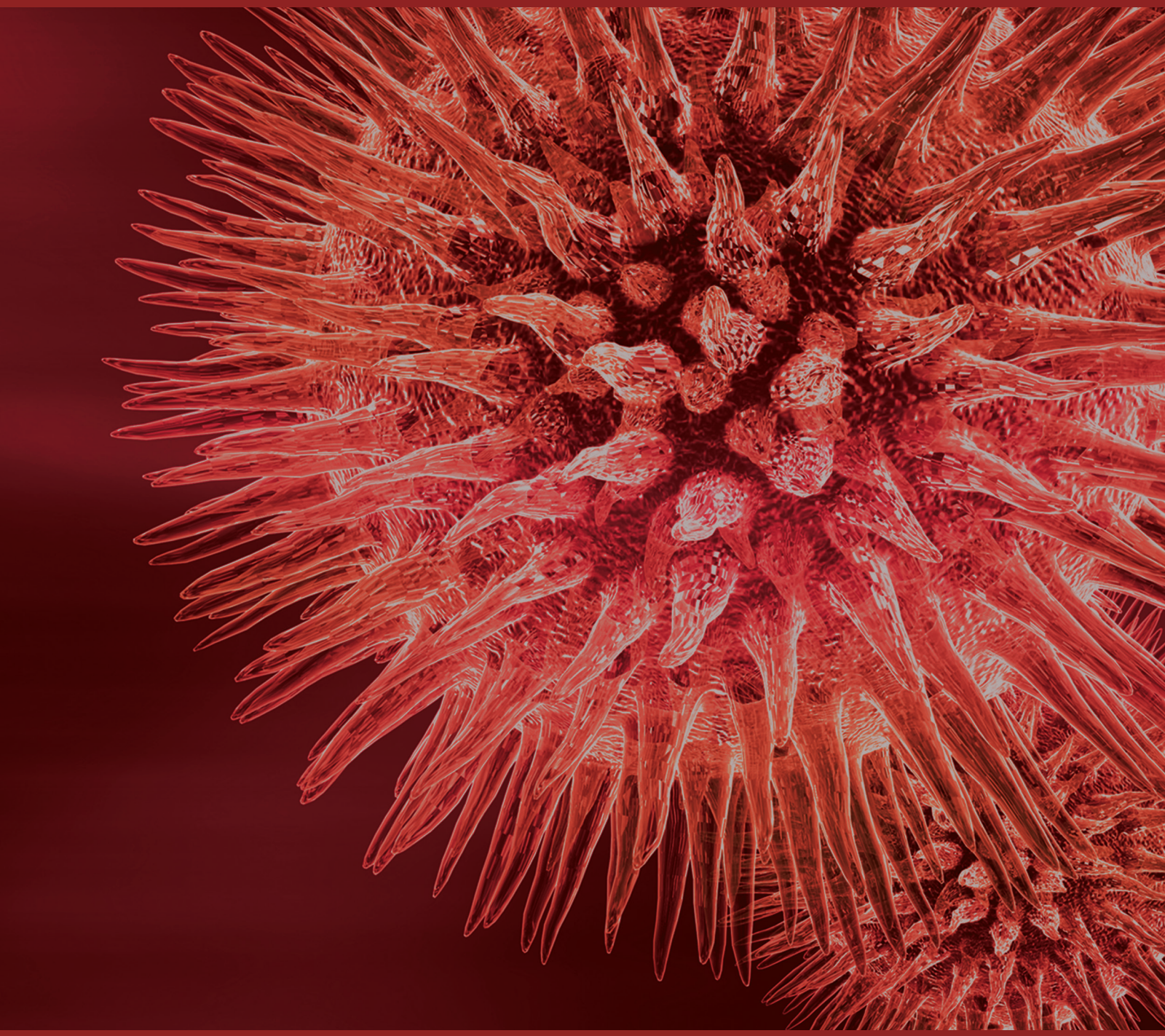
BioMed Research International

Novel Targets and Small Molecular Interventions for Liver Cancer

Guest Editors: Chunping Jiang, Youmin Wu, Jian Zhou, and Jingmin Zhao



Novel Targets and Small Molecular Interventions for Liver Cancer

BioMed Research International

Novel Targets and Small Molecular Interventions for Liver Cancer

Guest Editors: Chunping Jiang, Youmin Wu, Jian Zhou, and Jingmin Zhao

Contents

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 148783, 2 pages
http://dx.doi.org/10.1155/2014/148783

Editorial

Novel Targets and Small Molecular Interventions for Liver Cancer

Chunping Jiang,[1] Youmin Wu,[2] Jian Zhou,[3] and Jingmin Zhao[4]

[1] *Department and Institute of Hepatobiliary Surgery, The Affiliated Drum Tower Hospital, Medical School, Nanjing University, Nanjing, Jiangsu 210008, China*
[2] *Intra-Abdominal Transplant and Hepatobiliary Surgery, Westchester Medical Center of New York Medical College, Valhalla, NY 10595, USA*
[3] *Department of Liver Surgery and Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai 200032, China*
[4] *Department of Pathology and Hepatology, Beijing Institute of Infectious Diseases, Beijing 302 Hospital, Beijing 100039, China*

Correspondence should be addressed to Chunping Jiang; chunpingjiang@163.com

Received 10 July 2014; Accepted 10 July 2014; Published 4 August 2014

Liver cancer is currently the fifth most common solid tumor worldwide, causing almost 7000 deaths every year. Among primary liver cancers, hepatocellular carcinoma (HCC) represents the major histological subtype, accounting for 70% to 85% of the total liver cancer burden. To date, the major etiologies and risk factors for liver cancer development are well-defined. Due to the progress in clinical and biological fields, the primary carcinogenetic steps and molecular mechanisms have been elucidated in recent decades. The interventions for liver cancer patients vary depending on the stages. Surgical resection remains the most effective method for liver cancer patients of early stage. For patients of advanced stages, palliative therapies such as percutaneous ethanol injection, radiofrequency ablation, microwave ablation, transarterial chemoembolization (TACE), selective internal radiation therapy (SIRT), and sorafenib are recommended treatment choices for unresectable liver cancers. Despite the advances in diagnostic and therapeutic measures, the prognosis of liver cancer is not satisfying, with the 5-year survival rate being less than 12%. So the discovery of new interventions is still in demand. Recently, much attention has been paid to exploring new molecular mechanisms that might be involved in liver cancer biological behaviors. We believe further study of novel targets and small molecular interventions would be helpful to improve the prognosis of liver cancer.

In this current issue, we focus on recent advances in the field of novel targets and small molecular interventions for liver cancer which might help reveal the possible mechanism of tumorigenesis, progression, metastasis, and recurrence of liver cancer and contribute to emerging therapeutics for liver cancer. We present nine articles on novel targets and small molecular interventions for liver cancer of which six investigate the targets and mechanisms for hepatocarcinogenesis, progression, metastasis, recurrence, and HCC drug resistance, two introduce novel agents for HCC treatments, and one makes a comprehensive review on the novel molecular targets for future therapies of HCC.

The paper titled "*Reexpression of let-7g microRNA inhibits the proliferation and migration via K-Ras/HMGA2/snail axis in hepatocellular carcinoma*" by K. Chen et al. found that reexpression of let-7g inhibited the proliferation, migration, and invasion of HCC, and low expression of let-7g was significantly associated with poorer overall survival.

The work by H. Xiao et al. investigated BAG3 and HIF-1α expression in HCC tissues and analyzed the association between BAG3 and HIF-1α coexpression and prognosis following liver transplantation. They found that expression level of BAG3 and HIF-1α was efficient prognostic parameters in patients with HCC after liver transplantation.

The work by Z. Wang et al. analyzed the association between two common polymorphisms (miR-146a G>C and

miR-196a2 C>T) and risk of HCC by meta-analysis. MiR-146a G>C and miR-196a2 C>T were associated with decreased HCC susceptibility, especially in Asian population.

The work by Y.-X. Liu et al. analyzed the role of microRNA-24 in HCC related to aflatoxin B1 and revealed miR-24 was upregulated in HCC tumor tissues. MicroRNA-24 overexpression modified the recurrence-free survival and overall survival of HCC patients. The joint effects between miR-24 and AFB1 exposure on HCC prognosis were also observed.

The work by W. Chen et al. investigated tumor microenvironment on HCC cell's reaction to sorafenib. The oral multi-tyrosine kinase inhibitor sorafenib is the only approved systemic therapy for HCC patients in BCLC stage C with significant survival benefit. In this paper, Chen revealed that hepatic stellate cell- (HSC-) LX2 coculture induced sorafenib resistance in Huh7 through HGF/c-Met/Akt pathway and Jak2/Stat3 pathway which gave support to the theory that tumor microenvironment confers drug resistance to kinase inhibitors.

C. Peng et al. studied the function of tumor suppressor ZDHHC2 in HCC. Loss of heterozygosity on ZDHHC2 was associated with early metastatic recurrence following liver transplantation. Restoration of ZDHHC2 inhibited HCC proliferation, migration, and invasion.

In the work by Z. Wang et al., baicalein was found to exhibit prominent anti-HCC activity. This flavonoid induces apoptosis and protective autophagy via ER stress. Combination of baicalein and autophagy inhibitors may represent a promising therapy against HCC.

The work titled "*Dehydroabietic acid derivative QC2 induces oncosis in hepatocellular carcinoma cells*" by G. Zhang et al. investigated the inhibitory effect of a new dehydroabietic acid derivative QC2 on HCC. They found that QC2 induced HCC cell death by oncosis through activating oncosis related protein calpain.

The review article "*Hepatocellular carcinoma: novel molecular targets in carcinogenesis for future therapies*" by G. Bertino et al. made a general review on novel molecular targets in carcinogenesis for HCC.

In summary, development of novel systemic therapies for advanced liver cancer, including drugs, small molecular agents, and gene therapies, is of paramount importance. This special issue presents several intriguing achievements in the area of novel targets and small molecular interventions which we believe could be utilized in liver cancer therapy in the future.

Acknowledgments

We wish to express our appreciation to all the authors for their excellent contribution and to the editors for their hard work on this issue.

Chunping Jiang
Youmin Wu
Jian Zhou
Jingmin Zhao

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 682197, 11 pages
http://dx.doi.org/10.1155/2014/682197

Research Article

Dehydroabietic Acid Derivative QC2 Induces Oncosis in Hepatocellular Carcinoma Cells

Guang Zhang,[1] Chunping Jiang,[2] Zhongxia Wang,[2,3] Weibo Chen,[2,3] Wen Gu,[4] and Yitao Ding[1,2]

[1] *Department of Hepatobiliary Surgery, Drum Tower Clinical Medical College of Nanjing Medical University, Nanjing, Jiangsu 210008, China*
[2] *Department of Hepatobiliary Surgery, Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, China*
[3] *School of Medicine, Nanjing University, Nanjing, Jiangsu 210093, China*
[4] *College of Chemical Engineering, Nanjing Forestry University, Nanjing, Jiangsu 210037, China*

Correspondence should be addressed to Wen Gu; njguwen@163.com and Yitao Ding; yitao_ding@163.com

Received 27 March 2014; Accepted 8 June 2014; Published 6 July 2014

Academic Editor: Jian Zhou

Aim. Rosin, the traditional Chinese medicine, is reported to be able to inhibit skin cancer cell lines. In this report, we investigate the inhibitory effect against HCC cells of QC2, the derivative of rosin's main components dehydroabietic acid. *Methods.* MTT assay was used to determine the cytotoxicity of QC2. Morphological changes were observed by time-lapse microscopy and transmission electron microscopy and the cytoskeleton changes were observed by laser-scanning confocal microscopy. Cytomembrane integrity and organelles damage were confirmed by detection of the reactive oxygen (ROS), lactate dehydrogenase (LDH), and mitochondrial membrane potential ($\Delta\psi$m). The underlying mechanism was manifested by Western blotting. The oncotic cell death was further confirmed by detection of oncosis related protein calpain. *Results.* Swelling cell type and destroyed cytoskeleton were observed in QC2-treated HCC cells. Organelle damage was visualized by transmission electron microscopy. The detection of ROS accumulation, increased LDH release, and decreased ATP and $\Delta\psi$m confirmed the cell death. The oncotic related protein calpain was found to increase time-dependently in QC2-treated HCC cells, while its inhibitor PD150606 attenuated the cytotoxicity. *Conclusions.* Dehydroabietic acid derivative QC2 activated oncosis related protein calpain to induce the damage of cytomembrane and organelles which finally lead to oncosis in HCC cells.

1. Introduction

HCC, the fifth common cancer, causes the third most cancer-related death worldwide [1]. Although therapy strategies have been developed, the outcome of this fatal disease is unsatisfactory, with the five-year overall survival rate being less than 11% [2]. Various reasons might contribute to this dilemma situation among which the bad behavior of HCC might be the most important one. The general therapies for HCC are comprised of surgery and palliative treatments. Hepatectomy and liver transplantation remain the curative treatments for this fatal disease while only 30–40% patients are eligible [3]. For those end-stage patients, noncurative strategies including transcatheter arterial chemoembolization (TACE) and radiofrequency ablation (RFA) are available but only few-month survival time is prolonged [3]. Unlike other cancers, specific molecular targets in HCC are deficient which results in lacking of preferable molecular targeted drugs except for sorafenib [4, 5]. On the other hand, the overexpression of multidrug-resistance gene leads to the insensitivity of systemic chemotherapy [6]. As a result, it is urgent and significant to develop novel therapies for HCC.

Natural derived compounds and their derivatives have been developed into anticancer agents for decades. Newman

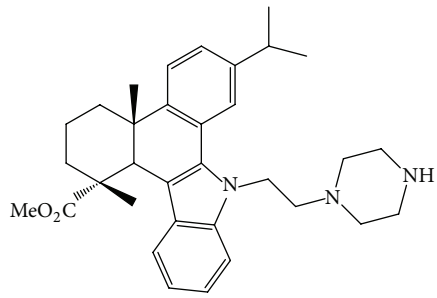


Figure 1: Structure of QC2 (2,3,4,4a,9,13c-hexahydro-7-isopropyl-1, 4a-dimethyl-9-(2-(piperazin-1-yl)ethyl)-1H-dibenzo[a,c]carbazole-1-carboxylic acid methyl ester).

et al. [7] analyzed 92 commercially available anticancer drugs. Of all the drugs, they found that 62% were related to the natural products. Natural compounds and their derivatives play a critical role in the discovery of new anticancer agents which can be proved by the approval of commonly used drugs in clinic. These agents include toposide (established from the podophyllin), paclitaxel (originated from the rind of *Taxus brevifolia*), and doxorubicin (extracted from the streptomycin). Rosin, the natural product of pine tree, is widely used in traditional Chinese medicine. Tanaka tested the anticancer effects of the main components of rosin and found that 4 out of 8 compounds were toxic to the two skin cancer cell lines [8]. Gu et al. did chemical modification to the dehydroabietic acid, an effective component of rosin, according to Tanaka's research and tested the antibacterial activity [9]. The newly synthesized compounds showed potential inhibitory activity to different bacteria. Nevertheless, the anticancer effect remained unknown.

In this study, we used an N-substituted 1H-dibenzo[a,c]carbazole derivative from dehydroabietic acid, which was granted by Gu, and focused on the anticancer effects and the underlying mechanisms.

2. Methods

2.1. Synthesis of Dehydroabietic Acid Derivative QC2. QC2 (Figure 1) is an N-substituted 1H-dibenzo[a,c]carbazole derivative synthesized from dehydroabietic acid. The synthetic route of compound QC2 is illustrated in Scheme 1 and the detailed methods could be found in the paper of Gu and coworkers [10].

2.2. Cell Culture and Reagents. Human HCC cell lines Hep3B, HepG2, and Huh7 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China); SMMC-7721 and human hepatocyte LO2 cells were purchased from Cell Bank of Xiangya Central Experiment Laboratory of Central South University (Changsha, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Gaithersburg, MD) containing 10% fetal bovine serum (FBS) (GIBCO BRL, Gaithersburg, MD), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured in 5% CO_2 at 37°C. Purified QC2 was dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mg/mL and stocked at −20°C. 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium (MTT) and anti-tubulin-Cy3 antibody were purchased from Sigma Aldrich (St. Louis, MO). Alexa Fluor 594 phalloidin and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Life Technologies (Invitrogen, Carlsbad, CA). Calpain, caspase-3, actin, and tubulin antibody were obtained from Cell Signaling Technology (Danvers, MA, USA).

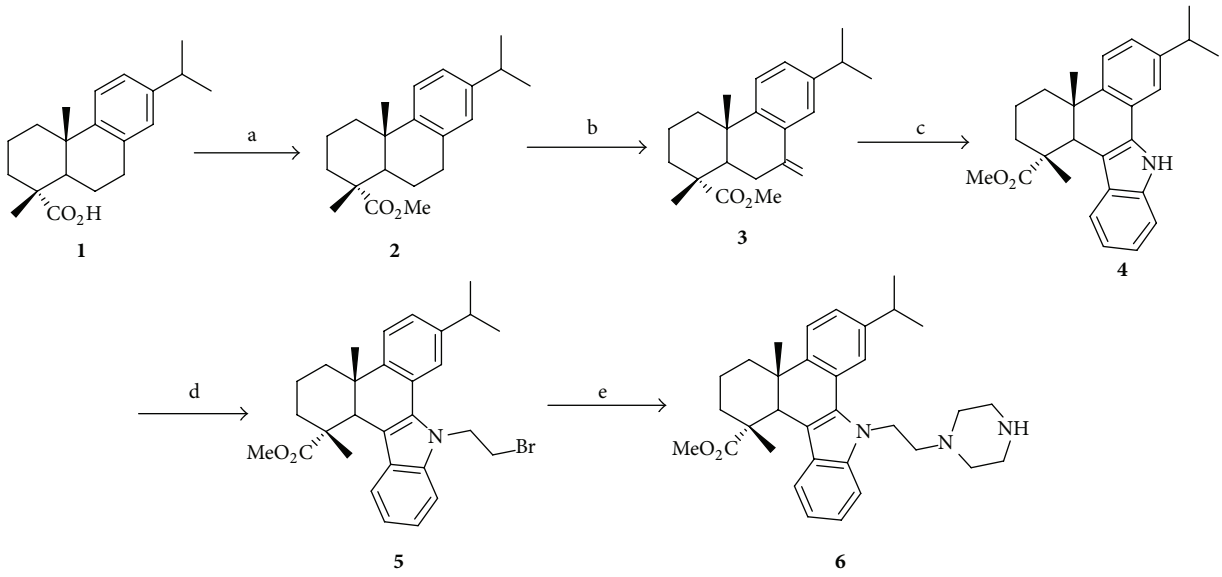
2.3. MTT Assay. Cytotoxicity was determined by MTT assay. Generally, 5×10^3 cells were seeded into 96-well plates and cultured for 24 hours. After being treated with indicated reagents for different times, cells were incubated with 20 μL MTT (5 mg/mL) for another 4 hours. Then, the culture medium was discarded and 150 μL DMSO was added to dissolve the MTT formazan precipitates. The optical density was measured using a microplate reader (Molecular Devices, Sunnyvale, CA) at 490 nm. The cytotoxicity of QC2 to each cell line was determined by the preceding results.

2.4. Annexin V-FITC/PI Staining. Cells were collected after being treated with different concentration of QC2. The cell aggregates were resuspended with the binding buffer and incubated with Annexin V-FITC for 30 minutes at room temperature. The supernatants were discarded after centrifugation. Cells were resuspended with the binding buffer and incubated with PI and subjected to flow cytometry analysis.

2.5. Western Blotting. To determine the level of indicated proteins, QC2-treated cell lysates were prepared as described [11]. Twenty μg proteins was analyzed by Western blot as described. The PVDF membranes with transferred proteins were incubated with primary antibodies at 4°C overnight and HRP-conjugated secondary antibodies at room temperature for 2 hours. The signal was developed by the enhanced chemiluminescence (ECL) reagent (Millipore, Bedford, MA) and visualized by FlourChem FC2 Imaging System (Alpha Innotech, San Leandro, CA).

2.6. Transmission Electron Microscopy. Cells treated with QC2 were digested by trypsin and then centrifuged at 1000 r/min. Thereafter, cells were fixed with 4% glutaraldehyde and postfixed with 0.15 mol/L phosphate buffer with 3% OsO_4 both for 1 hour. Dehydration was carried out in alcohol-water solutions of varying concentrations and 100% propylene oxide. Then, cells were embedded by a mixture of propylene oxide and araldite at the ratio of 1 : 3 (v/v) concentrations overnight after been embedded at 1 : 1 for 1 hour. After treated with undiluted resin for 1 h, cells were polymerized at 60°C for 3-4 days. Ultrathin sections were obtained and stained with 1% toluidine blue. Ultrastructure was observed by a transmission electron microscope (JEM-1010, Japan).

2.7. Fluorescence Microscopy. Cells were seeded on the cover slides in 24-well plates. Fixation was performed with 4%



Scheme 1: Synthetic route of compound QC2 (**6**) from dehydroabietic acid (**1**). Reagents and conditions: (a) (i) $SOCl_2$, benzene, reflux, and 3 h; (ii) MeOH, reflux, and 2 h; (b) CrO_3, AcOH, Ac_2O, 0°C to rt, and 12 h; (c) phenylhydrazine hydrochloride, EtOH, conc. HCl, reflux, and 3 h; (d) 1,2-dibromoethane, TBAB, NaOH, benzene, rt, and 12 h; (e) piperazine, K_2CO_3, KI, MeCN, reflux, and 8 h.

paraformaldehyde for 10 minutes and permeabilization was carried out with 0.1% Triton X-100 for 5 minutes. After washing for 5 times, cells were preincubated with PBS containing 1% BSA for 30 minutes. Staining solution was added after discarding the 1% BSA. Incubation time was according to the manufacturer's instructions followed by washing with PBS for 5 times. When nuclear staining was required, DAPI or PI was used afterwards. Cover slides were mounted onto glass slides and observed by a fluorescence microscope.

2.8. Lactate Dehydrogenase (LDH) Release Assay. 5×10^3 cells were seeded into 96-well plates. After being treated with 5 mg/mL QC2 for indicated time, the plates were centrifuged to get the supernatants. The LDH level of the supernatants was detected by a LDH assay kit (Beyotime, Nantong, China). The optical density was measured using a microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm.

2.9. Detection of Intracellular ATP. As a parameter of Na^+, K^+-ATPase activity, the intracellular ATP was detected. We carried out this detection using a commercial detection kit (Beyotime, Nantong, China) according to the manufacturer's instructions. Luciferase activity was measured as the instruction of ATP level by Dual-Luciferase Reporter Assay System.

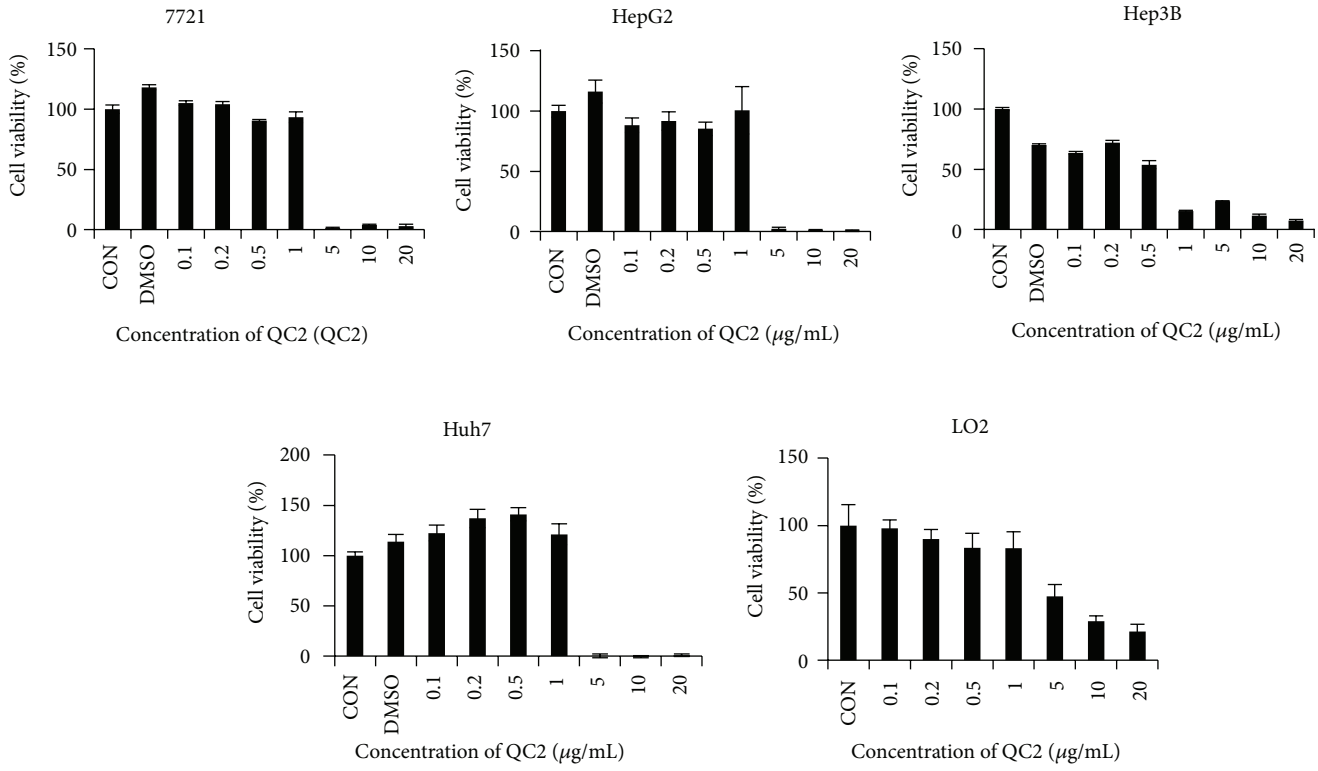
2.10. Mitochondrial Membrane Potential Measurement. Changes of mitochondrial membrane potential were the earliest indication of cell death but not the distinction between apoptosis and oncosis. We measured the mitochondrial membrane potential using a commercial kit (Beyotime, Nantong, China) according to the manufacturer's protocols. Cells were stained by the kit and fluorescence intensities were detected by flow cytometry. The $\Delta\psi m$ was calculated through the fluorescence intensities using the Cell Quest software (Becton Dickinson, Bedford, MA).

2.11. Reactive Oxygen Species Assay. Intracellular ROS level was measured using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Pretreated cells were reacted with DCFH-DA according to the manufacture's protocol. Cellular fluorescence was measured by flow cytometry.

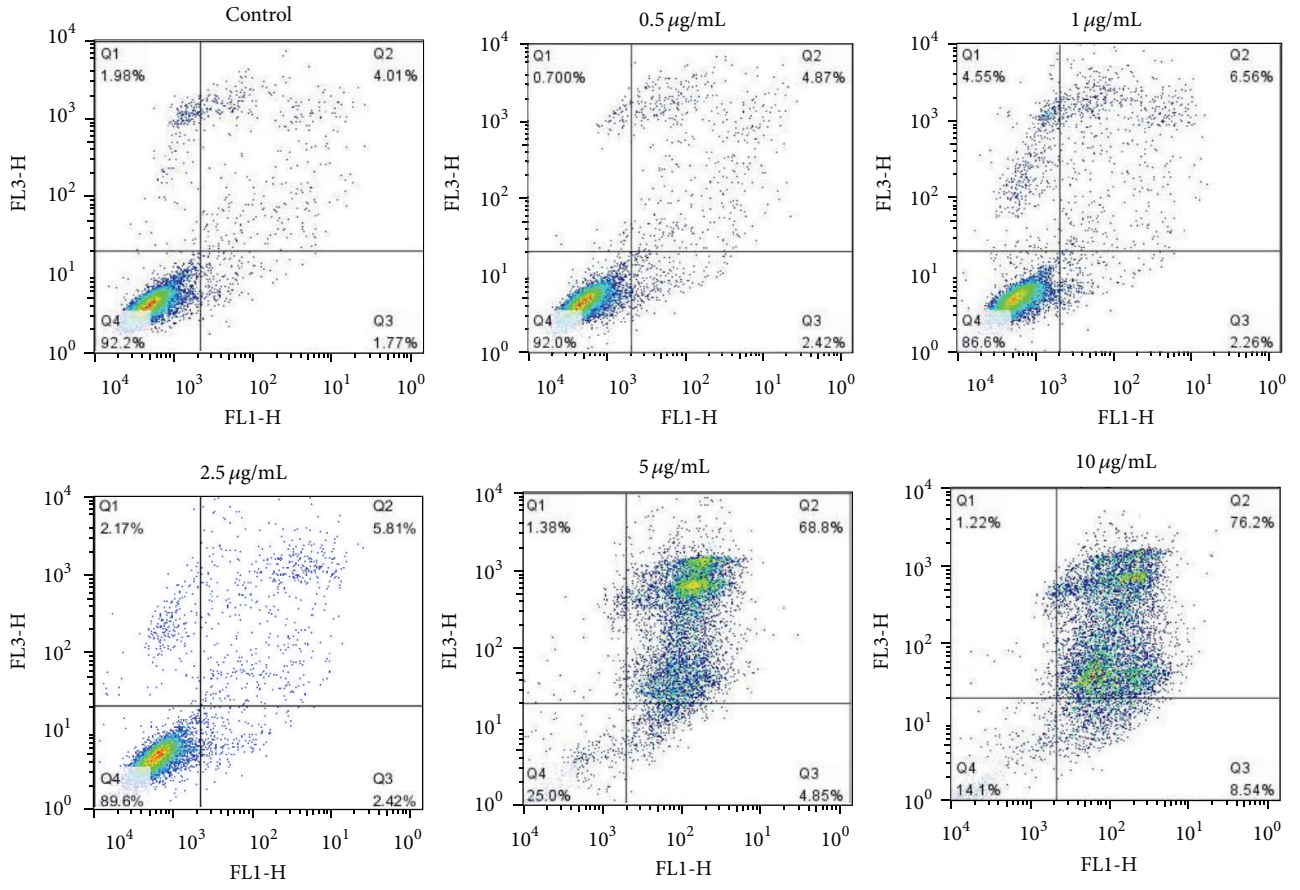
2.12. Statistical Analysis. Numeric data were shown as means ± SD. Statistical significance between two groups was analyzed by two-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. QC2 Exerted Cytotoxicity against HCC Cell Lines as well as Human Hepatocytes. We studied the cytotoxicity of QC2 on SMMC-7721, Hep3B, HepG2, and Huh7 by MTT assay and flow cytometry (Figures 2(a) and 2(b)). Of all the four HCC cell lines, QC2 showed high cytotoxicity that 5 μg/mL QC2 was lethal to most HCC cells. The IC_{50} values of four HCC cell lines were 0.37 μg/mL for Hep3B cells, 1.17 μg/mL for HepG2 cells, 3 μg/mL for Huh7 cells, and 2.67 μg/mL for SMMC-7721 cells, respectively. The IC_{50} value for LO2 cells was higher: 4.22 μg/mL. Something interesting was that Huh7 cells and SMCC-7721 died at the concentration of 5 μg/mL while nearly all cells survived at 2.5 μg/mL. During the cytotoxicity determination, we also observed the morphologic change. Time-lapse microscopy showed that cells membrane became incomplete and cells swelling happened after plasma membrane blebbing appeared (Figure 2(c)).

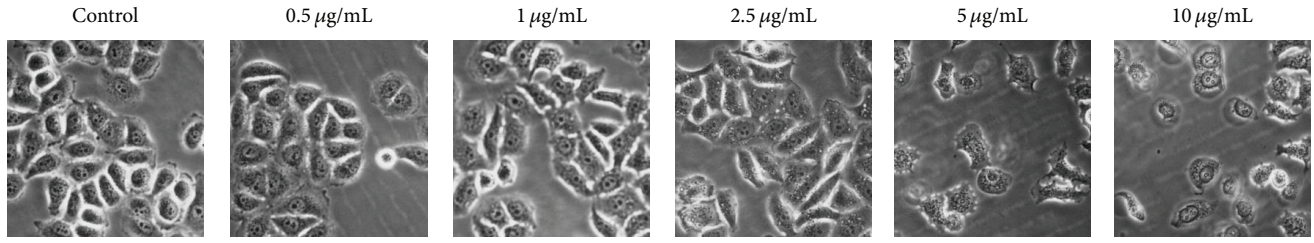


(a)



(b)

Figure 2: Continued.



(c)

Figure 2: QC2 induced cell death in HCC and human hepatocytes. (a) QC2 exerted cytotoxicity in four HCC cell lines as well as in human hepatocytes; the IC_{50} values were 0.37 μg/mL, 1.17 μg/mL, 3 μg/mL, 2.67 μg/mL, and 4.22 μg/mL, respectively. (b) QC2 induced cell death in SMMC-7721 cells was testified by flow cytometry. (c) QC2 dose-dependently induced morphological changes in SMMC-7721 cells.

3.2. QC2 Ravaged the Integrity of Cell Membrane. LDH would be released into the medium and the nucleus would become easily stained when the cell membrane is destructed. Hence, we evaluated the integrity of cell membrane by LDH release assay and PI uptake. We treated cells with 10 μg/mL QC2 from 0.25 hour to 4 hours. As shown in Figure 3(a), LDH level stayed low during the first 1 hour and increased at 2 hours which suggested a membrane rupture. The dose-dependent PI uptake was in accordance with the LDH release assay. As shown in Figure 3(b), cells retained smooth morphology and no fluorescence was seen in the control group or the low dose groups while cells became swelled and red fluorescence stained by PI in the groups over 2.5 μg/mL.

3.3. Ultrastructure Examination Confirmed Oncotic Cell Death. Morphology changes were the direct distinction between apoptosis and oncosis. Due to the extraordinary morphology changes observed by time-lapse microscopy, we examined the ultrastructure of QC2-treated cells (Figure 3(c)). After being treated with QC2 for 1 hour, pseudopodia disappeared because of cell swelling and organelles injured apparently which might lead to membrane blebbing. Nucleus remained integrated but dilated as well as the whole cell. Two hours later, plasma membrane got destroyed and organelles became fuzzy. Chromosomes condensation was observed in the 2-hour group. The above ultrastructure changes confirmed our hypothesis that QC2 induced oncosis in HCC cells.

3.4. Cytoskeleton Was Destroyed by QC2. As ultrastructure examination showed fuzzy morphology at the end of QC2 treatment, we speculated that the cytoskeleton might also be affected. So we testified the cytoskeleton changes by fluorescence microscopy and Western blotting. As shown in Figure 4(a), the staining of microtubules and microfilaments weakened with the extended response time. Actin and tubulin protein were also detected and both suffered a significant decrease (Figure 4(b)).

3.5. QC2 Induced Oncosis Was Not Reversed by Caspase-3 Inhibitor z-VAD-fmk or Necrosis Inhibitor Necrostatin. As an executioner caspase, caspase-3 plays a crucial role both in extrinsic (death ligand) and intrinsic (mitochondrial) apoptosis pathways [12, 13]. Blockage of caspase-3 by its inhibitor z-VAD-fmk could abolish the apoptotic events; the same thing would happen in necrosis by necrostatin [14]. To further distinguish oncosis from apoptosis and necrosis, we pretreated cells with z-VAD-fmk (50μM) or necrostatin (500 nM) before QC2 treatment. Results came out that no statistical difference was detected when using z-VAD-fmk or necrostatin (Figures 5(a) and 5(b)). During the protein detection, carboplatin was set as the positive control for activated caspase-3 detection but no such activated caspase-3 was found in the QC2 groups (Figure 5(c)). All these results suggested that the oncosis induced by QC2 in SMMC-7721 cells was different from apoptosis and necrosis.

3.6. QC2 Caused ATP Depletion, ROS Generation, and Mitochondrial Membrane Potential Changes in HCC Cells. Researchers have found that oncosis started with ATP depletion; then, ion pumps were influenced and $\Delta\psi$m collapsed [15]. As an indicator of mitochondria damage, ROS was also detected. ATP decreased time-dependently as well as $\Delta\psi$m (Figures 6(a) and 6(b)). Compared to the positive control, 5 mg/mL Rosup, the QC2-treated cells showed a similar tendency of ROS accumulation (Figure 6(c)). ROS generation and mitochondrial membrane changes were also key events in apoptosis and necrosis; however, ATP depletion emerged only in oncosis. The ATP depletion detected in our research confirmed QC2 induced oncosis in HCC cells.

3.7. Oncosis Related Protein Calpain Activated in QC2-Treated HCC Cells. Various studies have found that calpain, a kind of calcium-dependent thiol proteinase, mediated the oncotic event in different cells. Researchers have found that calpain increased before membrane damage and inhibition of calpain could protect cells from cell death [15], but whether calpain-1 or calpain-2 was involved remained unknown. In our study, we confirmed the activation of calpain-1 in QC2-treated cells by detecting the level of this protein. As shown in Figure 7(a), calpain-1 increased and the protein autolyzed from the molecular weight of 80 kDa to 76 kDa when treated with QC2. The increased calpain-1 level might imply the activation of this protein during the oncotic cell death. In the subsequent experiment, the calpain inhibitor PD150606 was adopted. To our delight, 100 μM PD150606 partially

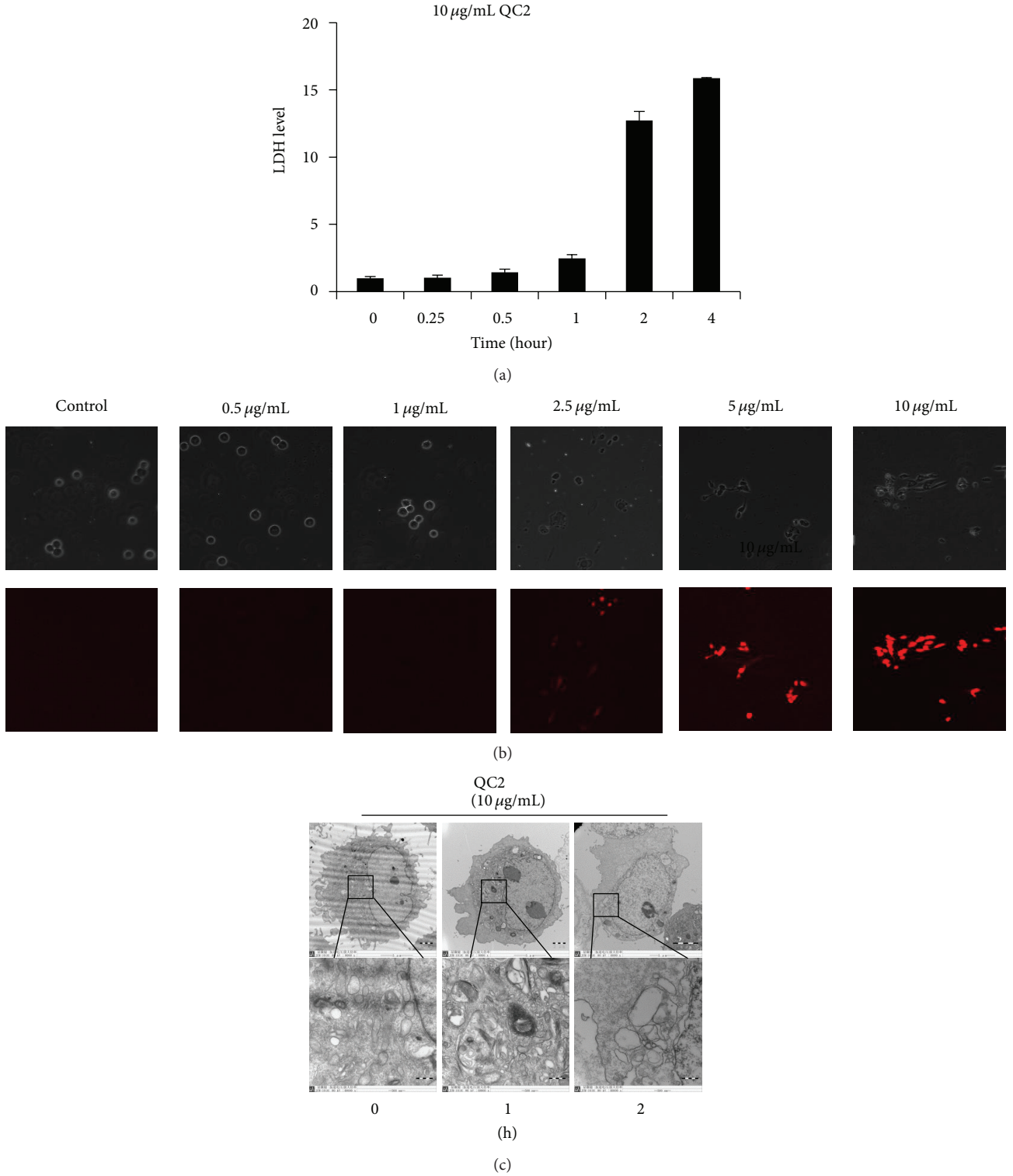


Figure 3: QC2 induced destruction of cytomembrane and ultrastructural changes in SMMC-7721 cells. (a) LDH level time-dependently increased in the supernatants of QC2-treated cells. (b) PI uptake of SMMC-7721 cells accumulated along with increase of QC2 concentration. (c) Electron micrographs of QC2-treated SMMC-7721 cells showed ultrastructure changes.

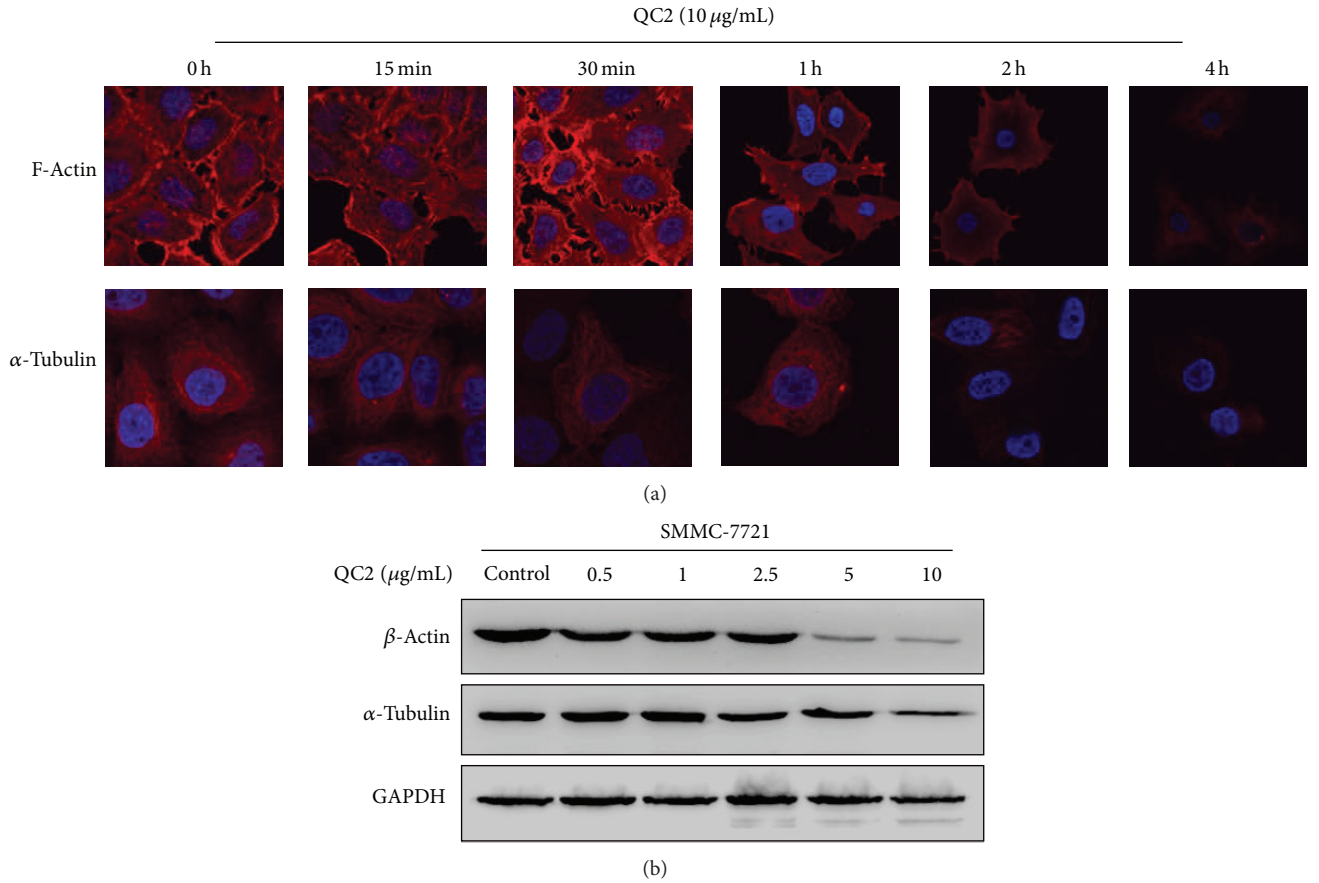


Figure 4: QC2 destroyed the cytoskeleton of SMMC-7721 cells. (a) Immunostaining of cytoskeleton proteins actin and tubulin. (b) Expression of actin and tubulin in SMMC-7721 cells treated with or without QC2; GAPDH was set as internal standard to normalize loadings.

abrogated the cytotoxicity of QC2 at the concentration of 3 μg/mL (Figure 7(b)).

4. Discussion

HCC is one of the tumors with poor prognosis. The dilemma of HCC might lie in deficiency of effective drugs. According to the BCLC staging system, patients with advanced stage tumors are recommended to receive palliative treatments such as TACE, sorafenib, and symptomatic treatment [3]. Sorafenib is the only molecular-targeted drug that has been proved to have survival benefit for HCC patients although it can only prolong the median survival time for few months. Systemic chemotherapy has been proved to be useless for HCC but TACE, a kind of local chemotherapy, partially improves the survival time [16, 17]. During TACE, tumor tissues are perfused with chemotherapeutics like doxorubicin and cisplatin and the tumor supplying blood vessels are embolized. As a result, tumor tissues are suffering from ischemia and chemotherapeutics that HCC cells die of both apoptosis and necrosis. This therapeutic tragedy could suppress the tumor growth. However, it could not thoroughly remove the tumor cells that most patients would experience the tumor recurrence. Neovascularization and resistance to chemotherapeutics might be the reason [11]. Thus, more effective chemotherapeutics need to be discovered.

Cell death is a topic which has been debated for decades. Therapeutic drugs are also developed based on their cytotoxicity. In the past, apoptosis and accidental cell death are the main cell death types that have been widely studied [18]. Apoptosis is a programed process while accidental cell death is unpredictable [19]. Autophagic cell death is a recently discovered cell death type [20]. Of all these types of cell death, apoptosis is the most widely studied and its molecular mechanisms are well revealed. Apoptosis starts with damage of nuclear chromatin; then the cytoplasm condensates and DNA degrades, and at last cell membrane blebs and the whole cell fragmentates into apoptotic bodies [18]. Oncosis, which is originated from the word "swell," is one type of accidental cell death and is earlier known for being common in ischemia issues. During the process of oncosis, cells suffer from the deletion of intracellular ATP caused by ischemia or other stuff; then, cytomembrane becomes unstable and cellular swelling happens [21]. As a result, the morphological changes of oncosis turn out to be different: the incompleteness of cytomembrane, blebbing of plasma membrane, clumping of nuclear chromatin, and dilation of endoplasmic reticulum (ER) [19]. Researchers also found that oncosis was accompanied with rearrangement

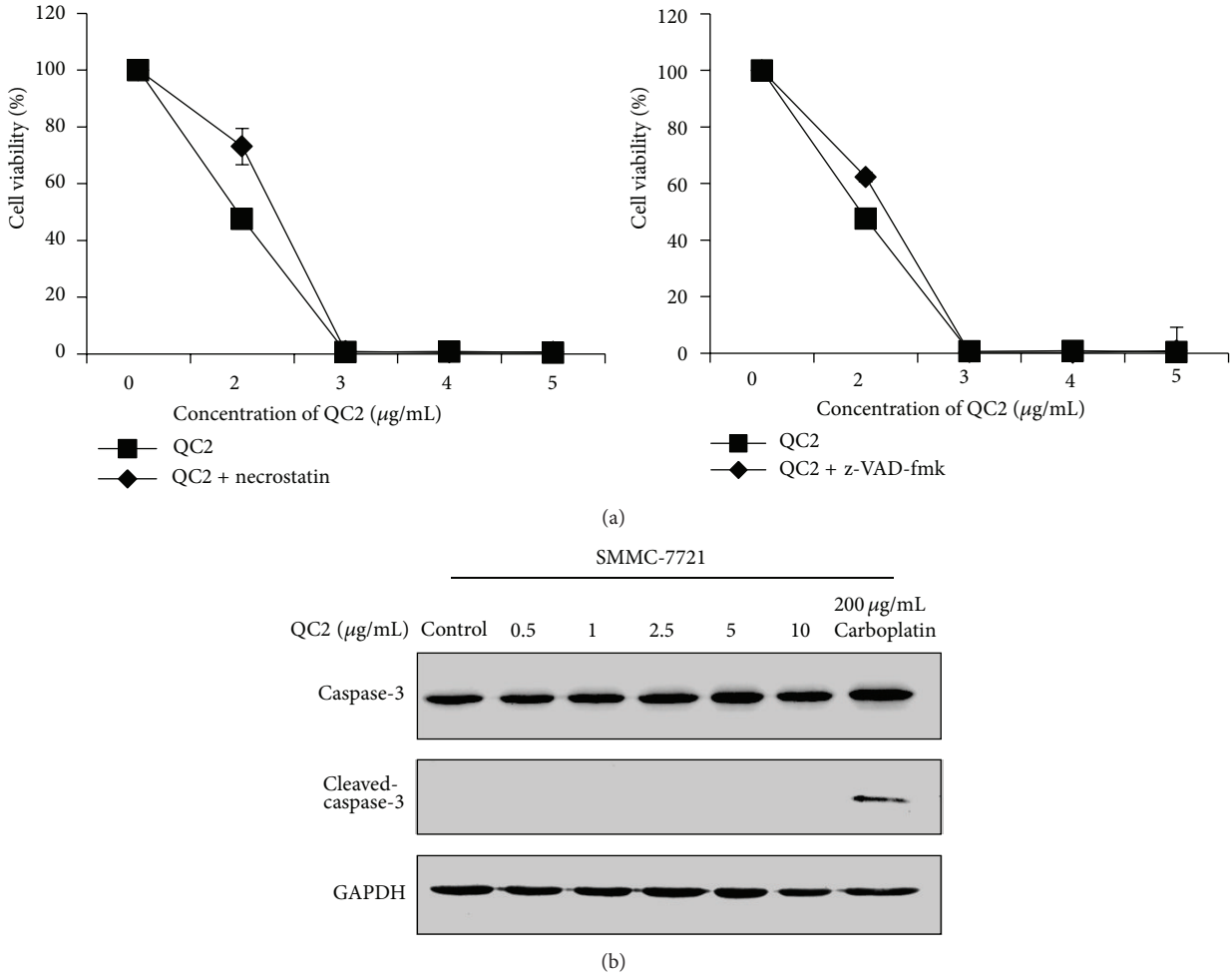


Figure 5: QC2 induced oncosis was not interfered by neither apoptosis inhibitor nor necrosis inhibitor and no caspase-3 activation was detected under QC2 treatment. (a) No significant change of cell viability was detected when SMMC-7721 cells were pretreated with or without z-VAD-FMK or necrostatin ($P > 0.05$). (b) As QC2 concentration increased, no cleaved-caspase-3 was detected while it appeared in 200 μg/mL carboplatin treated cells.

of cytoskeleton [22]. Meanwhile, these two processes did not distinguish completely between each other; for example, the mitochondrial membrane potential changes and ROS accumulates and they both share the same ending, necrosis.

Despite the fact that the procedure of oncosis has been known for decades, the mechanisms were not fully understood. ATP deletion is thought to be the origin of this passive death type. Lieberthal et al. [23] used antimycin and 2-deoxyglucose to reduce ATP in mouse proximal tubular cells. They found that oncosis happened when 85% or more ATP was deleted but cells would survive or apoptosis would happen when less ATP was deleted. Liu et al. [15] found that the oncotic cell death could not be restored when the respiration and ion homeostasis were totally destroyed. In oncosis, the decrease in intracellular ATP (ATP[i]) resulted in the inactivation of Na^+, K^+-ATPase at the membrane; then, the intracellular Na^+, Ca^{2+}, and Cl^- arise along with water influx which leads to the cellular swelling. At the same time, the rapid increase of Ca^{2+}[i] stimulates many signals and also results in the dysfunction of mitochondria after which the intracellular ATP synthesis is further influenced [24]. This positive feedback exacerbates cell death and the cytoskeleton also collapses as a result of Ca^{2+}[i] increase.

Recently, oncosis regained researchers' attention as some agents exhibited anticancer activity via this process and certain novel anticancer agents were also established based on induction of oncotic cell death. Ma et al. [25] focused on an antibiotic isolated from *Pseudomonas jinjuensis*, which was previously proved to have anticancer potential, and found that this antibiotic could induce oncosis in breast cancer cells. Steroidal alkaloidal saponins were demonstrated to be natural anticancer agents as they induced apoptosis in cancer cells [26]. However, oncosis was also inducted by the solamargine, one of steroidal alkaloidal saponins [27]. Mechanisms were not mentioned in preceding studies. Artesunate was also a native compound which could induce oncosis in cancer cells. Zhou et al. [28] found that the increase of calpain-2 level was accompanied with the oncosis event induced by artesunate

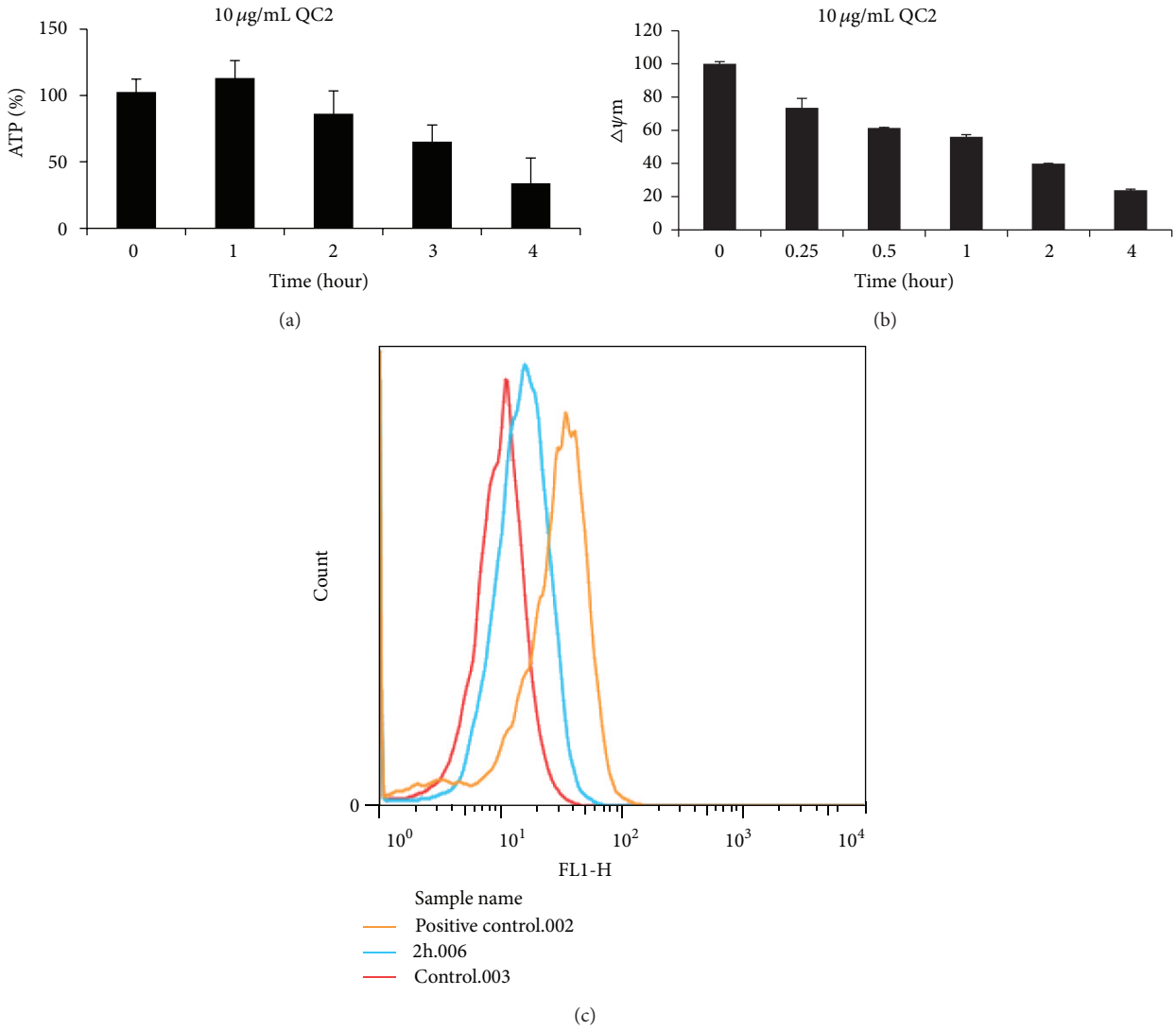


Figure 6: ATP deletion, $\Delta\psi$m collapse, and ROS generation were observed in oncotic cells. ((a) and (b)) ATP and $\Delta\psi$m level generally decreased in 10 μg/mL QC2-treated SMMC-7721 cells time-dependently. (c) ROS accumulation was detected in QC2-treated cells.

and they partly elucidated the mechanisms based on calpain-2 activation. The underlying mechanism of oncosis might be related to activation of calpain.

In our study, the QC2-treated cells showed a significant ATP decreasing level, while the ATP depletion was considered to be the initiation of oncosis [15]. The cytomembrane was destroyed due to the inactivation of ion pumps caused by ATP depletion. At the same time, ROS accumulated and $\Delta\psi$m collapsed as the marker of cellular damage. Oncotic cell death was irreversible when mitochondrial function and ion homeostasis were totally destroyed [29]. Calpain activation also played a key role in this process but its autolysis was controversial [30]. For example, Molinari et al. [31] reported that calpain was activated at 80 kDa form but Kulkarni et al. [32] thought that the activation was accompanied with the autolysis. We studied the protein level of calpain-1 and found that its upregulation was accompanied with its autolysis as well as the oncotic events while its inhibition partially rescued the cells. As a calcium-activated neutral protease, calpain could hydrolyze many substrates including cytoskeletal proteins. The morphological changes and the destruction of cytoskeletal proteins might be related to the activation of calpain. Nevertheless, relationships between calpain and oncosis should be further illuminated.

Liver dysfunction is always accompanied with HCC [33]. The diseased liver may not sustain the cytotoxicity of chemotherapeutic agents. The cytotoxicity of QC2 to normal cells was also considered. We testified the sensitivity with a normal liver cell line LO2. The IC_{50} value for LO2 was higher than for HCC cell lines; however, the cytotoxicity should not be ignored. Further study on decreasing cytotoxicity against normal hepatocytes in vivo should be carried out.

In conclusion, QC2 showed a potential anticancer effect in HCC cell lines through inducing oncotic cell death. It might provide a novel therapeutic strategy for HCC and the safety needs to be further studied.

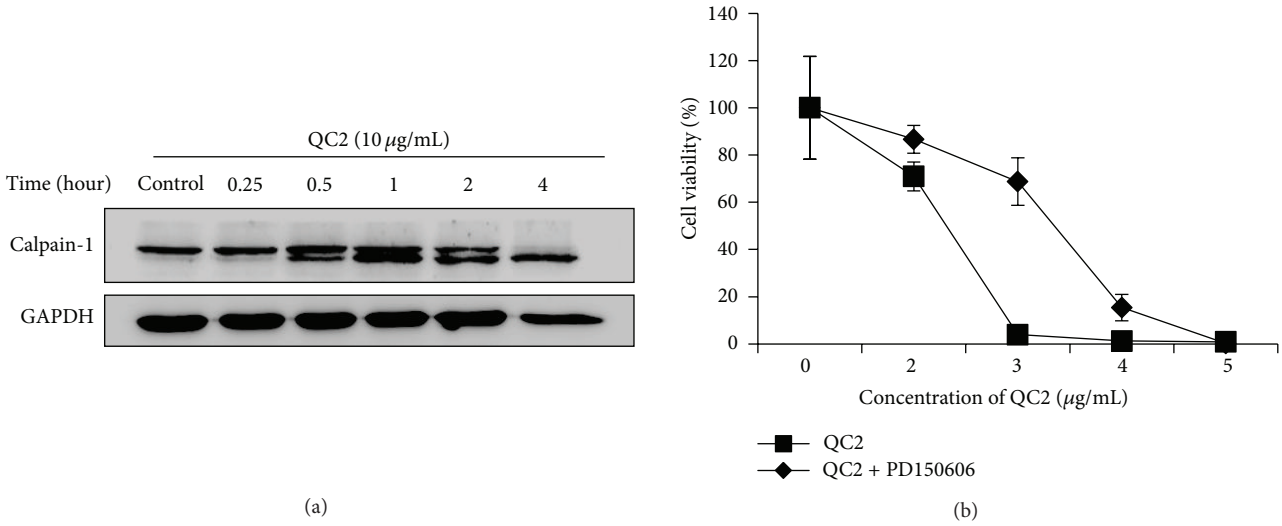


Figure 7: Calpain-1 increased during oncosis and its inhibition attenuated the cytotoxicity of QC2. (a) Calpain-1 increased and gradually autolyzed into 76 kDa fragments under QC2 treatment. (b) Significant change of cell viability was observed in SMMC-7721 cells when pretreated with the calpain inhibitor PD150606 ($P < 0.05$).

Conflict of Interests

The authors declared no conflict of interests.

Authors' Contribution

Guang Zhang and Chunping Jiang contributed equally to this study.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. NSFC30801417); the Natural Science Foundation of Jiangsu Province (no. BK2009010); the Doctoral Fund of the Ministry of Education of China (no. RFDP200802841004); Key Project supported by Medical Science and Technology Development Foundation, Nanjing Department of Health (no. ZKX12030); and the Scientific Research Foundation of Graduate School of Nanjing University (no. 2013CL14).

References

[1] K. Schütte, J. Bornschein, and P. Malfertheiner, "Hepatocellular carcinoma-epidemiological trends and risk factors," *Digestive Diseases*, vol. 27, no. 2, pp. 80–92, 2009.

[2] B. Blechacz and L. Mishra, "Hepatocellular carcinoma biology," *Recent Results in Cancer Research*, vol. 190, pp. 1–20, 2013.

[3] J. Bruix and M. Sherman, "Management of hepatocellular carcinoma: an update," *Hepatology*, vol. 53, no. 3, pp. 1020–1022, 2011.

[4] J. M. Llovet, S. Ricci, V. Mazzaferro et al., "Sorafenib in advanced hepatocellular carcinoma," *The New England Journal of Medicine*, vol. 359, no. 4, pp. 378–390, 2008.

[5] A. Cheng, Y. Kang, Z. Chen et al., "Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial," *The Lancet Oncology*, vol. 10, no. 1, pp. 25–34, 2009.

[6] I. O. L. Ng, C. L. Liu, S. T. Fan, and M. Ng, "Expression of P-glycoprotein in hepatocellular carcinoma: a determinant of chemotherapy response," *The American Journal of Clinical Pathology*, vol. 113, no. 3, pp. 355–363, 2000.

[7] D. J. Newman, G. M. Cragg, and K. M. Snader, "The influence of natural products upon drug discovery," *Natural Product Reports*, vol. 17, no. 3, pp. 215–234, 2000.

[8] R. Tanaka, H. Tokuda, and Y. Ezaki, "Cancer chemopreventive activity of "rosin" constituents of Pinus spez. and their derivatives in two-stage mouse skin carcinogenesis test," *Phytomedicine*, vol. 15, no. 11, pp. 985–992, 2008.

[9] W. Gu, R. Wu, S. Qi, C. Gu, F. Si, and Z. Chen, "Synthesis and antibacterial evaluation of new N-acylhydrazone derivatives from dehydroabietic acid," *Molecules*, vol. 17, no. 4, pp. 4634–4650, 2012.

[10] W. Gu, C. Qiao, S. F. Wang, Y. Hao, and T. T. Miao, "Synthesis and biological evaluation of novel N-substituted 1H-dibenzo[a, c]carbazole derivatives of dehydroabietic acid as potential antimicrobial agents," *Bioorganic Medicinal Chemistry Letters*, vol. 24, no. 1, pp. 328–331, 2014.

[11] X. Ding, J. Wu, and C. Jiang, "ABCG2: a potential marker of stem cells and novel target in stem cell and cancer therapy," *Life Sciences*, vol. 86, no. 17-18, pp. 631–637, 2010.

[12] G. S. Salvesen, "Caspases: opening the boxes and interpreting the arrows," *Cell Death and Differentiation*, vol. 9, no. 1, pp. 3–5, 2002.

[13] L. Ribault, J. P. Gournier, and B. L. Barthe, "Volvulus of the pelvic colon. Apropos of 59 cases in the western Africa savannah area," *Chirurgie; Mémoires de l'Académie de Chirurgie*, vol. 115, no. 2, pp. 78–83, 1989 (French).

[14] A. Degterev, J. Hitomi, M. Germscheid et al., "Identification of RIP1 kinase as a specific cellular target of necrostatins," *Nature Chemical Biology*, vol. 4, no. 5, pp. 313–321, 2008.

[15] X. Liu, T. van Vleet, and R. G. Schnellmann, "The role of calpain in oncotic cell death," *Annual Review of Pharmacology and Toxicology*, vol. 44, pp. 349–370, 2004.

[16] J. M. Llovet, M. I. Real, X. Montaña et al., "Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial," *The Lancet*, vol. 359, no. 9319, pp. 1734–1739, 2002.

[17] C. Lo, H. Ngan, W. Tso et al., "Randomized controlled trial of transarterial Lipiodol chemoembolization for unresectable hepatocellular carcinoma," *Hepatology*, vol. 35, no. 5, pp. 1164–1171, 2002.

[18] G. Majno and I. Joris, "Apoptosis, oncosis, and necrosis: an overview of cell death," *The American Journal of Pathology*, vol. 146, no. 1, pp. 3–15, 1995.

[19] P. Weerasinghe and L. M. Buja, "Oncosis: an important non-apoptotic mode of cell death," *Experimental and Molecular Pathology*, vol. 93, no. 3, pp. 302–308, 2012.

[20] M. Petersen, D. Hofius, and S. U. Andersen, "Signaling unmasked: autophagy and catalase promote programmed cell death," *Autophagy*, vol. 10, no. 3, pp. 520–521, 2014.

[21] B. F. Trump and I. K. Berezesky, "Calcium-mediated cell injury and cell death," *The FASEB Journal*, vol. 9, no. 2, pp. 219–228, 1995.

[22] X. Liu and R. G. Schnellmann, "Calpain mediates progressive plasma membrane permeability and proteolysis of cytoskeleton-associated paxillin, talin, and vinculin during renal cell death," *Journal of Pharmacology and Experimental Therapeutics*, vol. 304, no. 1, pp. 63–70, 2003.

[23] W. Lieberthal, S. A. Menza, and J. S. Levine, "Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells," *The American Journal of Physiology—Renal Physiology*, vol. 274, no. 2, part 2, pp. F315–F327, 1998.

[24] B. F. Trump, I. K. Berezesky, S. H. Chang, and P. C. Phelps, "The pathways of cell death: oncosis, apoptosis, and necrosis," *Toxicologic Pathology*, vol. 25, no. 1, pp. 82–88, 1997.

[25] L. S. Ma, C. Y. Jiang, M. Cui et al., "Fluopsin C induces oncosis of human breast adenocarcinoma cells," *Acta Pharmacologica Sinica*, vol. 34, no. 8, pp. 1093–1100, 2013.

[26] Y. B. Ji, S. Y. Gao, C. F. Ji, and X. Zou, "Induction of apoptosis in HepG2 cells by solanine and Bcl-2 protein," *Journal of Ethnopharmacology*, vol. 115, no. 2, pp. 194–202, 2008.

[27] L. Sun, Y. Zhao, H. Yuan, X. Li, A. Cheng, and H. Lou, "Solamargine, a steroidal alkaloid glycoside, induces oncosis in human K562 leukemia and squamous cell carcinoma KB cells," *Cancer Chemotherapy and Pharmacology*, vol. 67, no. 4, pp. 813–821, 2011.

[28] X. Zhou, W. Sun, W. Wang et al., "Artesunate inhibits the growth of gastric cancer cells through the mechanism of promoting oncosis both in vitro and in vivo," *Anti-Cancer Drugs*, vol. 24, no. 9, pp. 920–927, 2013.

[29] J. H. Moran and R. G. Schnellmann, "Diverse cytoprotectants prevent cell lysis and promote recovery of respiration and ion transport," *Biochemical and Biophysical Research Communications*, vol. 234, no. 1, pp. 275–277, 1997.

[30] X. Liu, J. J. Rainey, J. F. Harriman, and R. G. Schnellmann, "Calpains mediate acute renal cell death: role of autolysis and translocation," *The American Journal of Physiology—Renal Physiology*, vol. 281, no. 4, pp. F728–F738, 2001.

[31] M. Molinari, J. Anagli, and E. Carafoli, "Ca2+-activated neutral protease is active in the erythrocyte membrane in its nonautolyzed 80-kDa form," *Journal of Biological Chemistry*, vol. 269, no. 45, pp. 27992–27995, 1994.

[32] S. Kulkarni, T. C. Saido, K. Suzuki, and J. E. B. Fox, "Calpain mediates integrin-induced signaling at a point upstream of rho family members," *The Journal of Biological Chemistry*, vol. 274, no. 30, pp. 21265–21275, 1999.

[33] N. Yamanaka, E. Okamoto, E. Kawamura et al., "Dynamics of normal and injured human liver regeneration after hepatectomy as assessed on the basis of computed tomography and liver function," *Hepatology*, vol. 18, no. 1, pp. 79–85, 1993.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 764981, 10 pages
http://dx.doi.org/10.1155/2014/764981

Research Article

Hepatic Stellate Cell Coculture Enables Sorafenib Resistance in Huh7 Cells through HGF/c-Met/Akt and Jak2/Stat3 Pathways

Weibo Chen,[1,2] Junhua Wu,[3] Hua Shi,[3] Zhongxia Wang,[1,2] Guang Zhang,[2,4] Yin Cao,[1,2] Chunping Jiang,[1,2,4] and Yitao Ding[1,2,4]

[1] *Department of Hepatobiliary Surgery, Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing, Jiangsu 210008, China*
[2] *Jiangsu Province's Key Medical Center for Hepatobiliary Surgery, 321 Zhongshan Road, Nanjing, Jiangsu 210008, China*
[3] *School of Medicine, Nanjing University, Nanjing, Jiangsu 210093, China*
[4] *Department of Hepatobiliary Surgery, Drum Tower Clinical College of Nanjing Medical University, Nanjing, Jiangsu 210008, China*

Correspondence should be addressed to Chunping Jiang; chunpingjiang@163.com and Yitao Ding; yitao_ding@163.com

Received 27 March 2014; Revised 28 May 2014; Accepted 4 June 2014; Published 25 June 2014

Academic Editor: Jian Zhou

Purpose. Tumor microenvironment confers drug resistance to kinase inhibitors by increasing RKT ligand levels that result in the activation of cell-survival signaling including PI3K and MAPK signals. We assessed whether HSC-LX2 coculture conferred sorafenib resistance in Huh7 and revealed the mechanism underlying the drug resistance. *Experimental Design*. The effect of LX2 on sorafenib resistance was determined by coculture system with Huh7 cells. The rescue function of LX2 supernatants was assessed by MTT assay and fluorescence microscopy. The underlying mechanism was tested by administration of pathway inhibitors and manifested by Western blotting. *Results*. LX2 coculture significantly induced sorafenib resistance in Huh7 by activating p-Akt that led to reactivation of p-ERK. LX2 secreted HGF into the culture medium that triggered drug resistance, and exogenous HGF could also induce sorafenib resistance. The inhibition of p-Akt blocked sorafenib resistance caused by LX2 coculture. Increased phosphorylation of Jak2 and Stat3 was also detected in LX2 cocultured Huh7 cells. The Jak inhibitor tofacitinib reversed sorafenib resistance by blocking Jak2 and Stat3 activation. The combined administration of sorafenib and p-Stat3 inhibitor S3I-201 augmented induced apoptosis even in the presence of sorafenib resistance. *Conclusions*. HSC-LX2 coculture induced sorafenib resistance in Huh7 through multiple pathways: HGF/c-Met/Akt pathway and Jak2/Stat3 pathway. A combined administration of sorafenib and S3I-201 was able to augment sorafenib-induced apoptosis even in the presence of LX2 coculture.

1. Introduction

Hepatocellular carcinoma (HCC) is currently the fifth most common solid tumor worldwide, causing almost 700,000 deaths every year, making it the third leading cause of cancer-related death [1]. Surgical resection remains the major therapy for HCC patients of early stage. However, HCC is often diagnosed at an advanced stage, when most potentially curative therapies are of limited functions. Recently, much attention has been paid to the discovery of novel therapeutics for HCC patients of advanced stage. Despite all the progress, the prognosis of HCC is still not satisfying, with the total 5-year survival rate being at 12% [2, 3].

Sorafenib is a multitargeted tyrosine kinase inhibitor that blocks the Ser/Thr kinase Raf and several receptor tyrosine kinases including vascular endothelial growth factor receptor- (VEGFR-) 2 and -3 and platelet-derived growth factor receptor- (PDGFR-) β to inhibit tumor cell proliferation and tumor angiogenesis. Sorafenib has been approved by the Food and Drug Administration (FDA) for the treatment of unresectable HCC since late 2007 based on the results from a phase III clinical trial that demonstrated that sorafenib improves overall survival and is safe for patients with advanced HCC.

Although sorafenib is currently the only approved pharmacological therapy for HCC, the overall tumor response rates are unfortunately low. The antitumor efficiency of sorafenib correlates with the inhibition of MAPK signaling and the reactivation of ERK has been discovered to cause

sorafenib resistance. It has been reported that cancer cells typically express multiple receptor tyrosine kinases (RTKs) that mediate the upregulation of cell-survival effectors, generally, phosphatidylinositol-3-OH kinase (PI3K) and mitogen-activated protein kinase (MAPK) [4]. The increase of RTK-ligands, either from autocrine or paracrine, was able to confer resistance to kinase inhibitors [5]. Lately, tumor microenvironment was found to confer innate resistance to targeted agents. By coculture system, Ravid et al. found that stromal cell could secret hepatocyte growth factor (HGF) that resulted in the resistance to RAF inhibitors in melanoma and breast cancer cells [6].

In this study, by using coculture system we aimed to discover whether the stromal cells in HCC microenvironment could confer sorafenib resistance and to reveal the underlying mechanism beneath the drug resistance.

2. Materials and Methods

2.1. Cells and Reagents. The human HCC cell lines Huh7 and PLC/PRF/5 were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Human hepatic stellate cell (HSC) line HSC-LX2 was purchased from Cell Bank of Xiangya Central Experiment Laboratory of Central South University (Changsha, China). MHCC-97H and MHCC-97L cell lines were obtained from the Liver Cancer Institute, Fudan University (Shanghai, China). Huh7 and HSC-LX2 were supported with Dulbecco's Modified Eagle Medium (DMEM, WISENT, CA) containing 10% fetal bovine serum (FBS) (ExCell Bio, China). Both cells were incubated in 5% CO_2 at 37°C. Sorafenib tosylate, Met inhibitor crizotinib (PF-02341066), the p-Akt inhibitor MK-2206 2HCl, the Janus kinase (Jak) inhibitor tofacitinib (CP-690550), and the signal transducer and activator of transcription 3 (Stat3) inhibitor S3I-201 were purchased from Selleck (Selleck Chemicals, China). MTT (3-(4,5-dimethylthiazol) 2, 5-diphenyltetrazolium) was purchased from Sigma Aldrich (St. Louis, MO). The antibodies for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), p-Met, p-Akt, Akt, p-ERK, ERK, p-Stat3, Stat3, caspase-9, caspase-3, poly (ADP-ribose) polymerase (PARP), and p-Jak2 were obtained from Cell Signaling Technology (Beverly, MA). The antibodies for Bcl-2, Mcl-1, and Bax were purchased from Enogene (Nanjing, China). The antibodies for VEGFR-3 and c-kit were obtained from Bioworld Technology Inc. (Bioworld, USA). The antibody for PDGFR-β was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). ELISA kit for human HGF detection was purchased from ExCell Bio (Shanghai, China).

2.2. Cell Coculture Model. For LX2 cell coculture, 6-well 0.4 μm Millicell hanging cell culture inserts (Switzerland) were used. Generally, 5×10^5 Huh7 cells were placed in 6-well plate while 1×10^5 LX2 cells were placed in hanging cell inserts. For coculture in 96-well plates, the culture supernatants of LX2 after incubating for 48 h were collected and centrifuged at 5000 rpm before it was distributed for Huh7 culture for 72 h.

2.3. Cell Viability Assay. Cell viability was monitored using MTT assay. Generally, 5×10^3 cells were allowed to grow in 96-well plates. After incubation with sorafenib for 48 h, 20 μL MTT solution (0.5%) was added to the medium for further incubation for 4 h. 150 μL DMSO was added to every well to dissolve the insoluble formazan product after removing the medium. The absorbance of the colored solution was measured at 570 nm with a spectrophotometer. All experiments were performed in triplicates.

2.4. Western Blotting. Cell lysates prepared after 48 h of administration of sorafenib were immunoblotted following published protocol [7]. The signal was developed with ECL (Millipore, Switzerland) after incubation with appropriate second antibody.

2.5. ELISA Assay. HGF concentration in LX2 supernatants was detected by human HGF ELISA kit (ExCell, China) following the manufacturer's instruction. Briefly, after cultivating HSC-LX2 for 48 h, the medium was collected and centrifuged at 5000 rpm for 5 min. Total medium with 10% FBS was set as control.

2.6. Real-Time PCR. ETS-1 expression level was detected by real-time PCR. Briefly, total RNA was extracted by using TRIzol reagent (TaKaRa, Japan) and reverse transcription was performed using Primescript RT master mix (TaKaRa, Japan). The cDNA was subjected to quantitative real-time PCR using the SYBR Green PCR Kit (TaKaRa, Japan) and the assay was performed on ABI PRISM 7300 Sequence Detector. B-actin was used as the internal control. The relative expression level was calculated by $2^{-\Delta\Delta Ct}$ method. The primer sequences for ETS-1 and β-actin were as follows: ETS-1 forward: 5′-GTTAATGGAGTCAACCCAGCCTATCC-3′; ETS-1 reverse: 5′-GGGGTGACGACTTCTTGTTTGATAGC-3′; β-actin forward: 5′-CAGGCACCAGGGCGTGATGG-3′; β-actin reverse: 5′-CTGTAGCCGCGCTCGGTGAG-3′.

2.7. DAPI Staining. Cell apoptosis was detected by 4′,6-diamidino2-phenylindole (DAPI) staining which allowed identification of apoptotic nuclear changes. Briefly, cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. After being washed with PBS, cells were stained with 10 μg/mL DAPI for 5 min. Cells on slides were subjected to fluorescence microscopic examination.

2.8. Statistical Analysis. All the data were expressed as mean ± SD from three individual experiments. Differences between groups were determined by using the Student's t-test and two-way ANOVA with Bonferroni correction. $P < 0.05$ was considered significant.

3. Results

3.1. HSC-LX2 Coculture Induced Sorafenib Resistance in Huh7. To investigate whether HSC-LX2 coculture induced sorafenib

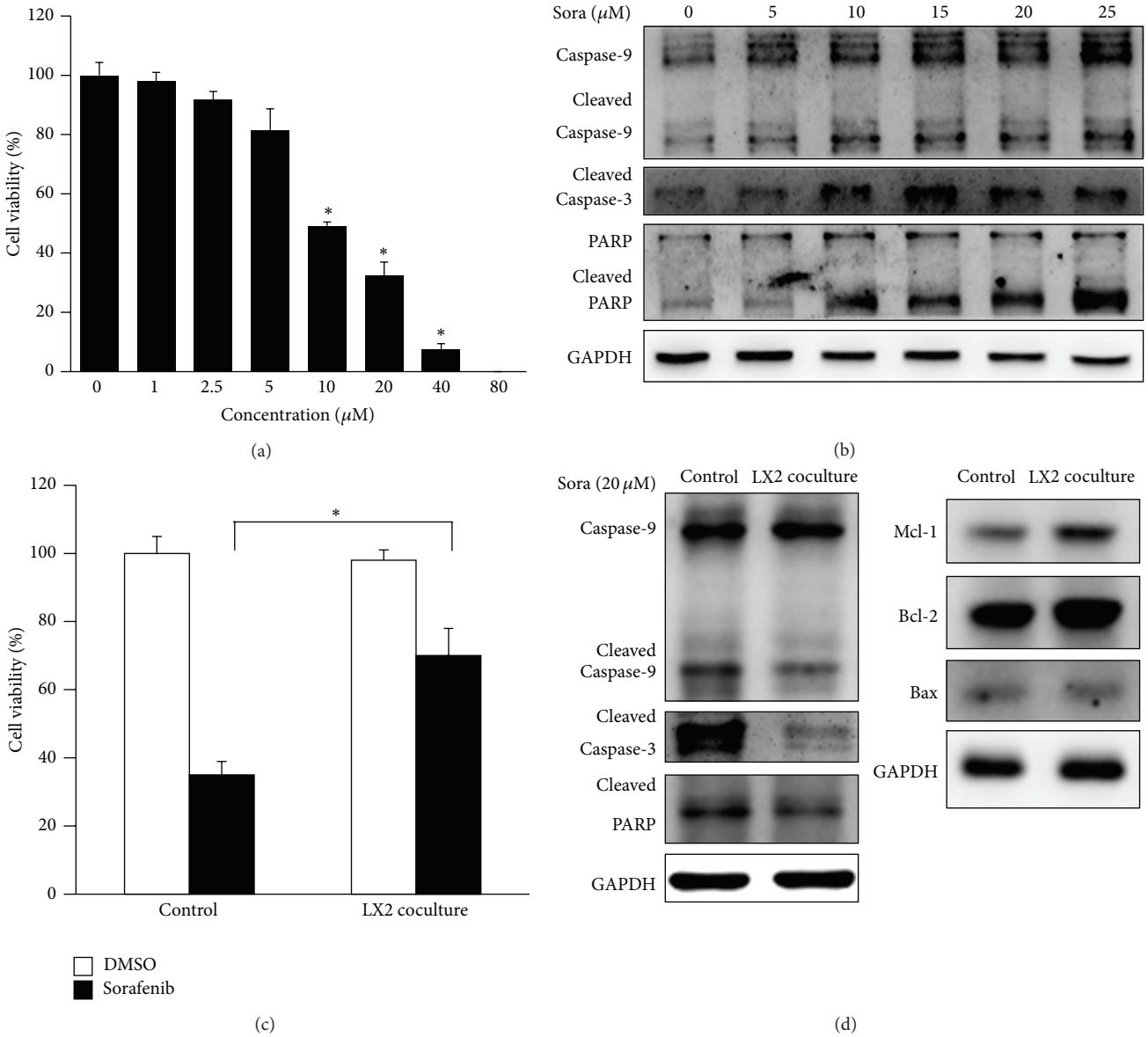


Figure 1: HSC-LX2 coculture induced sorafenib resistance in Huh7. (a) The inhibitory effect of sorafenib on Huh7 cells. After 48 h of sorafenib exposure at different concentrations, cell viability was assessed by MTT assay. Sorafenib could impair Huh7 cell viability at the concentration equal to and more than 10 μM. (b) Sorafenib-induced apoptosis in Huh7. After 48 h of sorafenib exposure at different concentrations, cell apoptosis was assessed by Western blotting. (c) HSC-LX2 coculture attenuated sorafenib-induced cell suppression. Huh7 cells were cocultured with LX2 for 48 h before the administration of sorafenib for another 48 h. Cell viability was assessed by MTT assay which showed attenuated cell death in cocultured group. (d) HSC-LX2 coculture attenuated sorafenib-induced apoptosis by increasing antiapoptotic proteins. Cell lysates were subjected to Western blotting after sorafenib administration for 48 h. HSC-LX2 coculture decreased cleavage of caspases and PARP (left panel). HSC-LX2 coculture increased expression levels of antiapoptotic proteins Mcl-1 and Bcl-2 (right panel) ($^*P < 0.05$; sora: sorafenib).

resistance in HCC cell lines, we cocultured several HCC cell lines with LX2 supernatants for 48 h and then assigned the cells to administration of sorafenib. We found that LX2 coculture impaired cell viability of MHCC-97H, MHCC-97L, and PLC/PRF/5, but not Huh7, so we selected Huh7 cells for further investigations (data not shown). The impaired cell viability might be the results of LX2 coculture induced epithelial-mesenchymal transition (EMT) [8]. We first confirmed that sorafenib suppressed proliferation and induced apoptosis in Huh7 as reported [9]. The effect of sorafenib on cell proliferation was measured by MTT assay. Sorafenib inhibited cell viability dose-dependently in Huh7, with the inhibition rate being at about 50% for 10 μM and about 60% for 20 μM (Figure 1(a)). The IC50 value of sorafenib for Huh7 was 11.25 μM. Furthermore, sorafenib administration induced elevated protein level of cleaved caspase-9, cleaved caspase-3, and cleaved PARP dose-dependently (Figure 1(b)).

We utilized MTT assay to verify the induced sorafenib resistance in Huh7. LX2 coculture for 48 h significantly attenuated sorafenib-induced cell viability suppression (Figure 1(c)). What was more, LX2 coculture also reversed the cleavage of caspase family, as the cleaved form of caspase-9, caspase-3, and PARP decreased in LX2 cocultured Huh7 after sorafenib administration. We also detected an increased level of the antiapoptotic proteins Mcl-1 and Bcl-2; however, no obvious change was detected as to proapoptotic protein Bax (Figure 1(d)).

3.2. Elevated HGF in LX2 Coculture Supernatant Contributed to Sorafenib Resistance. To find out what was the compound in LX2 supernatants that caused sorafenib resistance, we screened the concentration of HGF in LX2 supernatants which was reported to cause elotinib resistance in breast cancer [6, 10]. Interestingly, the results indicated that HGF concentration in LX2 supernatants increased more than 5 times compared to control medium (Figure 2(a)). Because the stimulated expression of c-Met in human endothelial cells by HGF was largely dependent on the induction of essential transcription factors (ETS), especially ETS-1(11), we further checked the expression level of ETS-1 in LX2 coculture Huh7, and elevated ETS-1 mRNA level was detected (Figure 2(b)). To further confirm that HGF reverted resistance to sorafenib, crizotinib (PF234106) which was a potent c-Met inhibitor was adopted for further studies. Crizotinib abrogated LX2 coculture induced sorafenib resistance by decreasing the cell viability (Figure 2(c)). Changes in the phosphorylation of Met, ERK, Akt, and Stat3 and cleavage of caspase-9 and PARP were determined by Western blotting to evaluate the effect of crizotinib on c-Met pathway and apoptosis. Crizotinib inhibited the phosphorylation of Met significantly and also inhibited p-ERK and p-AKt at the dose of 500 nM. No change of p-Stat3 was detected upon crizotinib administration. Crizotinib also increased the level of cleaved form of PARP and caspase-9 which turned to be the reversal of sorafenib resistance (Figure 2(d)).

3.3. Exogenous HGF Could Also Trigger Sorafenib Resistance in Huh7. Having established that it was HGF secreted by LX2 that was responsible for sorafenib resistance, we sought to determine whether exogenous HGF could also trigger sorafenib resistance. Therefore Huh7 cells were pretreated with HGF at different concentrations (10 ng/mL and 20 ng/mL) for 2 h before the treatment of 20 μM sorafenib for 48 h. The cell viability was subsequently assessed using MTT assay. The results showed that HGF of 20 ng/mL attenuated sorafenib-induced cell death significantly (Figure 3(a)). Cell death was also assessed by DAPI staining and Western blotting which turned out to be consistent in demonstrating the rescue effect of HGF in saving Huh7 cells from sorafenib-induced cell death (Figures 3(b) and 3(c)).

The above data showed that HGF secreted by LX2 into the supernatants was responsible for LX2 coculture induced sorafenib resistance in Huh7, and the pretreatment with exogenous HGF could also induce sorafenib resistance.

3.4. Inhibition of p-Akt Reversed LX2 Coculture Induced Sorafenib Resistance. To characterize the mechanism responsible for sorafenib resistance in Huh7, we first focused on the MAPK, AKT, and Stat3 signaling pathway, because sorafenib inhibited the phosphorylation of ERK in HCC cells and induced apoptosis [11]. The activation of PI3 K/Akt signaling pathway mediated the acquired resistance to sorafenib in HCC [9]. Sorafenib could downregulate p-Stat3 in a dose- and time-dependent manner in HCC cells, while p-Stat3 activation was discovered to be the cause of recombinant tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance. Therefore, the phosphorylation of ERK, Akt, and Stat3 was assessed by Western blotting. The results indicated an increased level of phosphorylation of ERK, Akt, and Stat3 in LX2 cocultured Huh7 (Figure 4(a)). However, we did not find the change in the protein level of RTKs like c-kit, PDGFR-β, and VEGFR-3 (Figure 4(b)).

We hypothesized that the reactivation of ERK by LX2 coculture was due to the activation of p-Akt, and p-Stat3 played a role in mediating the drug resistance. Thus, we validated the role of Akt in the effects of sorafenib resistance by blocking Akt phosphorylation with MK-2206, an Akt inhibitor. Our data showed that the presence of MK-2206 was sufficient to reverse LX2 coculture induced sorafenib resistance at the dose of 10 μM (Figure 4(c)). The combination of sorafenib and MK-2206 significantly increased the percentage of apoptotic cells in LX2 cocultured Huh7 while MK-2206 alone affected no cell viability at the concentration of 10 μM. In addition, downregulation of p-Akt sensitized Huh7 cells to sorafenib-induced apoptosis, as showed by the activation of caspase-9 and PARP cleavage (Figure 4(d)).

3.5. Inhibition of Jak2/Stat3 Reserved LX2 Coculture Induced Sorafenib Resistance. Jak inhibitor tofacitinib was also adopted in our research which showed that tofacitinib at the concentration of 0.25 μM reversed sorafenib resistance by decreasing the cell viability while it alone did not impact cell viability (Figure 5(a)). Jak2 but not Jak3 activation was responsible for Stat3 activation. The inhibition of Jak2 blocked Stat3 activation and induced the activation of PARP cleavage (Figure 5(b)).

We then further investigate whether the direct inhibition of p-Stat3 could reverse sorafenib resistance. We used S3I-201 to inhibit p-Stat3 activity. S3I-201 was a direct Stat3 inhibitor that blocks Stat3 dimerization and DNA-binding and transcriptional activities. The therapeutic effect of S3I-201 in xenografts of HCC cell line Huh7 was that it inhibited Stat3 tyrosine phosphorylation and tumor growth at a dose of 5 mg/kg given every other day [12]. Based on the above research, we first tested the inhibitory effect of S3I-201 on Huh7. We found that S3I-201 inhibited Huh7 cell viability dose-dependently, and it inhibited p-Stat3 activation also in a dose-dependent manner. At the optimized dose of 80 μM, S3I-201 could significantly inhibit Stat3 phosphorylation and impair Huh7 cell viability. Treatment of S3I-201 alone could activate the cleavage of caspase-9 (Figures 5(c) and 5(d)). As the inhibition of p-Stat3 alone could induce apoptosis in Huh7, we next tried to figure out whether the combination

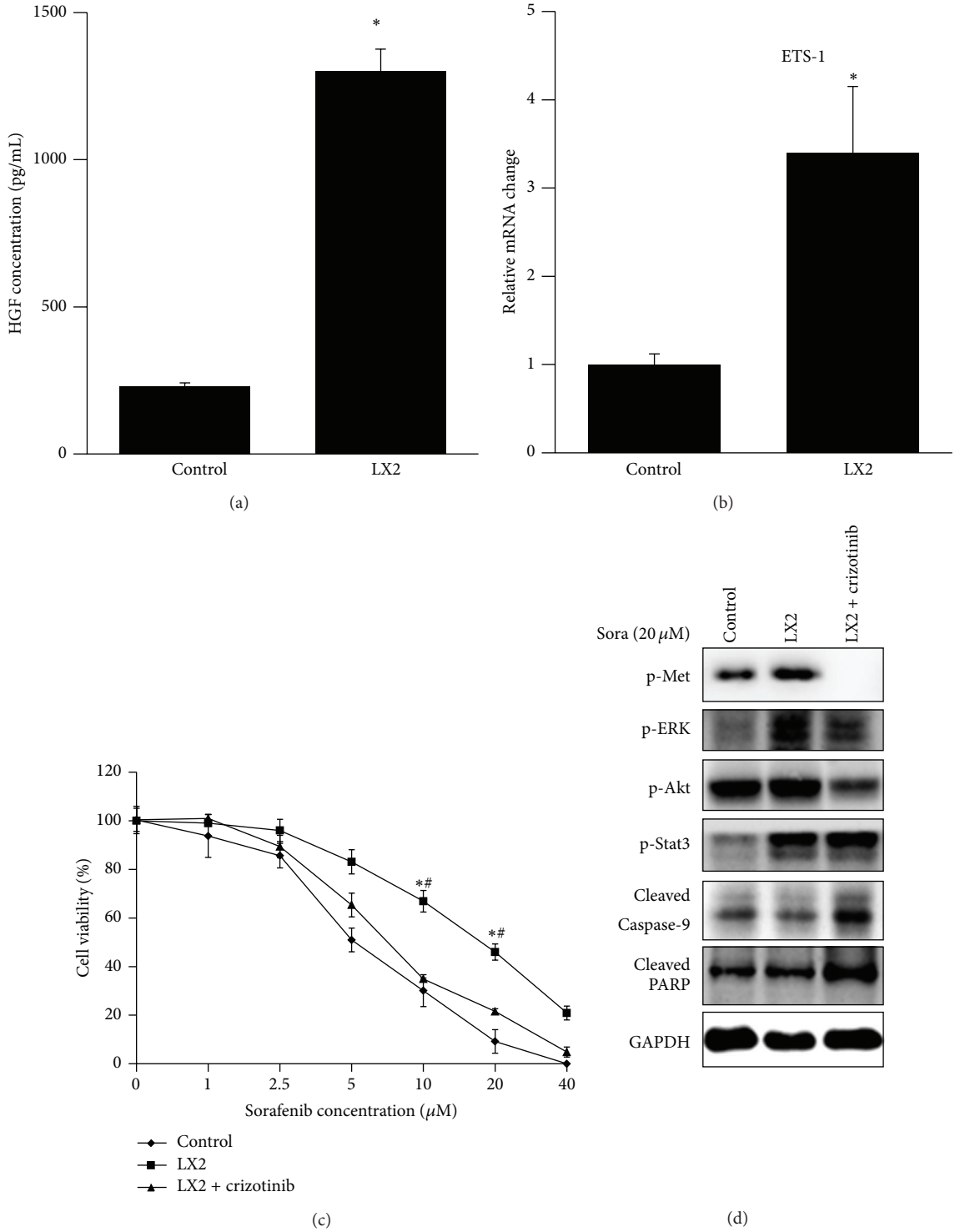


Figure 2: Elevated HGF in LX2 coculture supernatant contributed to sorafenib resistance. (a) HGF increased in LX2 supernatants. HGF concentration in LX2 supernatants was determined by ELISA kits. HGF in LX2 supernatants increased more than 5 times than control medium to the concentration of almost 1300 pg/mL. (b) ETS-1 expression level in LX2 cocultured Huh7 was determined by real-time PCR. ETS-1 mRNA level increased about 3 times than control. (c) Administration of crizotinib reversed sorafenib resistance. 500 nM crizotinib was added to the coculture system for 48 h when administrating sorafenib. Cell viability was assessed by MTT assay. Crizotinib reversed coculture induced cell survival while it alone impaired no cell viability. (d) Crizotinib reversed inactivation of caspase cleavage by inhibiting p-Met, p-ERK, and p-Akt. Crizotinib inhibited phosphorylation of Met, ERK, and Akt while it did not affect Stat3 ($^{*}P < 0.05$ LX2 versus control; $^{\#}P < 0.05$ LX2 versus LX2 + crizotinib. sora: sorafenib).

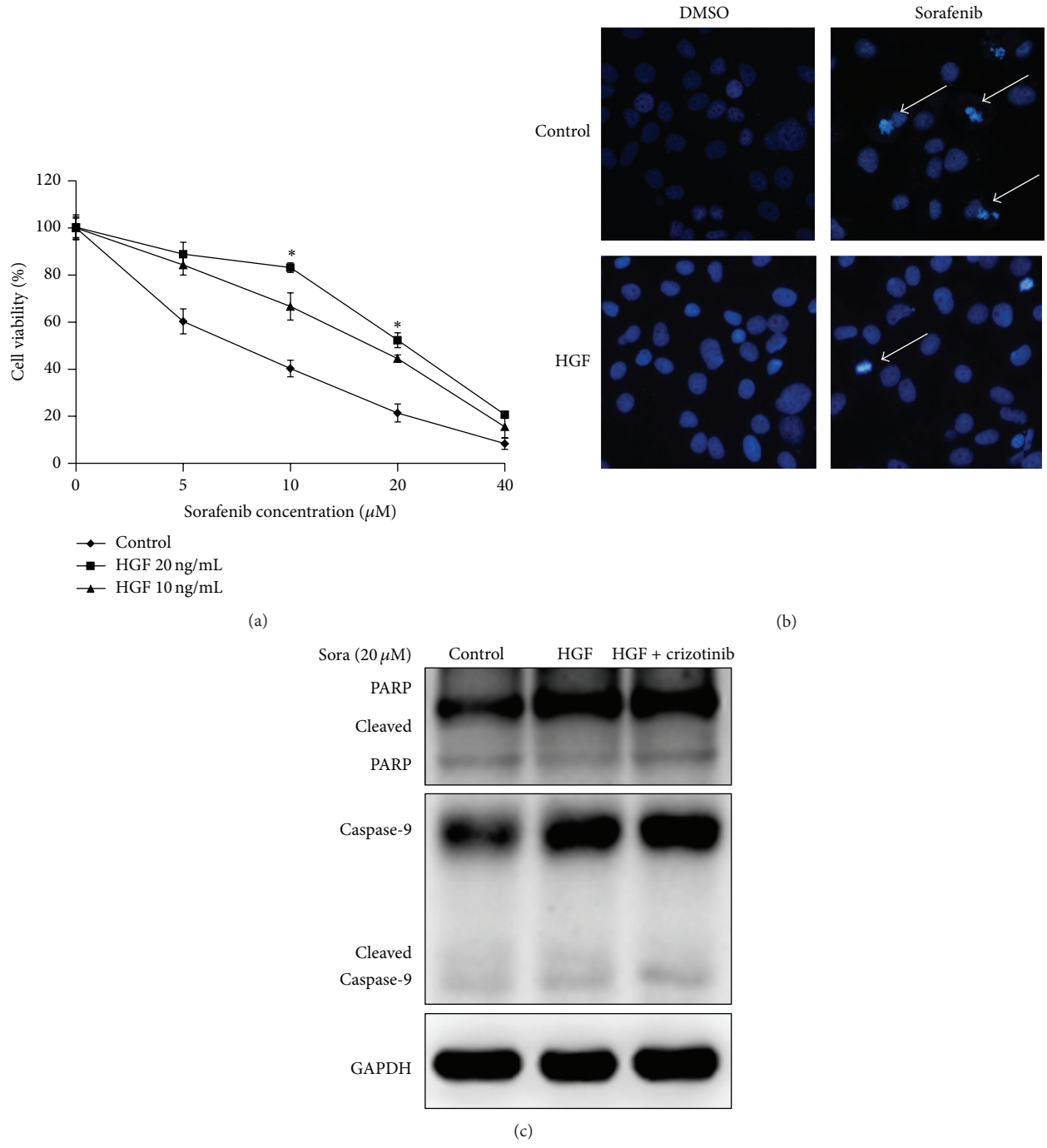


Figure 3: Exogenous HGF could also trigger sorafenib resistance in Huh7. (a) HGF of indicated concentration was administrated 2 h before sorafenib administration. HGF of 20 ng/mL rescued Huh7 from cell death. (b) DAPI staining was adopted for the manifestation of cell death. HGF of 20 ng/mL decreased sorafenib-induced cell death. (c) Crizotinib reversed HGF-induced cell survival. Crizotinib activated the cleavage of PARP and caspase-9 ($^{*}P < 0.05$ HGF 10 ng/mL versus control; sora: sorafenib).

of sorafenib and S3I-201 could promote the apoptosis in Huh7 in the presence of LX2 supernatants. To our delight, S3I-201 could induce severer cell death than sorafenib even in the presence of LX2 coculture, and the combination of sorafenib and S3I-201 augmented the induced apoptosis (Figure 5(e)).

4. Discussion

Sorafenib is a multitargeted tyrosine kinase inhibitor that blocks the Ser/Thr kinase Raf and several receptor tyrosine kinases including VEGFR-2 and -3 and PDGFR-β to inhibit tumor cell proliferation and tumor angiogenesis. It has been approved by FDA for the treatment of unresectable HCC

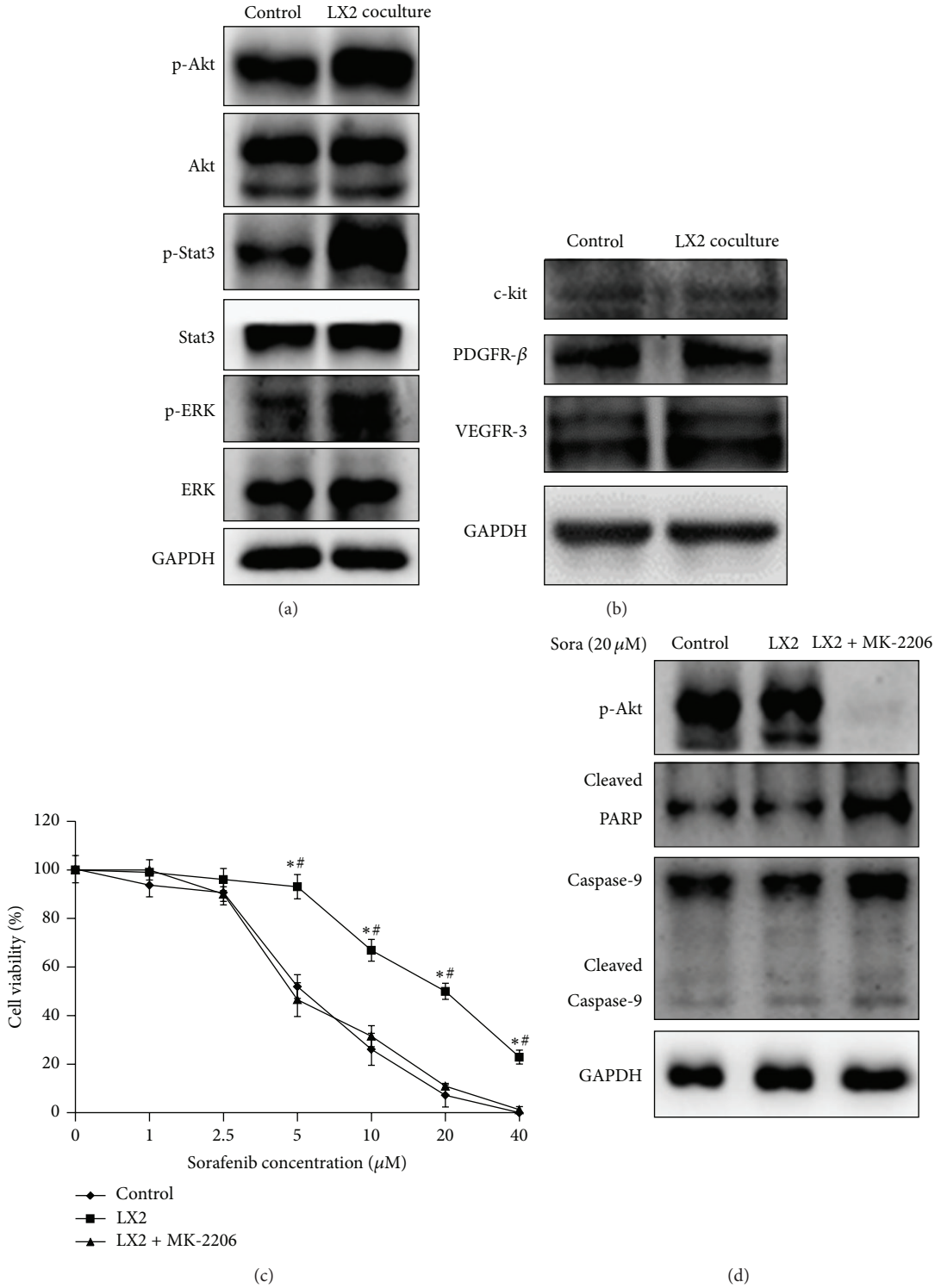


Figure 4: Inhibition of p-Akt reversed LX2 coculture induced sorafenib resistance. (a) Signaling pathway changed in LX2 cocultured Huh7. p-ERK, p-Akt, and p-Stat3 increased in LX2 cocultured Huh7. (b) LX2 coculture did not impact c-kit, PDGFR-β, and VEGFR-3 protein level. (c) Inhibition of p-Akt by MK-2206 reversed LX2 coculture induced sorafenib resistance. 10 μM MK-2206 was added to the coculture system for 48 h when administrating sorafenib. Cell viability was assessed by MTT assay. MK-2206 reversed coculture induced cell survival while it alone impaired no cell viability. (d) MK-2206 reversed inactivation of caspase cleavage by inhibiting p-Akt. MK-2206 significantly inhibited p-Akt and reactivated PARP and caspase-9 cleavage ($^{*}P < 0.05$ LX2 versus control; $^{\#}P < 0.05$ LX2 versus LX2 + MK-2206; sora: sorafenib).

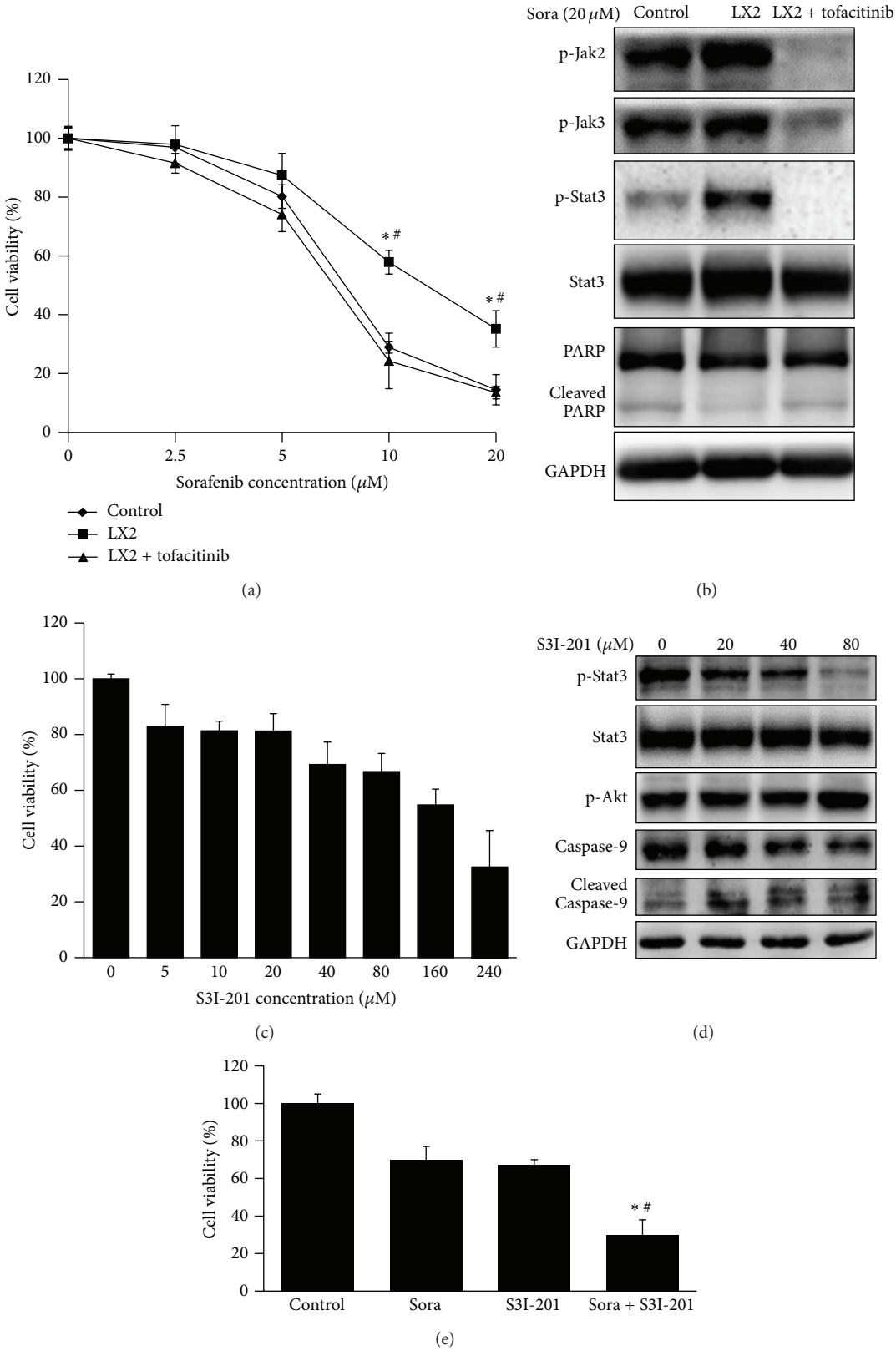


Figure 5: Inhibition of p-Stat3 reserved LX2 coculture induced sorafenib resistance. (a) Jak inhibitor tofacitinib reversed LX2 coculture induced sorafenib resistance. 0.25 μM tofacitinib was added to the coculture system for 48 h when administrating sorafenib. Cell viability was assessed by MTT assay. Tofacitinib reversed coculture induced cell survival while it alone impaired no cell viability. (b) Activation of Jak2/Stat3 induced sorafenib resistance whose inhibition activated sorafenib-induced apoptosis. (c) Stat3 inhibitor S3I-201 inhibited cell viability dose-dependently. (d) S3I-201 inhibited phosphorylation of Stat3 and induced apoptosis dose-dependently. (e) Combined administration of sorafenib (20 μM) and S3I-201 (80 μM) augmented cell apoptosis in the presence of LX2 coculture ($^{*}P < 0.05$ sora + S3I-201 versus sora; $^{\#}$ sora + S3I-201 versus S3I-201; sora: sorafenib).

based on the results from a phase III clinical trial that demonstrated its benefits for overall survival and the safety for patients with advanced HCC. However, sorafenib resistance is a major obstacle in improving therapeutic efficacy. Stromal cells in tumor microenvironment have been known to be capable of secreting RTK ligands to confer resistance to kinase inhibitors. In this report, we discovered that HSC-LX2, the stromal cell in HCC microenvironment, was able to confer sorafenib resistance in Huh7 cells by secreting HGF into the culture medium which could increase the phosphorylation of Met, Akt, ERK, and Stat3. Exogenous HGF was also able to trigger sorafenib resistance. LX2 coculture induced sorafenib resistance could be reversed by the administration of crizotinib, MK-2206, and tofacitinib, the potent inhibitors of Met, Akt, and Jak, respectively. In addition, we found that when combining administration of sorafenib and S3I-201, the inhibitor of Stat3 augmented induced apoptosis even in the presence of sorafenib resistance.

Tumor microenvironment has been demonstrated to confer drug resistance that leads to relapse and incurability of cancers. Soluble factors as cytokines and growth factors secreted by stromal cells are a major cause of acquired resistance [13]. Among the various factors, HGF is found to confer substantial resistance to RAF and MEK inhibition by activation of the RTK Met in melanoma and breast cancers [6]. Activation of HGF/c-Met axis elicits multiple cellular responses regulating cell survival [14]. The study by Yu et al. shows that HSC-LX2 cell secreted HGF contributes to the chemoresistance of HCC. The elevated HGF level instead of TGF-β activates Met in HCC cell Hep3B [8]. In our study, increased level of HGF was also detected which contributed to sorafenib resistance. The blockage of HGF/c-Met axis by crizotinib reversed drug resistance. However, there was a big gap between the HGF concentration in LX2 supernatants and the concentration we used for exogenous HGF stimulation experiments. We supposed that the different HGF exposure time might be the reason for the gap.

PI3K/Akt signaling is a vital survival and proliferative pathway involving many growth factors, cytokines, and activation of receptors. Chen et al. have reported that activation of PI3K/Akt signaling pathway mediates acquired resistance to sorafenib in HCC, and the combined administration of sorafenib and MK-2206 overcomes the resistance [9]. In our study, LX2 coculture increased the activation of p-Akt and thus decreased the activation of caspase and PARP cleavage and increased the antiapoptotic protein Mcl-1 and Bcl2 levels. While under the administration of crizotinib and MK-2206, when both inhibitors inhibit the phosphorylation of Akt indirectly or directly, the rescue effect was reversed. As sorafenib blocks the RAF/MEK/ERK pathway to inhibit tumor angiogenesis and induce apoptosis in HCC cells, the reactivation of ERK signaling confers acquired resistance to BRAF and MEK inhibitors [11, 15]. As shown in Figures 2(d) and 4(a), p-ERK increased in LX2 cocultured Huh7 cells and no c-kit, PDGFR-β, or VEGFR-3 protein level increase was detected (Figure 4(b)); we assumed that ERK activation might be the direct reason for induced resistance. Because the inhibition of p-Met by crizotinib can partially block the activation of p-ERK, crizotinib reverses sorafenib resistance (Figure 2(d)). So in LX2 cocultured induced sorafenib resistance in Huh7, LX2 secreted HGF into the tumor microenvironment which activated HGF/c-Met axis and thus activates p-Akt and then reactivated p-ERK somehow which finally led to the increased level of antiapoptotic protein and decreased caspase cleavage.

Because crizotinib did not affect Stat3 activation as shown in Figure 2(d), we assumed that c-Met activation was not responsible for Stat3 activation. We observed Jak2 activation but not Jak3 activation in LX2 cocultured Huh7, and Jak inhibitor tofacitinib reversed sorafenib resistance by inhibiting Jak2 and Stat3 activation. We also found that the direct inhibition of p-Stat3 induced apoptosis. The inhibition of p-Met affected no p-Stat3 activation and inhibition of p-Stat3 affected no p-Akt, either. There seemed no cross-talk between p-Akt and p-Stat3 signaling pathway. Stat3 is associated with cell proliferation and survival and it is crucial in the prognosis of many cancers and a potential target for anticancer therapy [16, 17]. It has been reported that Stat3 is a major kinase-independent target of sorafenib in HCC and sorafenib downregulates p-Stat3 and reduces the expression levels of Stat3-related proteins as Mcl-1, survivin, and cyclin D1 by upregulating SHP-1 activity [18–21]. The Stat3 inhibitor, S3I-201, has been demonstrated to inhibit Stat3 dimerization and DNA-binding activity as well as inhibit cell proliferation and tumor growth [22, 23]. Stat3 inhibition combined with targeted therapy has been proved to significantly suppress cancer cell growth in pancreatic cancers [24]. In our study, the combined therapy of S3I-201 and sorafenib synergistically suppressed Huh7 cell growth and induced apoptosis in the presence of sorafenib resistance.

In summary, HSC-LX2 coculture induced sorafenib resistance in Huh7 through two independent pathways: HGF/c-Met/Akt pathway and Jak2/Stat3 pathway. A combined administration of sorafenib and S3I-201 was able to augment sorafenib-induced apoptosis even in the presence of sorafenib resistance.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Weibo Chen and Junhua Wu participated in designing the research and conducting the experiments and wrote the paper. Hua shi, Zhongxia Wang, Guang Zhang, and Yin Cao participated in the study design and data analysis. Chunping Jiang and Yitao Ding conceived the study, participated in its design, and gave final approval of the version to be published. All authors have read and approved the final paper. Weibo Chen and Junhua Wu contributed equally to the work as co-first authors.

Acknowledgment

This study was supported by the Medical Science and Technology Development Foundation, Nanjing Department of Health (no. ZKX12030).

References

[1] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA: Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[2] H. B. El-Serag and K. L. Rudolph, "Hepatocellular carcinoma: epidemiology and molecular carcinogenesis," *Gastroenterology*, vol. 132, no. 7, pp. 2557–2576, 2007.

[3] H. B. El-Serag, "Hepatocellular carcinoma," *The New England Journal of Medicine*, vol. 365, no. 12, pp. 1118–1127, 2011.

[4] A. Moritz, Y. Li, A. Guo et al., "Akt-RSK-S6 kinase signaling networks activated by oncogenic receptor tyrosine kinases," *Science Signaling*, vol. 3, no. 136, p. ra64, 2010.

[5] T. R. Wilson, J. Fridlyand, Y. Yan et al., "Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors," *Nature*, vol. 487, no. 7408, pp. 505–509, 2012.

[6] R. Straussman, T. Morikawa, K. Shee et al., "Tumour microenvironment elicits innate resistance to RAF inhibitors through HGF secretion," *Nature*, vol. 487, no. 7408, pp. 500–504, 2012.

[7] M. W. Nasser, J. Datta, G. Nuovo et al., "Down-regulation of micro-RNA-1 (miR-1) in lung cancer: suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by miR-1," *The Journal of Biological Chemistry*, vol. 283, no. 48, pp. 33394–33405, 2008.

[8] G. Yu, Y. Jing, X. Kou et al., "Hepatic stellate cells secreted hepatocyte growth factor contributes to the chemoresistance of hepatocellular carcinoma," *PloS ONE*, vol. 8, no. 9, Article ID e73312, 2013.

[9] K.-F. Chen, H.-L. Chen, W.-T. Tai et al., "Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib in hepatocellular carcinoma cells," *Journal of Pharmacology and Experimental Therapeutics*, vol. 337, no. 1, pp. 155–161, 2011.

[10] J. Bean, C. Brennan, J. Shih et al., "MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 52, pp. 20932–20937, 2007.

[11] L. Liu, Y. Cao, C. Chen et al., "Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5," *Cancer Research*, vol. 66, no. 24, pp. 11851–11858, 2006.

[12] G. He and M. Karin, "NF-κB and STAT3- key players in liver inflammation and cancer," *Cell Research*, vol. 21, no. 1, pp. 159–168, 2011.

[13] Z. Li and W. S. Dalton, "Tumor microenvironment and drug resistance in hematologic malignancies," *Blood Reviews*, vol. 20, no. 6, pp. 333–342, 2006.

[14] F. Cecchi, D. C. Rabe, and D. P. Bottaro, "Targeting the HGF/Met signalling pathway in cancer," *European Journal of Cancer*, vol. 46, no. 7, pp. 1260–1270, 2010.

[15] R. B. Corcoran, J. Settleman, and J. A. Engelman, "Potential therapeutic strategies to overcome acquired resistance to BRAF or MEK Inhibitors in BRAF mutant cancers," *Oncotarget*, vol. 2, no. 4, pp. 336–346, 2011.

[16] F. Yang, C. Brown, R. Buettner et al., "Sorafenib induces growth arrest and apoptosis of human glioblastoma cells through the dephosphorylation of signal transducers and activators of transcription 3," *Molecular Cancer Therapeutics*, vol. 9, no. 4, pp. 953–962, 2010.

[17] S. Huang and F. A. Sinicrope, "Sorafenib inhibits STAT3 activation to enhance TRAIL-mediated apoptosis in human pancreatic cancer cells," *Molecular Cancer Therapeutics*, vol. 9, no. 3, pp. 742–750, 2010.

[18] K. Chen, W. Tai, T. Liu et al., "Sorafenib overcomes TRAIL resistance of hepatocellular carcinoma cells through the inhibition of STAT3," *Clinical Cancer Research*, vol. 16, no. 21, pp. 5189–5199, 2010.

[19] C. Liu, L. Tseng, J. Su et al., "Novel sorafenib analogues induce apoptosis through SHP-1 dependent STAT3 inactivation in human breast cancer cells," *Breast Cancer Research*, vol. 15, article R63, 2013.

[20] F.-M. Gu, Q.-L. Li, Q. Gao et al., "Sorafenib inhibits growth and metastasis of hepatocellular carcinoma by blocking STAT3," *World Journal of Gastroenterology*, vol. 17, no. 34, pp. 3922–3932, 2011.

[21] W. Tai, A. Cheng, C. Shiau et al., "Signal transducer and activator of transcription 3 is a major kinase-independent target of sorafenib in hepatocellular carcinoma," *Journal of Hepatology*, vol. 55, no. 5, pp. 1041–1048, 2011.

[22] X. Wang, D. Goldstein, P. J. Crowe, and J.-L. Yang, "S3I-201, a novel STAT3 inhibitor, inhibits growth of human soft tissue sarcoma cell lines," *World Journal of Cancer Research*, vol. 1, no. 1, pp. 61–68, 2013.

[23] L. Lin, R. Amin, G. I. Gallicano et al., "The STAT3 inhibitor NSC 74859 is effective in hepatocellular cancers with disrupted TGF-β signaling," *Oncogene*, vol. 28, no. 7, pp. 961–972, 2009.

[24] S. Jaganathan, P. Yue, D. C. Paladino, J. Bogdanovic, Q. Huo, and J. Turkson, "A functional nuclear epidermal growth factor receptor, SRC and Stat3 heteromeric complex in pancreatic cancer cells," *PLoS ONE*, vol. 6, no. 5, Article ID e19605, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 203693, 15 pages
http://dx.doi.org/10.1155/2014/203693

Review Article

Hepatocellular Carcinoma: Novel Molecular Targets in Carcinogenesis for Future Therapies

Gaetano Bertino,[1] Shirin Demma,[1] Annalisa Ardiri,[1] Maria Proiti,[1] Salvatore Gruttadauria,[2] Adriana Toro,[3] Giulia Malaguarnera,[4] Nicoletta Bertino,[5] Michele Malaguarnera,[4] Mariano Malaguarnera,[4] and Isidoro Di Carlo[3]

[1] *Hepatology Unit, Department of Medical and Pediatric Sciences, University of Catania, Policlinic, Via Santa Sofia No. 78, 95123 Catania, Italy*
[2] *Abdominal Transplantation Unit, ISMETT Palermo, UPMC–Italy, Via Ernesto Tricomi No. 5, 90127 Palermo, Italy*
[3] *Department of Surgical Sciences, Organ Transplantation and Advanced Technologies, University of Catania, Via Messina No. 829, 95126 Catania, Italy*
[4] *Research Centre "The Great Senescence", University of Catania, Via Messina No. 829, 95126 Catania, Italy*
[5] *Faculty of Pharmacy, University of Catania, Viale Andrea Doria No. 6, 95123 Catania, Italy*

Correspondence should be addressed to Gaetano Bertino; gaetanobertinounict@libero.it

Received 4 April 2014; Revised 29 May 2014; Accepted 30 May 2014; Published 25 June 2014

Academic Editor: Jingmin Zhao

Background. Hepatocellular carcinoma is one of the most common and lethal malignant tumors worldwide. Over the past 15 years, the incidence of HCC has more than doubled. Due to late diagnosis and/or advanced underlying liver cirrhosis, only limited treatment options with marginal clinical benefit are available in up to 70% of patients. During the last decades, no effective conventional cytotoxic systemic therapy was available contributing to the dismal prognosis in patients with HCC. A better knowledge of molecular hepatocarcinogenesis provides today the opportunity for targeted therapy. *Materials and Methods*. A search of the literature was made using cancer literature, the PubMed, Scopus, and Web of Science (WOS) database for the following keywords: "hepatocellular carcinoma," "molecular hepatocarcinogenesis," "targeted therapy," and "immunotherapy." *Discussion and Conclusion*. Treatment decisions are complex and dependent upon tumor staging, presence of portal hypertension, and the underlying degree of liver dysfunction. The knowledge of molecular hepatocarcinogenesis broadened the horizon for patients with advanced HCC. During the last years, several molecular targeted agents have been evaluated in clinical trials in advanced HCC. In the future, new therapeutic options will be represented by a blend of immunotherapy-like vaccines and T-cell modulators, supplemented by molecularly targeted inhibitors of tumor signaling pathways.

1. Introduction

Over the past 15 years, the incidence of hepatocellular carcinoma (HCC) has more than doubled. Every year there are 500,000 new cases in the Asia-Pacific region, often due to chronic hepatitis B virus (HBV) infection [1]. More than 60% of the total number of HCC cases occurs in China alone, and an estimated 360,000 patients residing in Far East countries, including China, Japan, Korea, and Taiwan, die from this disease each year. In Japan hepatitis C virus-(HCV-) related HCC represents 70% of all cases [2] In addition, in the USA and Europe, an increased incidence of HCV has led to an increased incidence of HCC [3]. A relevant risk factor for the high incidence of nonalcoholic fatty liver disease is obesity and diabetes, which can promote the development of liver cancer [4]. This involves a poor diagnosis and a low level of survival (5-year survival rate: less than 5%) in patients with advanced HCC at diagnosis. For a correct and effective treatment strategy in patients with cirrhosis, it is necessary to perform a liver ultrasound twice a year. Recently, the role of AFP serum levels has been discussed to be less useful than previously assumed [5, 6]. Furthermore, in addition

to AFP there are other biomarkers [7–9]: *Lens culinaris* agglutinin-reactive AFP (AFP-L3), des-carboxyprothrombin (DCP), glypican-3 (GPC-3), osteopontin (OPN), and several other biomarkers (such as squamous cell carcinoma antigen-immunoglobulin M complexes, alpha-1-fucosidase (AFU), chromogranin A (CgA), human hepatocyte growth factor, and insulin-like growth factor (IGF)) have been proposed as markers for the early detection of HCC [7–11]. None of them is optimal; however, when used together, their sensitivity in detecting HCC is increased. Recent developments in gene-expressing microarrays and proteomics promise even more potential diagnostic options [7–14].

While the endpoint of an early diagnosis is achieved quite easily in most patients with >1 cm HCC by computed tomography (CT) or magnetic resonance imaging (MRI) demonstrating the specific pattern of an intense contrast uptake during the arterial phase (wash-in) and contrast wash-out during the venous/delayed phase, nodules <1 cm in size are more difficult to diagnose, almost invariably requiring an enhanced follow-up with three monthly examinations with US until they grow in size or change their echo pattern. Owing to the lack of robust controlled evidence demonstrating a clinical benefit of surveillance, the real support for screening for liver cancer comes from the striking differences in response to therapy between screened populations in whom HCC is diagnosed and treated at early stages and patients with more advanced, incidentally detected tumors [15].

With the recent dramatic advances in diagnostic modalities, the diagnosis of HCC is primarily based on imaging. Ultrasound plays a crucial role in HCC surveillance. Dynamic multiphasic multidetector-row CT (MDCT) and magnetic resonance imaging (MRI) are the standard diagnostic methods for the noninvasive diagnosis of HCC [16].

Treatment decisions are complex and dependent upon tumor staging, presence of portal hypertension, and the underlying degree of liver dysfunction, as well as local expertise, as indicated by the National Comprehensive Cancer Network (NCCN), Asian Pacific Association for the Study of the Liver (APASL), American Association for the Study of Liver (AASLD), Barcelona-Clinic Liver Cancer (BCLC), European Association for the Study of the Liver (EASL), and Italian Association of Study of the Liver (AISF) guidelines.

Only 30–40% of HCC patients at initial diagnosis are at an early stage (0 or A) according to the BCLC classification, which defines patients who may be treated with local ablation (particularly radiofrequency ablation: RFA), resection, or orthotopic liver transplantation [17].

Unfortunately, most patients will not be candidates for either surgery or transplant. For patients in the intermediate stage (asymptomatic multifocal HCC without vascular localization or metastasis: BCLC stage B), TACE is considered the standard of care, achieving partial response (PR) in 20–50% of patients and an expansion of median survival for up to 20 months throughout the development of new vector systems (polymers) and more accurate patient selection methods [18]. Unfortunately, HCC is diagnosed at an advanced stage. In this case the therapeutic option is the systemic therapy. In the last decade, no effective conventional cytotoxic systemic therapy was available [18, 19], which has contributed to the dismal prognosis in patients with HCC [17]. Systemic chemotherapy has marginal activity and frequent toxicity and is not associated with improved survival.

In fact, HCC is highly refractory to cytotoxic chemotherapy and, until now, no conventional systemic chemotherapy has provided response rates >25% and prolonged survival in patients with advanced HCC [20]. Patients with HCC have been observed to need high rates of chemotherapy sessions due to tumor drug resistance mechanisms. The intrinsic drug resistance of tumor cells is mediated by enhanced cellular drug efflux mechanisms in association with an increase in a drug transporter family (ATP-binding cassette proteins containing MDR1 and P-gp) [21]. Furthermore, resistance is also determined by p53 mutations and overexpression of DNA topoisomerase IIa. In addition, we must consider that the liver cirrhosis and hepatic dysfunction complicate administration of systemic therapy due to pharmacokinetic properties [22–24]. Altogether, no systemic therapy could be considered a standard of care for patients with advanced HCC in the preera of targeted therapy [25].

Agents such as octreotide, tamoxifen, and antiandrogens are ineffective.

In the pivotal phase III study, sorafenib, a small molecule multikinase inhibitor, was shown to extend overall survival by almost three months [26]. Thus, current guidelines suggest its use in patients with advanced HCC (BCLC C) [25].

During the last years, several molecular targeted agents have been evaluated in clinical trials in advanced HCC. In the future, new therapeutic options will be represented by a blend of immunotherapy-like vaccines and T-cell modulators, supplemented by molecularly targeted inhibitors of tumor signaling pathways. The identification of the key molecules/receptors/signaling pathways and the assessment of their relevance as potential targets will be the main future challenge.

However, a better knowledge of molecular hepatocarcinogenesis today provides the opportunity for targeted therapy [27]. The knowledge of the mechanisms of hepatocarcinogenesis in HCC is crucial.

2. Hepatocarcinogenesis

Hepatocarcinogenesis is known to be a highly complex multistep process and nearly every pathway involved in carcinogenesis is altered to some degree in HCC [28]. Therefore, there exists no single dominant or pathognomonic molecular mechanism in HCC.

Hepatocarcinogenesis is considered a process originating from hepatic stem cells (however the role of liver stem cells as HCC cells of origin is under debate) [20] or mature hepatocytes and evolving from chronic liver disease driven by oxidative stress, chronic inflammation, cell death followed by unrestricted restricted proliferation/regeneration, and permanent liver remodeling. Chronic liver injury caused by HBV, HCV, chronic alcohol consumption, nonalcoholic steatohepatitis (NASH), hereditary hemochromatosis, primary biliary cirrhosis (PBC), and alpha-1 antitrypsin deficiency leads to permanent hepatocyte damage followed by a massive

compensatory cell proliferation and regeneration in response to cytokine stimulation (Table 1). Finally, fibrosis and cirrhosis develop in this setting of permanent liver remodeling, particularly driven by the synthesis of extracellular matrix components from hepatic stellate cells. In this carcinogenic environment, the development of hyperplastic and dysplastic nodules as preneoplastic conditions is only a question of time. However, the suspected sequential accumulation of molecular events at different stages of liver disease (normal liver tissue, chronic hepatitis, cirrhosis, hyperplastic and dysplastic nodules, and cancer) is only partially understood. Therefore, it is necessary to consider the likely molecular mechanisms that can explain the hepatocarcinogenesis.

During last years the identification of several new signaling pathways, in addition to those already known, has led to the development of new agents.

Table 1: At-risk group for HCC.

Patients at increased risk of HCC	
Without cirrhosis	Cirrhosis
Hepatitis B carrier	(i) Hepatitis B
(i) Family history of HCC	(ii) Hepatitis C
(ii) Africans	(iii) Alcoholic cirrhosis
(iii) Asian males > 40 years	(iv) Nonalcoholic steatohepatitis
(iv) Asian females > 50 years	(v) Genetic hemochromatosis
	(vi) Primary biliary cirrhosis
	(vii) Alpha-1 antitrypsin deficiency

2.1. Molecular Pathogenesis of HCC. The molecular pathogenesis of HCC involves different genetic/epigenetic aberrations and alterations in multiple signaling pathways leading to the known heterogeneity of the disease in terms of its biologic and clinical behavior [29]. The most prevalent molecular aberrations in HCC are as follows:

(a) alterations in gene expression,

(b) somatic mutations,

(c) genomic instability,

(d) epigenetic alterations,

which can be considered as potential therapeutic targets.

(a) Genome-Wide Alterations. Several candidate genes in hepatocarcinogenesis have emerged: c-myc (8q), cyclin A2 (4q), cyclin D1 (11q), Rb1 (13q), AXIN1 (16p), p53 (17p), IGFR-II/M6PR (6q), p16 (9p), E-cadherin (16q), SOCS (16p), and PTEN (10q). Chromosomal amplification or deletions are identified in almost all tumors, being the most prevalent amplifications of 1q (58%–78%), 6p, 8q, 17q, and 20q, and deletions in 4q, 8p, 13q, 16q, and 17p. High-level amplifications have been detected in 11q13 (5% of cases, regions encoding cyclin D1) and 6p21 (4%–6% of cases, regions encoding VEGFA). Further research is needed to identify key oncogenes in HCC [30].

(b) Somatic Mutations. Few somatic mutations have been described in patients with HCC, an area that is expected to advance in the near future with the introduction of high-throughput sequencing technology. TP53, a tumor suppressor gene involved in cell cycle control, DNA repair, apoptosis, and differentiation, is mutated in 27% of cases (range 0%–67%). Aflatoxin B exposure in Africa and Asia is associated with p53 G-to-T mutation at the third position of codon 249. Mutations in beta-catenin in exon 3 are present in 17% of cases of HCC and in 60% of cases of hepatoblastoma. Less frequent somatic mutations have been described in AXIN1, phosphoinositol 3-kinase A (PI3KA), and K-Ras. Conversely, prevalent mutations in other cancers, such as epidermal growth factor receptor (EGFR), Her2/nu phosphatase and tensin homolog (PTEN), or H-Ras, are marginal in HCC, while germline mutations have not been described [27].

(c) Genomic Instability. This has been described associated with telomere shortening, aberrant methylation, and aberrations in mismatch repair genes. Telomerase activity is increased in nearly 90% of human HCCs and results from HBV integration in the telomerase reverse transcriptase (TERT) locus, amplification of the gene encoding the telomerase RNA component (TERC), or allelic loss of chromosome 10p, a region encoding a telomerase repressor [31]. High genomic instability has been associated with the proliferation subclass of HCC and is more prevalent in HBV-related HCCs.

(d) Epigenetic Alterations. Epigenetic silencing of gene expression occurs by abnormal methylation of gene promoter regions. Liver cancer cells have certain areas of dense hypermethylation over a background of global hypomethylation. Hypermethylation affects CpG islands localized in promoter regions of tumor suppressor genes like p16INK4a, E-cadherin, NORE1A, RASSF1, IGFR-II/MP6, and BRCA1 [27].

Demethylation agents allow reexpression of these genes and restoration of their antineoplastic functions.

Genomic instability, disturbances in cell cycle regulation, angiogenesis, apoptosis resistance, and reactivation of telomerase reverse transcriptase (TERT) caused by genetic (chromosomal amplification/deletion, point mutations, microsatellite instability, loss of heterozygosity, and activation of telomerases inhibiting telomere shortening) and epigenetic (aberrant hypermethylation of gene promoter regions and gene silencing by histone deacetylation) aberrations represent the molecular basis for most HCCs. Besides these common molecular themes in most HCCs independent of etiology, there exist some special features of hepatocarcinogenesis depending on the type of chronic liver injury. HBV encodes proteins (such as HBx) with oncogenic properties [32], by binding and inactivating the tumor suppressor gene p53 and inducing oxidative stress. Moreover, HBV genome integration has been associated with chromosomal instability. HCV-induced hepatocarcinogenesis is particularly driven by apoptosis and subsequent regeneration caused by the strong immunological response. Apart from direct interactions with components of the mitogen-activated protein kinase

(MAPK) signaling pathway or with p53, an oxidative stress-mediated mechanism is involved, as it is established also in alcohol-induced hepatocarcinogenesis [33, 34].

Finally, the accumulation of genetic and epigenetic alterations leads to an activation of oncogenes and inhibition of tumor suppressor genes accompanied by an escalation of genetic instability and the disruption of signaling pathways related to the main promoters of hepatocarcinogenesis, namely, cell proliferation and neoangiogenesis. However, as known from gene expression studies (microarray analyses), there exists no single dominant or pathognomonic receptor or signaling pathway in HCC, and the concept of a multistep process explains why single-targeted agents will not achieve complete response (CR) in HCC [35, 36].

2.2. Growth/Angiogenic Factors and Signaling Pathways in HCC. Although HCCs are phenotypically and genetically heterogeneous tumors, several signaling pathways such as the Ras/Raf/MEK/ERK (MAPK) pathway, the phosphoinositol 3-kinase (PI3k)/Akt/mammalian target of rapamycin (mTOR) pathway, and the Wnt/beta catenin pathway have been repeatedly identified as important for HCC cell proliferation and angiogenesis. In addition, the relevance of growth and angiogenic factors/receptors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and particularly epidermal growth factor (EGF) receptor was recognized [27, 29].

The MAPK pathway, frequently upregulated in HCC [37], includes a cascade of phosphorylation of four major cellular kinases, Ras, Raf, mitogen-activated protein extracellular kinase (MEK), and extracellular signal-regulated kinase (ERK) [38].

Several members of the EGF family, in particular EGF, and transforming growth factor-alpha have been shown to play a crucial role in HCC proliferation. EGF receptor (EGFR) is frequently expressed in HCC and its overexpression has shown to be an independent negative prognostic factor for early tumor recurrence and extrahepatic metastasis. EGFR can be targeted either by the use of the monoclonal antibody cetuximab or by small molecules that inhibit the intracellular tyrosine kinase (erlotinib, gefitinib, and lapatinib). The ligands EGF, hepatocytes growth factor (HGF), VEGF, and PDGF among others activate this pathway and induce transcription of genes of the AP-1 family, such as c-fos and c-jun involved in HCC proliferation and differentiation. The currently most promising molecular targeted agent within this pathway is the Raf kinase inhibitor sorafenib that additionally targets the tyrosine kinases of VEGFR and PDGFR. HCC is a hypervascularized tumor depending on neoangiogenesis and both cell proliferation and neoangiogenesis contribute to initiation and progression of HCC. Neoangiogenesis plays a crucial role in each step of hepatocarcinogenesis [39], providing the rationale for angiogenic inhibitors as a new class of relevant therapeutics in this malignancy.

Hence, targeted therapy of HCC has to include both aspects and hence VEGF/EGFR receptor signaling and the MAPK pathway have been addressed in clinical settings. VEGF as central mediator of angiogenesis is frequently expressed in HCC [39] and VEGF levels correlate with angiogenic activity, tumor progression, and poor prognosis [39–41]. Its effects are mediated via its interaction with tyrosine kinase receptors, namely, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/Kdr), and VEGFR-3 (Flt-4), which are located on endothelial cells [42]. Angiogenesis and particularly VEGFR signaling can be targeted either by the monoclonal antibody bevacizumab or by inhibiting downstream intracellular tyrosine kinases by small molecules such as sorafenib or sunitinib. Other relevant factors for angiogenesis are platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), HGF, and angiopoietins. The PI3k/Akt/mTOR pathway which is located downstream of various tyrosine kinase receptors such as EGFR is upregulated in a subset of HCC patients and controls cell proliferation, cell cycle progression, and apoptosis [43]. Other signaling pathways potentially responsible for hepatocarcinogenesis have been identified: c-mesenchymal-epithelial transition factor (Met)/HGF signaling, insulin-like growth factor signaling, nuclear factor-κB, and the extrinsic/intrinsic apoptotic pathways [44] (Figure 1).

2.3. The Role of DCP and GPC-3. DCP is a novel type of VEGF, having mitogenic and migratory activities in the angiogenesis of HCC. DCP is an abnormal prothrombin induced by the absence of vitamin K2, which is increased in the serum of patients with HCC. In hepatoma cells, genetic alterations, the inability of membrane receptors to uptake labeled low-density lipoprotein, cytoskeletal changes, and hepatocyte cytoplasmic transfers involved in vitamin K metabolism could play an important role in producing detectable DCP serum levels. DCP is not exclusively a diagnostic or prognostic biomarker for HCC but is also a novel type of VEGF, with mitogenic and migratory roles in the angiogenesis of HCC. DCP might stimulate HCC cell growth through the DCP-Met-JAK1-STAT3 signaling pathway. DCP was found to increase HCC invasion and metastasis through activation of matrix metalloproteinase (MMPs) and the ERK1/2 MAPK signaling pathway. DCP might stimulate the formation of angiogenesis through the DCP-KDR-PLC-γ-MAPK signaling pathway.

In HCC patients, DCP production is independent of vitamin K deficiency, although pharmacological doses of vitamin K can transiently suppress DCP production in some tumors. In an *in vitro* model, DCP production was observed in HepG2 cells and inhibited by introducing additional vitamin K2 into the treated cells. In addition to the decrease in DCP production, there has been a reduction of the growth and invasiveness of carcinoma cells. Therefore, administration of vitamin K2, associated with therapies of proven efficacy, could be considered a promising option for the treatment of HCC [7, 13]. GPC-3 is a heparan sulfate proteoglycan. Recent studies have shown that GPC3 levels are increased in HCC patients. It is thought that GPC3 stimulates the growth of HCC cells by upregulating autocrine/paracrine canonical Wnt signaling. GPCs stimulate both the canonical and noncanonical pathways and regulate migration, adhesion, and actin cytoskeleton organization in tumor cells through Wnt signaling modulation [7]. The principal factors

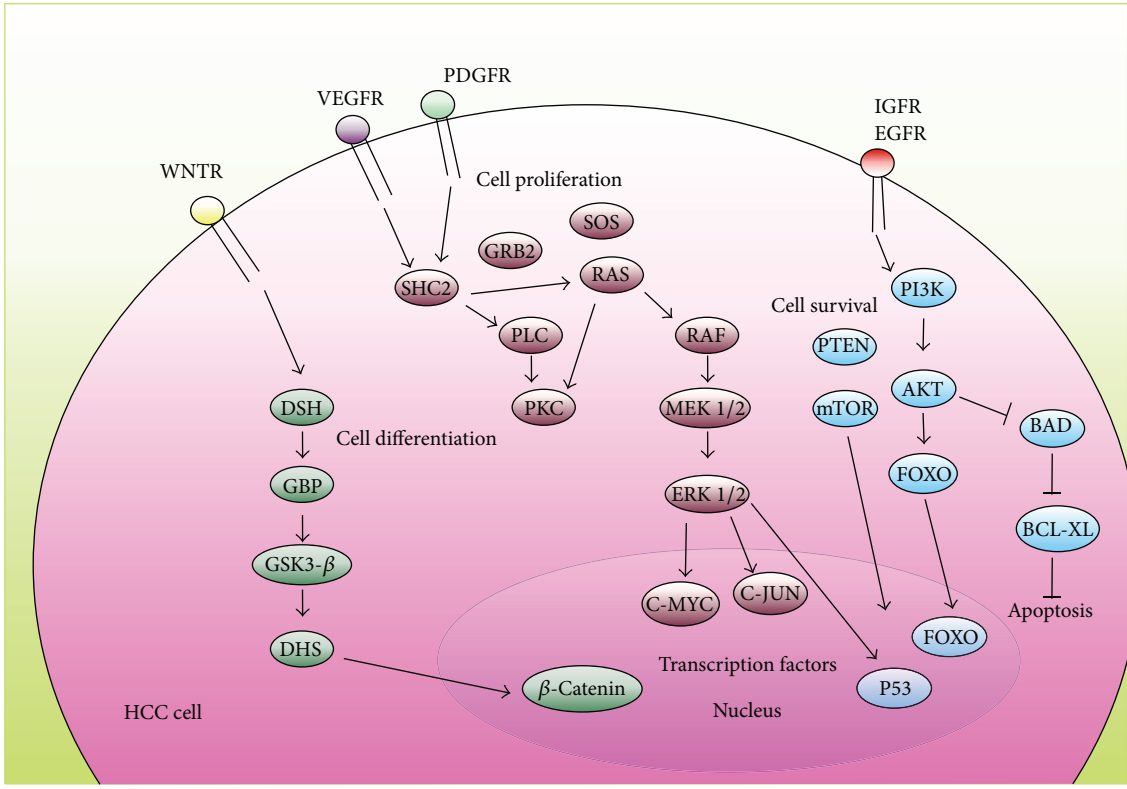


Figure 1: Cellular signaling pathways implicated in the pathogenesis of hepatocellular carcinoma.

in the development of HCC and in hepatocarcinogenesis are the heparan sulfate chains of GPC-3 combined with other heparin-binding growth factors. Therefore, GPC-3 could be an interesting molecular therapy [45]. In addition, the tyrosine kinase inhibitor of IGF-1R (NUP-AEW541) expressing HCC cell line (PLC/PRF/5) has interestingly been recently reported on [46]. A new cancer therapy could be a combination of the anti-GPC-3 antibody and molecular therapy targeting GPC-3 related molecules, such as FGFR.

2.4. Molecular Immunological Targets. The rationale for immunotherapy for HCC is based on the finding that patients with tumours containing infiltrating, presumably tumour-specific effector T cells, had a reduced risk of tumour recurrence following liver transplantation [47]. Moreover, anti-CD3 and IL-2 stimulated autologous T lymphocytes infused in HCC patients significantly improved postsurgical recurrence-free survival [48]. These data imply a central role of T cells in modulating tumour progression and provide strong justification for T-cell immunotherapy. A prerequisite for the successful development of T-cell-based immunotherapeutic approaches is the identification and characterization of immune responses to tumour-associated antigens (TAAs). Six HCC-specific TAAs that are targeted by T cells have been identified: AFP, GPC3, NY-ESO-1, SSX-2, melanoma antigen gene-A (MAGE-A), and telomerase reverse transcriptase (TERT) [49–53].

In patients with HCC, there is an alteration of different pattern of the immunological system.

Several mechanisms could contribute to the weak and often inefficient TAA-specific CD8+ T-cell responses in HCC patients.

(i) The regulatory T cells (Tregs) are the best characterized suppressor cells and have been shown to suppress tumour immunity in numerous studies. The Tregs were found to produce the immunosuppressive cytokine IL-10; IL-10 suppresses both CD4+ and CD8+ and, consequently, Tregs play a major role in the inhibition of tumour-specific T-cell responses in HCC [54].

(ii) Myeloid-derived suppressor cells (MDSCs) comprise a mixture of monocytes/macrophages, granulocytes, and dendritic cells (DCs) at different stages of differentiation. In patients with HCC, blood MDSCs have recently been shown to induce Foxp3 and IL-10 expression in CD4+ T cells via arginase activity [55]. There is currently no specific drug or antibody available that selectively targets MDSCs. Since inhibition of arginase activity can cause side effects, due to the critical role of this enzyme in the urea cycle, it will be important to identify additional specific markers to target these cells.

(iii) Impairment of TAA processing and presentation: several studies have shown that expression of HLA class I molecules and B7 costimulatory molecules is downregulated in HCC tissue [56] and HCC cell lines [57, 58]. Such downregulation is likely to lead to impaired processing of TAAs. Moreover, it has been shown that circulating myeloid DCs in HCC patients were decreased in numbers and had reduced cytokine production, raising the possibility that TAA presentation by DCs may also be impaired.

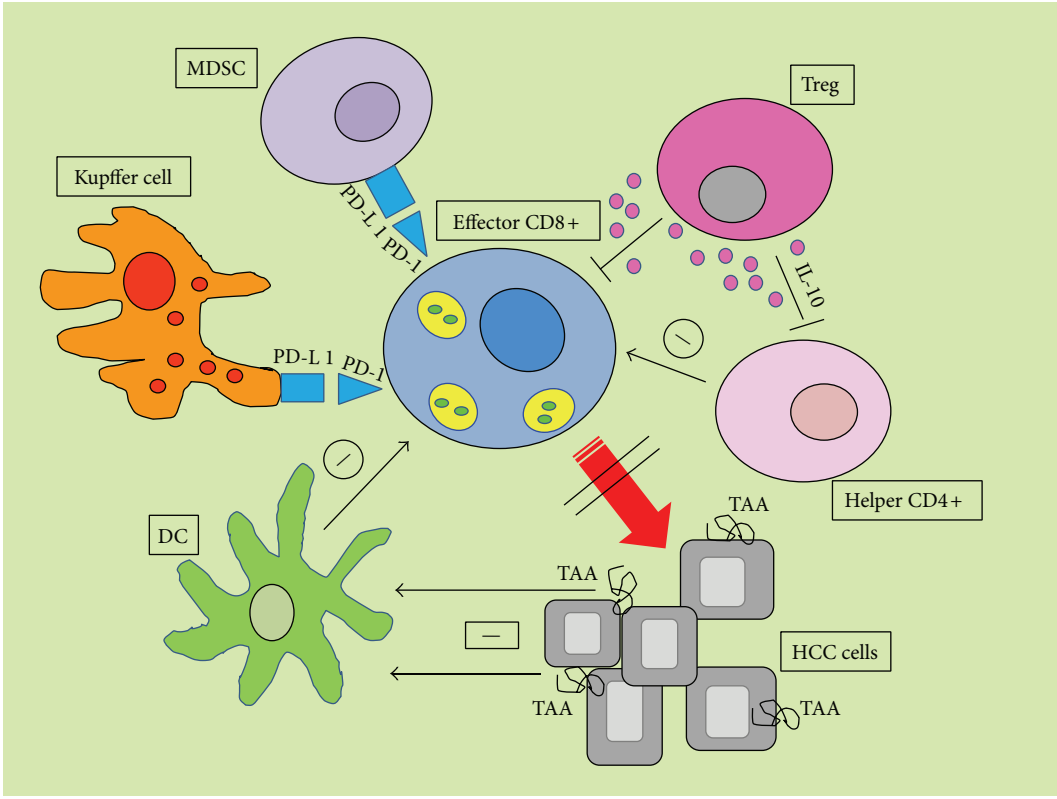


Figure 2: Mechanisms responsible for inefficient T-cell responses in HCC. Failure of TAA processing and presentation; suppression of CD4+ and CD8+ cells by Treg; insufficient levels of CD4 help; negative regulation by PD-1/PDL1 pathway.

(iv) Inhibitory receptors: many human cancers express PD-L1, the ligand for the inhibitory receptor programmed cell death-1 (PD-1). Tumour-associated PD-L1 has been shown to induce apoptosis of effector T cell and is thought to contribute to immune evasion by cancers. In HCC, tumour infiltrating CD8+ T cells are characterized by an increase in PD-1 expression [59]. Intratumor Kupffer cells have been shown to upregulate PD-L1 and decrease the effector function of PD-1-expressing CD8+ T cells in HCC patients [29, 30]. It should be noted that cells with a MDSC phenotype also upregulated PD-L1 in these studies and exerted a similar inhibitory effect on T-cell activation, raising the possibility of MDSC-mediated T-cell suppression. These data suggest that the inhibition of PD-1 may be a potential strategy in the boosting of HCC-specific immunity.

(v) Lack of CD4+ T-cell responses: it is known that a lack of CD4+ T-cell help may lead to CD8+ T-cell exhaustion. In fact, in HCC, AFP-specific CD4+ T-cell responses were only present in patients with early stage disease and became exhausted as the disease progressed [60]. Thus, for the activation of fully functional cytotoxic T lymphocytes it will be important to identify TAA-derived CD4 T helper cell epitopes and include them in a vaccine along with CD8 T-cell epitopes.

In conclusion, multiple mechanisms may limit the TAA -specific CD8+ T-cell responses in HCC: failure of TAA processing and presentation; insufficient levels of CD4 help; suppression of both CD4+ and CD8+ T cells by Tregs; and negative regulation by PD-1/PD-L1 pathway [61, 62] (Figure 2).

2.5. Immunosuppression Induced by Chronic HBV and HCV Infection. Chronic HBV or HCV infections are well known to induce a chronic proinflammatory hepatic and systemic state associated with immunosuppressive and immunomodulatory effects [33, 34, 63–65].

In HCC, there is chronic hepatitis B virus and hepatitis C virus-mediated immunosuppression. Chronic HBV or HCV infections are well known to induce a chronic proinflammatory hepatic and systemic state associated with immunosuppressive and immunomodulatory effects [33, 34, 63–66].

Dysfunctional T-cell responses to both virus-specific and unrelated antigens, characterized by impaired proliferation and IL-2 production, are observed in chronic hepatitis B virus-infected patients. Chronic infection with hepatitis C virus negatively regulates both the innate and adaptive arms of the immune system. There are many mechanisms of HCC immune escape; the peripheral blood of HCC patients shows impaired IL-12 production and reduced allostimulatory activity [67]. In HCC numerous regulatory mechanisms involving nearly all cellular subsets of the immune system contribute to tumor development and progression.

An impairment of natural killer cell production and activity has also been described in HCC patients [68, 69].

3. Molecular Targeted Therapies: Present and Future

3.1. Multikinase Inhibitors. Sorafenib (BAY43-9006) is currently the only approved systemic treatment for HCC. It has been approved for the therapy of asymptomatic HCC patients with well-preserved liver function who are not candidates for potentially curative treatments, such as surgical resection or liver transplantation, and it is the first FDA approved systemic therapy for patients with advanced HCC. In clinical practice, the failure of locoregional therapy, such as TACE, led to the use of sorafenib. Its efficacy and safety in HCC patients were demonstrated by the SHARP trial in western patients [70, 71].

Most cases of hepatocellular carcinoma occur in the Asia-Pacific region, where chronic hepatitis B infection is an important aetiological factor. In the Cheng et al. study [72] a multinational phase III, randomised, double-blind, placebo-controlled trial has been done to assess the efficacy and safety of sorafenib in patients from the Asia-Pacific region with unresectable or metastatic (advanced) HCC. The authors conclude that sorafenib is effective for the treatment of advanced HCC in patients from the Asia-Pacific region and is well tolerated. Taken together with data from the Sorafenib Hepatocellular Carcinoma Assessment Randomised Protocol (SHARP) trial, sorafenib seems to be an appropriate option for the treatment of advanced HCC.

Several pathways are now implicated in hepatocarcinogenesis, and agents that target these pathways continue to be developed and effective drugs can be synthesized and used to target a specific HCC subgroup.

Sunitinib malate (SU11248, Sutent; Pfizer, NY, USA) is an oral multikinase inhibitor that targets several tyrosine kinases receptors, such as VEGF-1/2 and PDGFR-a/b, and is implicated in HCC proliferation and angiogenesis. In addition, it was defined as an inhibitor of c-kit, Fit-3, and RET [73]. It has already demonstrated preliminary antitumoral activity and an acceptable safety profile in different phase II trials for patients with advanced HCC [74, 75]. However, despite the other tumor types, sunitinib seems to have more side effects for its toxicity in HCC. Due to more treatment-related toxicities using the 50 mg dose, in most planned trials, a 37.5 mg dose has been used. In the Huynh et al. study [76] (xenograft models), the authors wanted to compare the effectiveness of sunitinib relatively to sorafenib, both strong inhibitors of tyrosine-kinases proteins involved in tumor growth, angiogenesis, and metastasis, reporting suppressed tumor growth, angiogenesis, cell proliferation, and induced apoptosis in both HCC models, orthotopic and the ectopic, for both the drugs.

However the antitumoral effectiveness of 50 mg/kg of sorafenib was greater than that of 40 mg/kg of sunitinib. The sorafenib inhibited p-elF4E Ser 209 and p-p 38 Thr180/Tyr182 and reduced survivin expression.

Not the same was with sunitinib. In addition the antitumoral effect and the apoptosis of sorafenib, which is associated with upregulation of fast migrating Bin and ASK1 and the downregulation of the survivin, were greater than sunitinib. These observations explained the apparently more efficacy of sorafenib in antitumoral activity, if one compared it with sunitinib.

Huynh concludes that the antitumoral effect of sunitinib is inferior to sorafenib, in both ectopic and orthotopic models of human HCC. However, these observations should be verified in humans. Concomitant liver function, liver disease, and the local liver environment have a huge impact on treatment outcomes. Sunitinib antiproliferative action on HCC cell lines, either *in vitro* or in xenograft and orthotopic models, was studied by Bagi et al. [77] in order to evaluate the effect of the local liver vasculature on drug efficacy. Drug exposure and treatment regimen were the same in both tumors. Comparing sunitinib effect on models, the *in vivo* results show that it is much less effective against intrahepatic tumors compared with xenograft. Sunitinib affects large solid intrahepatic tumors, as shown by histological data, but unopposed local growth of the small tumors and the development of distant micrometastases seem to be a problem with these kinds of VEGF inhibitors. No doubt both xenograft and orthotopic models are limited.

Thus recently sunitinib efficacy/safety assessment studies have been suspended due to an unfavourable risk-benefit relationship of its administration (SUN 1170 Phase III open label study), in comparison to sorafenib [78].

Linifanib (ABT-869) is a multitargeted tyrosine kinase inhibitor that inhibits multiple members of the VEGFR and PDGFR families [62]. In a xenograft model of HCC, ABT-869 significantly reduced tumor burden. Interim phase II results in patients with advanced HCC showed a median TTP of 3.7 months with ABT-869 treatment and a safety profile consistent with angiogenesis inhibition. In the Toh et al. study, the authors demonstrate that linifanib as a single agent was found to be clinically active in patients with advanced HCC, with an acceptable safety profile [79].

In the open-label, global phase III trial linifanib versus sorafenib as first-line therapy in patients with advanced Child-Pugh A (CPA) HCC (NCT01009593) was evaluated. Patients were randomized 1 : 1 to linifanib 17.5 mg QD or sorafenib 400 mg BID and stratified by region (non-Asia/Japan/rest of Asia), ECOG performance status (0/1), vascular invasion or extrahepatic spread (yes/no), and HBV infection (yes/no). The primary efficacy endpoint was overall survival (OS); both noninferiority (margin 1.0491) and superiority hypotheses were to be tested. Secondary efficacy endpoints included time to progression (TTP) and ORR, using RECIST v1.1. Adverse events (AEs) severity was graded using NCI-CTCAE v4.0.1035. Patients (median age 60 y, 68% Asian, 65% ECOG 0, 49% HBV, 70% vascular invasion or extrahepatic spread) were randomized at 149 sites in 26 countries. Hazard ratio (HR) for OS was 1.046 (95% CI: 0.896, 1.221). Median OS (95% CI) was 9.1 months (m) (8.1, 10.2) on linifanib and 9.8 m (8.3, 11.0) on sorafenib. For all prespecified subgroup analyses, OS HRs ranged from 0.793 to 1.119, and the 95% CI contained 1.0. TTP HR was 0.759 (95% CI: 0.643, 0.895; $P = 0.001$) favoring linifanib. Median TTP (95% CI) was 5.4 m (4.2, 5.6) on Lin and 4.0 m (2.8, 4.2) on sorafenib. ORR was 13.0% on linifanib and 6.9% on sorafenib. Grade 3/4 AEs, serious AEs, and AEs leading to discontinuations, dose interruptions, and reductions were

more frequent on linifanib versus sorafenib (all $P < 0.001$). In conclusion linifanib and sorafenib resulted in similar OS in advanced HCC. Predefined superiority and noninferiority OS boundaries were not met for linifanib. Secondary endpoints (TTP and ORR) favored linifanib while safety results favored sorafenib [80].

3.2. MET Inhibitors. Tivantinib (ARQ 197) is a new oral selective MET inhibitor that acts by blocking growth and inducing apoptosis in human tumor cell lines that express MET. MET is a tyrosine kinase receptor involved in tumor development and metastatic progression, which is encoded by a MET protooncogene. When binding to HGF, MET activates the RAS-MAPK and PI3 K-AKT signaling pathways [81, 82]. Tivantinib antitumor activity was demonstrated in murine xenograft models and its efficacy was confirmed in a panel of HCC cell lines [83].

Tivantinib may provide an option for second-line treatment in patients affected by advanced HCC with well-compensated cirrhosis, especially if they have MET high tumors. MET can represent an important prognostic and predictive biomarker in this type of patient. MET overexpression is associated with poor prognosis in HCC patients.

Tivantinib demonstrated a manageable safety profile and preliminary antitumor activity in patients with HCC and Child's A or B cirrhosis. Further studies of tivantinib in a biomarker-selected patient population are warranted [84].

Enrollment for this phase III clinical trial (ARQ 197-A-U303, NCT01755767) has begun. Eligible patients must present with Child-Pugh A; ECOG performance score <1; inoperable RECIST 1.1 measurable disease; adequate bone marrow, liver, and kidney functions; and no prior liver transplant. Pts must have progressed after or not tolerated one prior line of systemic therapy including eligible. Approximately 303 patients are randomized 2 : 1 to receive tivantinib 240 mg PO twice daily or placebo. Patients are stratified by vascular invasion, metastases, and alpha-fetoprotein level, and they are evaluated by CT or MRI scan at 8-week intervals. The primary endpoint is overall survival (OS). Secondary endpoints include progression-free survival and safety. Treatment continues until confirmed disease progression or unacceptable toxicity. Patients discontinued from study treatment will be followed for survival. Participating centers are located in Europe, Australia, New Zealand, and the Americas. This trial is expected to complete enrollment by mid-2015, and an interim analysis is planned when approximately 60% of OS events are reached [85].

3.3. Antiangiogenic Agents. In addition to these drugs new antiangiogenic agents have been studied: bevacizumab, brivanib, and ramucirumab.

Bevacizumab (Avastin; Genentech, CA, USA), a recombinant, humanized monoclonal antibody that targets VEGF, is one of the central drugs of colorectal tumor treatment [86]. In addition to inhibiting tumor growth, growth factor release, and metastasis, it can enhance chemotherapeutic agent delivery by normalizing tumor vasculature [87].

Brivanib (BMS-582664), a selective dual inhibitor of FGF and VEGF signaling, has recently been shown to have activity as a first-line treatment for patients with advanced HCC [88]. In the phase II open-label study by Finn et al., brivanib was assessed as a second-line therapy in patients with advanced HCC who had not been successfully treated with prior antiangiogenic treatment [89, 90]. The authors conclude that brivanib had a manageable safety profile and is one of the first agents to show promising antitumor activity in advanced HCC patients treated with prior sorafenib. Nevertheless, recent data showed that patients administrated with brivanib did not reach the primary endpoint (OS) both in first- and second-line therapy [90].

Llovet and colleagues studied brivanib in patients with advanced hepatocellular carcinoma who were intolerant to sorafenib or for whom sorafenib failed (randomized phase III BRISK-PS study) [91].

This multicenter, double-blind, randomized, placebo-controlled trial assessed brivanib in patients with HCC who had been treated with sorafenib. In all, 395 patients with advanced HCC who progressed on/after or were intolerant to sorafenib were randomly assigned (2 : 1) to receive brivanib 800 mg orally once per day plus best supportive care (BSC) or placebo plus BSC. The primary endpoint was overall survival (OS). Secondary endpoints included time to progression (TTP), objective response rate (ORR), and disease control rate based on modified Response Evaluation Criteria in Solid Tumors (mRECIST) and safety. Median OS was 9.4 months for brivanib and 8.2 months for placebo (hazard ratio [HR], 0.89; 95.8% CI, 0.69 to 1.15; $P = 0.3307$). Adjusting treatment effect for baseline prognostic factors yielded an OS HR of 0.81 (95% CI, 0.63 to 1.04; $P = 0.1044$). Exploratory analyses showed a median time to progression of 4.2 months for brivanib and 2.7 months for placebo (HR, 0.56; 95% CI, 0.42 to 0.76; $P < 0.001$) and an mRECIST ORR of 10% for brivanib and 2% for placebo (odds ratio, 5.72). Study discontinuation due to treatment-related adverse events (AEs) occurred in 61 brivanib patients (23%) and nine placebo patients (7%). The most frequent treatment-related grade 3 to 4 AEs for brivanib included hypertension (17%), fatigue (13%), hyponatremia (11%), and decreased appetite (10%). In conclusion, in patients with HCC who had been treated with sorafenib, brivanib did not significantly improve OS. The observed benefit in the secondary outcomes of TTP and ORR warrants further investigation [91].

Consequently, two large phase III studies of brivanib in hepatocellular carcinoma were launched, the BRISK-FL study in first-line therapy and the BRISK-PS study in patients whose disease progressed on or who were intolerant of sorafenib. Unfortunately, no one study met its endpoint to demonstrate activity of brivanib in HCC.

Ramucirumab (Cyramza, Eli Lilly and Company), a monoclonal antibody, is a specific inhibitor of VEGFR-2. A phase II study of 42 patients with advanced HCC and primarily well-preserved liver function (75% Child-Pugh A status) showed that first-line ramucirumab monotherapy produced a DCR of 50% and a median PFS of 4.3 months [72]. This positive study prompted the initiation of the Phase III REACH trial in HCC, which compares ramucirumab/supportive

care with placebo/supportive care for second-line treatment after sorafenib. REACH Phase III trial enrollment has been completed but no results are available yet. It will be very interesting to see the results and the final endpoints.

In addition to these drugs, *Cabozantinib (Cometriq, Exelixis Inc.)* acts as a dual c-Met/VEGFR2 inhibitor, inhibiting the tyrosine kinase activity of RET, MET, VEGFR-1, -2, and -3, KIT, TRKB, FLT-3, AXL, and TIE-2. The Verslype et al. study also demonstrated early evidence of antitumor activity in a randomized discontinuation phase II study. Interestingly, the clinical benefits were observed regardless of whether patients had received prior sorafenib treatment. Cabozantinib is undergoing additional evaluation in HCC to better assess its efficacy and safety profile in several ongoing clinical trials [92]. Phase III studies should evaluate the effectiveness of cabozantinib versus placebo and OS in patients with advanced HCC who have already been treated. OS was the primary endpoint, and the objective response rate and PFS for RECIST 1.1 were the secondary endpoints. Additional endpoints were safety and tolerability of cabozantinib; pharmacokinetics (PK); change from baseline tumor biomarker levels in the serum; and health-related quality of life as assessed by the EuroQol Health questionnaire.

3.4. mTOR Inhibitors. *Temsirolimus* and *Everolimus* are two inhibitors of mTOR.

In the phase 3 study of everolimus for advanced HCC that progressed during or after sorafenib (EVOLVE-1 NCT01035229), Zhu and colleagues assessed the efficacy and safety of everolimus for advanced HCC after sorafenib failure [93].

Patients aged ≥18 y with BCLC stage B or C HCC and Child-Pugh A liver function whose disease progressed during or after sorafenib or who were sorafenib intolerant were randomized 2 : 1 to everolimus 7.5 mg/d or placebo. All patients received best supportive care. Randomization was stratified by region (Asia versus rest of world) and macrovascular invasion (yes versus no). Study drug was given continuously until disease progression or intolerable toxicity. CT/MRI was performed every 6 wk. Primary endpoint was OS. Secondary endpoints were TTP, disease control rate (DCR; percentage of pts with best overall response of CR, PR, or SD per RECIST 1.0), and safety. Final analysis was performed when 454 deaths occurred. 546 patients from 18 countries enrolled from April 2010 to March 2012 (everolimus = 362, placebo = 184). Baseline characteristics were balanced between arms; median age was 66.0 y, 84.8% of pts were male, 86.3% had BCLC stage C disease, 16.7% were from Asia, 32.8% had macrovascular invasion, and 74.0% had extrahepatic disease. Prior sorafenib was discontinued for disease progression in 80.8% of pts and intolerance in 19.0%. Median OS was 7.56 mo with everolimus and 7.33 mo with placebo (HR 1.05; 95% CI 0.86–1.27; $P = 0.675$). Median TTP was 2.96 mo and 2.60 mo, respectively (HR 0.93; 95% CI 0.75–1.15). DCR was 56.1% and 45.1%, respectively ($P = 0.010$). The most common grade 3/4 AEs with everolimus (v placebo) were anemia (7.8% v 3.3%), asthenia (7.8% v 5.5%), decreased appetite (6.1% v 0.5%), and hepatitis B viral load increase or reappearance (6.1% v 4.4%). No patients experienced HCV flare. HBV reactivation was experienced by 39 pts (29 everolimus, 10 placebo); all cases were asymptomatic, but 3 everolimus recipients discontinued therapy. Unfortunately everolimus did not improve OS in patients with advanced HCC whose disease progressed on or after sorafenib or who were sorafenib intolerant. The safety profile was consistent with that previously observed with everolimus [93].

There is strong rationale to combine an m-TOR inhibitor (temsirolimus) with a VEGF inhibitor (bevacizumab) as a potentially active and well-tolerated treatment for HCC. Both agents have shown modest single agent activity in HCC and so evaluated in a phase II trial. Knox and colleagues [94] participated in the phase II trial of temsirolimus (TEM) and bevacizumab (BEV) in patients with advanced HCC. A modified 2-stage Simon design planned 25 or 50 patients to test the null hypothesis that true tumor response rate is at most 10% and true 6 mo progression-free survival rate (PFS) (by RECIST) is at most 65%, or no better than single agent BEV (6 mo PR >2 pts or PFS 6 mo >18 out of 25). Toxicity, TTP, PFS, and survival were 2nd endpoints. Eligible patients had confirmed HCC with disease unresectable or amenable to other localised therapies, Child-Pugh A liver status, and no prior systemic therapy involving the VEGF or m-TOR class of agents. TEM was administered at starting dose 25 mg IV d1, 8, 15, 22 with BEV at 10 mg/kg IV d 1, 15, all q 28 days (1 cycle). Imaging was q 8 wks. From 09/09 to 09/11, 27 eligible patients were enrolled with 25 evaluable for toxicity and efficacy. Med age 59 yrs, 85% male, PS 0/1: 35/65, 58% metastatic, >85% BCLC stage C. With med 6 cycles (range 1–14) delivered, most patients (88%) experienced grade 3+ adverse events. Common grade 3 adverse events related to treatment included thrombocytopenia (40%), neutropenia (20%), leucopenia (12%), fatigue (8%), anemia, mucositis, dyspnea, diarrhea, bleeds, fistula, and infections (4% each). There was one possible treatment related death. Per protocol dose reductions/discontinuation for TEM-related adverse events were most common. There were 2 confirmed PRs and 16 patients progression-free by 6 mos. A third patient developed a late PR at cycle 13. Median TTP on study was 6 mos, median PFS was 7.4 mos, and median survival was 8.3 mos, with 13 patients still alive. Accrual closed at end of stage 1 as neither the number of responses nor the PFS at 6 mos passed the futility stopping rule set for this combination. This multicenter study is the first HCC trial evaluating the BEV/TEM doublet. Despite manageable toxicity, the ORR and 6 mo PFS did not surpass assumptions based on single agent BEV in HCC. Further study of BEV/TEM combination in this advanced HCC population is not recommended [94].

The University of California, San Francisco, is conducting a phase II trial of the combination of temsirolimus and sorafenib in advanced hepatocellular carcinoma. This phase II trial is being developed following the completion of a phase I study of the combination of temsirolimus and sorafenib in 25 first-line therapy patients with advanced hepatocellular carcinoma (December 2009 through April 2012). The maximum tolerated dose (MTD) and recommended phase II dose (RP2D) of the combination of temsirolimus are 10 mg IV weekly plus sorafenib 200 mg (oral, twice daily).

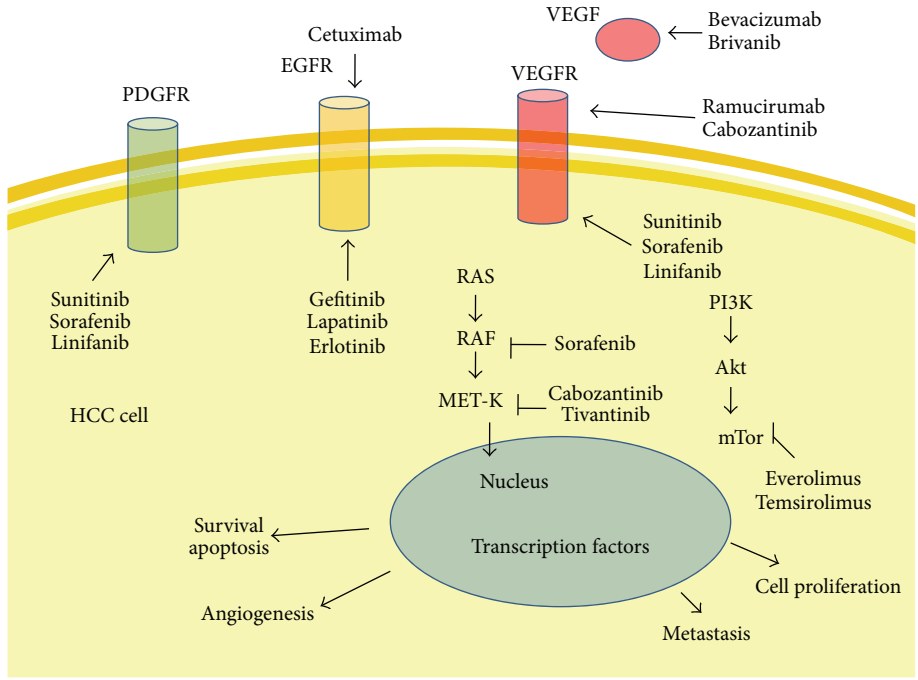


Figure 3: Molecular sites of action of active biochemical agents in hepatocellular carcinoma treatment.

The hypothesis of this single-arm phase II study is that the combination of temsirolimus and sorafenib will achieve a clinically meaningful median time to progression (TTP) of at least 6 months, with null hypothesis of less than or equal to 3 months, in first-line systemic therapy for patients with advanced HCC. A randomized trial would be required to formally compare the efficacy of this combination to sorafenib alone and will be indicated if this phase II study achieves a median TTP of at least 6 months. An interim safety analysis will employ stopping rules after 30% of planned patients have been treated with at least one dose of protocol therapy to ensure the combination does not confer excessive toxicity. A key aspect of this study will be the requirement of histologic confirmation along with adequate archival tissue for correlative tissue analyses to explore new biomarkers of response to mTOR inhibition. Circulating biomarker data including enumeration of circulating tumor cells (CTC) and measurement of the tumor marker AFP will be performed at specific time points to evaluate for predictive value. Specimen banking of tissue, serum, and peripheral blood mononuclear cells will be undertaken to enable future novel biomarker studies. Modified RECIST will be performed in addition to standard RECIST 1.1 to explore for improved imaging predictors of response.

The current primary outcome measures are median time to progression (TTP); median TTP will be calculated in months from date of first dose of protocol therapy to date of removal from study for progression. The current secondary outcome measures are response rate (RR), progression free survival (PFS), overall survival (OS), time to treatment failure (TTF), toxicity, and tolerability.

This study is currently recruiting participants; the estimated primary completion date is December 2015 (final data collection date for primary outcome measure) and completion date is December 2018 (Figure 3) (Table 2).

3.5. Current and Future Immunotherapy of HCC. Cellular immunotherapy would improve the immune state and has potential in enhancing the therapeutic outcome for HCC patients. Current attempts at harnessing the immune system to eliminate tumors have been focusing on vaccination, such as a dendritic cell (DC) vaccine, to increase the frequency of tumor specific cytotoxic T lymphocytes and adoptive transfer of effector T cells to promote tumor regression [95]. However, despite considerable success in preclinical studies, the outcome of immunotherapy is often disappointing when translated to clinical trials, which is at least in part due to the complexity of the immune escape mechanism of tumor cells.

Immune-based therapy can represent an improvement in outcomes for patients with HCC [95], as many clinical trials demonstrate. In a historical study, 150 patients were randomized to receive either IL-2 and anti-CD3-activated PBMC or observation after curative resection [48]. The results were encouraging, both with respect to time to relapse and disease-free survival ($P = 0.09$). A trial testing the administration of APC in HCC patients who received pulsed DC with autologous tumor lysate [96] showed an increase of 1-year survival (63 versus 10%; $P = 0.038$). Most recently, murine models have demonstrated that immunotherapy and DC in combination with IL-12 in an adjuvant setting activate T and natural killer cells and reduce HCC recurrence [69, 97, 98]. DCs could be used as a potential cellular adjuvant for the production of specific tumor vaccines. Recently, El Ansary and colleagues' study [69] evaluated the safety and efficacy of the autologous pulsed DC vaccine in advanced

Table 2: Targeting signaling in the treatment of HCC and phases of trials.

	Model of action	Target	Phases	Trials (*n.*)	State of trials
Multikinase inhibitors					
Sorafenib	TKI	VEGFR-2/-3, PDGFR-beta, Raf-1, B-Raf, Flt-3, cKIT, and RET	1, 1-2, 2, 3, 4	65	Closed
Sunitinib	TKI	VEGFR-1/-2, PDGFR-alpha, -beta, Flt-3, cKIT, and RET	2, 3	6	Closed
Linifanib	TKI	VEGFR and PDGFR family	2, 3	2	On-going
Cabozantinib	TKI	VEGFR2	1, 2	2	On-going
MET inhibitors					
Tivantinib	Inhibits growth and induces apoptosis in HCC c-MET positive	c-MET/HGF	1, 2, 3	4	On-going
Cabozantinib	Inhibits growth and induces apoptosis in HCC c-MET positive	c-MET	1, 2	2	On-going
Antiangiogenic agents					
Bevacizumab	MAB	VEGF	1, 1-2, 2	20	Closed
Brivanib	MAB	FGF and VEGF	1, 2, 3	6	On-going
Ramucirumab	MAB	VEGFR-2	3	1	On-going
mTOR inhibitors					
Everolimus	Inhibits cell replication	mTOR	3	1	Closed
Temsirolimus (+bevacizumab)	Inhibits cell replication	mTOR	2	2	Closed
Temsirolimus (+sorafenib)	Inhibits cell replication	mTOR	2	2	On-going

HGF = hepatocyte growth factor; FGF = fibroblast growth factor; MAB = monoclonal antibody; PDGF = platelet-derived growth factor; PDGF(R) = platelet-derived growth factor receptor; VEGF = vascular endothelial growth factor; and VEGF(R) = vascular endothelial growth factor receptor.

HCC patients in comparison with supportive treatment. Thirty patients with advanced HCC who were not suitable for radical or loco regional therapies were enrolled. Patients were divided into two groups; group I, consisting of 15 patients, received vaccination with mature autologous DCs pulsed *ex vivo* with a liver tumor cell line lysate. Group II (control group; $n = 15$) received supportive treatment. To generate DCs, 100 and 4 mL of venous blood were obtained from each patient. DCs were identified by CD80, CD83, CD86, and HLA-DR expressions using flow cytometry. Follow-up at 3 and 6 months after injection by clinical, radiological, and laboratory assessment was carried out. Improvements in OS were observed. Partial radiological response was obtained in two patients (13.3%), and stable course in nine patients (60%) and four patients (26.7%) showed progressive disease (died at 4 months after injection). Both CD8+ T cells and serum IFN-g were elevated after DC injection. The authors conclude that autologous DC vaccination in advanced HCC patients is safe and well tolerated. *Ex vivo* treatment with CTLA-4 blocking antibodies of T-cell CD8+, isolated from patients affected by HCC, showed an expanded antigen-specific T-cell repertoire, alluding that ipilimumab may possess a therapeutic potential in treating hepatocarcinoma [99]. A therapeutic advantage, regarding refractory solid tumors, can be obtained by an antibody-mediated block of PD-1; meanwhile the inhibition of Tim-3 signaling has been demonstrated to restore antitumor T-cell action in preclinical models [100]. Another approach has been described to overcome cancer-mediated immunosuppression, involving the reactivation of hyporesponsive tumor-specific T cells by supplying T-cell growth factors (IL-15 and IL-7) or costimulatory agonists (anti-4-1BB and anti-OX40) [101]. Other treatment options regarding tumor homing and penetration of T-effector cells are being evaluated because of the correlation between T-cell infiltration of hepatocarcinoma lesions and OS. Strategies are divided into two big groups: restoring the tumor vascularity and upregulation of chemokines and molecules of adhesion. Monoclonal antibodies against VEGF and its receptors, such as sorafenib or bevacizumab, appear to have a restricted therapeutic effect in clinical trials [26, 102]. In fact a hallmark of new vessel formation in HCC is their structural and functional abnormality; this leads to an abnormal tumor microenvironment characterized by low oxygen tension and low therapeutic agent levels [103]. Preclinical data sustain the idea that angiogenesis and tumor vascularity still represent a potential target that, through the generation of long-lived antivascular T-cell responses via VEGFR2 vaccine, can be suppressed via a T-cell dependent process [69]. Proinflammatory chemokines demonstrated their importance in HCC-specific T-cell immunity, such as

IFN-g-inducible chemokines CXCL9/Mig and CXCL10/IP-10, high levels of which correlated with the presence of CD8+ T cell in hepatocarcinoma. It is still unknown if this pattern of chemokine expression is correlated with a positive prognosis, as has been seen in patients with cervical/uterine tumors. Agents that can induce the expression of chemokines and adhesion molecules by vascular activation represent another promising approach [104].

4. Discussion and Conclusion

HCC treatment decisions are complex and dependent upon tumor staging. In patients with unresectable disease and tumor staging that falls within criteria, liver transplantation can be curative in a great majority of patients. Unfortunately, most patients will not be candidates for either surgery or transplant [105–107].

Cytotoxic chemotherapy, hormonal agents, and immunotherapy have been tested in HCC with marginal efficacy to date. Recent insights into the molecular pathogenesis of HCC have identified several aberrant signaling pathways that have served as targets for novel therapeutic agents. Several pathways are now implicated in hepatocarcinogenesis and agents that target these pathways continue to be developed.

The knowledge of molecular hepatocarcinogenesis broadened the horizon for patients with advanced HCC. During the last years, several molecular targeted agents have been evaluated in clinical trials in advanced HCC. Despite of only modest objective response rates according to the Response Evaluation Criteria in Solid Tumors (RECIST) [44], several studies showed encouraging results in terms of prolongation of the time to progression (TTP), disease stabilization (DS), and survival.

Cellular immunotherapy would improve the immune state and has potential in enhancing the therapeutic outcome for HCC patients. Immune-based therapy can represent an improvement in outcomes for patients with HCC, as many clinical trials demonstrate.

Therefore, in the future, new therapeutic options will be represented by a blend of immunotherapy-like vaccines and T-cell modulators, supplemented by molecularly targeted inhibitors of tumor signaling pathways [108, 109].

Molecular alterations may differ depending on the underlying risk factors and etiologies, potentially influencing patient responses to therapy. Thus, it will be necessary in the future to classify HCCs into subgroups according to their genomic and proteomic profiling. The identification of the key molecules/receptors/signaling pathways and the assessment of their relevance as potential targets will be the main future challenge. Defining molecular targeted agents effective for a specific subgroup will hopefully lead to personalized therapy [110, 111].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] C.-L. Lin and J.-H. Kao, "Risk stratification for hepatitis B virus related hepatocellular carcinoma," *Journal of Gastroenterology and Hepatology*, vol. 28, no. 1, pp. 10–17, 2013.

[2] T. Umemura, T. Ichijo, K. Yoshizawa, E. Tanaka, and K. Kiyosawa, "Epidemiology of hepatocellular carcinoma in Japan," *Journal of Gastroenterology*, vol. 44, supplement 19, pp. 102–107, 2009.

[3] J. A. Davila, J. R. Kramer, Z. Duan et al., "Referral and receipt of treatment for hepatocellular carcinoma in United States veterans: effect of patient and nonpatient factors," *Hepatology*, vol. 57, no. 5, pp. 1858–1868, 2013.

[4] G. Baffy, E. M. Brunt, and S. H. Caldwell, "Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace," *Journal of Hepatology*, vol. 56, no. 6, pp. 1384–1391, 2012.

[5] I. Di Carlo, M. Mannino, A. Toro et al., "Persistent increase in alpha-fetoprotein level in a patient without underlying liver disease who underwent curative resection of hepatocellular carcinoma. A case report and review of the literature," *World Journal of Surgical Oncology*, vol. 10, article 79, 2012.

[6] G. Bertino, A. M. Ardiri, M. M. Santonocito, and P. M. Boemi, "Some patients with HCC haven't abnornormal des-gamma-carboxy prothrombin and alpha-fetoprotein levels," *Panminerva Medica*, vol. 51, no. 2, pp. 133–134, 2009.

[7] G. Bertino, A. Ardiri, M. Malaguarnera, G. Malaguarnera, N. Bertino, and G. S. Calvagno, "Hepatocellualar carcinoma serum markers," *Seminars in Oncology*, vol. 39, no. 4, pp. 410–433, 2012.

[8] G. Bertino, S. Neri, C. M. Bruno et al., "Diagnostic and prognostic value of alpha-fetoprotein, des-γ-carboxy prothrombin and squamous cell carcinoma antigen immunoglobulin M complexes in hepatocellular carcinoma," *Minerva Medica*, vol. 102, no. 5, pp. 363–371, 2011.

[9] G. Bertino, A. M. Ardiri, G. S. Calvagno, N. Bertino, and P. M. Boemi, "Prognostic and diagnostic value of des-γ-carboxy prothrombin in liver cancer," *Drug News and Perspectives*, vol. 23, no. 8, pp. 498–508, 2010.

[10] A. Biondi, G. Malaguarnera, M. Vacante et al., "Elevated serum levels of Chromogranin A in hepatocellular carcinoma," *BMC Surgery*, vol. 12, supplement 1, article S7, 2012.

[11] G. Malaguarnera, I. Paladina, M. Giordano, M. Malaguarnera, G. Bertino, and M. Berretta, "Serum markers of intrahepatic cholangiocarcinoma," *Disease Markers*, vol. 34, no. 4, pp. 219–228, 2013.

[12] G. Bertino, A. Ardiri, S. Demma et al., "Rare benign tumors of the liver: still rare?" *Journal of Gastrointestinal Cancer*, vol. 45, no. 2, pp. 202–217, 2014.

[13] G. Bertino, A. M. Ardiri, P. M. Boemi et al., "A study about mechanisms of des-gamma-carboxy prothrombin's production in hepatocellular carcinoma," *Panminerva Medica*, vol. 50, no. 3, pp. 221–226, 2008.

[14] G. Bertino, A. M. Ardiri, G. S. Calvagno et al., "Carbohydrate 19.9 antigen serum levels in liver disease," *BioMed Research International*, vol. 2013, Article ID 531640, 6 pages, 2013.

[15] C. Della Corte and M. Colombo, "Surveillance for hepatocellular carcinoma," *Seminars in Oncology*, vol. 39, no. 4, pp. 384–398, 2012.

[16] N. Bolog, G. Andreisek, I. Oancea, and A. Mangrau, "CT and MR imaging of hepatocellular carcinoma," *Journal of Gastrointestinal and Liver Diseases*, vol. 20, no. 2, pp. 181–189, 2011.

[17] J. M. Llovet, C. Brú, and J. Bruix, "Prognosis of hepatocellular carcinoma: the BCLC staging classification," *Seminars in Liver Disease*, vol. 19, no. 3, pp. 329–337, 1999.

[18] P. Giunchedi, M. Maestri, E. Gavini, P. Dionigi, and G. Rassu, "Transarterial chemoembolization of hepatocellular carcinoma-agents and drugs: an overview. Part 2," *Expert Opinion on Drug Delivery*, vol. 10, no. 6, pp. 799–810, 2013.

[19] P. M. Lopez, A. Villanueva, and J. M. Llovet, "Systematic review: evidence-based management of hepatocellular carcinoma—an updated analysis of randomized controlled trials," *Alimentary Pharmacology and Therapeutics*, vol. 23, no. 11, pp. 1535–1547, 2006.

[20] M. A. Wörns, A. Weinmann, M. Schuchmann, and P. R. Galle, "Systemic therapies in hepatocellular carcinoma," *Digestive Diseases*, vol. 27, no. 2, pp. 175–188, 2009.

[21] C. Huang, D. Xu, Q. Xia, P. Wang, C. Rong, and Y. Su, "Reversal of P-glycoprotein-mediated multidrug resistance of human hepatic cancer cells by Astragaloside II," *Journal of Pharmacy and Pharmacology*, vol. 64, no. 12, pp. 1741–1750, 2012.

[22] T. Yau, P. Chan, R. Epstein, and R. T. Poon, "Evolution of systemic therapy of advanced hepatocellular carcinoma," *World Journal of Gastroenterology*, vol. 14, no. 42, pp. 6437–6441, 2008.

[23] C. Verhoef, H. Van Dekken, L. J. Hofland et al., "Somatostatin receptor in human hepatocellular carcinomas: biological, patient and tumor characteristics," *Digestive Surgery*, vol. 25, no. 1, pp. 21–26, 2008.

[24] S. Becker, V. Ardisson, N. Lepareur et al., "Increased Lipiodol uptake in hepatocellular carcinoma possibly due to increased membrane fluidity by dexamethasone and tamoxifen," *Nuclear Medicine and Biology*, vol. 37, no. 7, pp. 777–784, 2010.

[25] J. Bruix and M. Sherman, "American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update," *Hepatology*, vol. 53, no. 3, pp. 1020–1022, 2011.

[26] J. M. Llovet, S. Ricci, V. Mazzaferro et al., "Sorafenib in advanced hepatocellular carcinoma," *New England Journal of Medicine*, vol. 359, no. 4, pp. 378–390, 2008.

[27] A. Villanueva, P. Newell, D. Y. Chiang, S. L. Friedman, and J. M. Llovet, "Genomics and signaling pathways in hepatocellular carcinoma," *Seminars in Liver Disease*, vol. 27, no. 1, pp. 55–76, 2007.

[28] A. Patel and W. Sun, "Molecular targeted therapy in hepatocellular carcinoma: from biology to clinical practice and future," *Current Treatment Options in Oncology*, 2014.

[29] S. Tanaka and S. Arii, "Molecular targeted therapies in hepatocellular carcinoma," *Seminars in Oncology*, vol. 39, no. 4, pp. 486–492, 2012.

[30] D. Y. Chiang, A. Villanueva, Y. Hoshida et al., "Focal gains of VEGFA and molecular classification of hepatocellular carcinoma," *Cancer Research*, vol. 68, no. 16, pp. 6779–6788, 2008.

[31] P. A. Farazi, J. Glickman, S. Jiang, A. Yu, K. L. Rudolph, and R. A. DePinho, "Differential impact of telomere dysfunction on initiation and progression of hepatocellular carcinoma," *Cancer Research*, vol. 63, no. 16, pp. 5021–5027, 2003.

[32] S.-A. Ng and C. Lee, "Hepatitis B virus X gene and hepatocarcinogenesis," *Journal of Gastroenterology*, vol. 46, no. 8, pp. 974–990, 2011.

[33] C. Miroux, T. Vausselin, and N. Delhem, "Regulatory T cells in HBV and HCV liver diseases: implication of regulatory T lymphocytes in the control of immune response," *Expert Opinion on Biological Therapy*, vol. 10, no. 11, pp. 1563–1572, 2010.

[34] D. Petrovic, Z. Stamataki, E. Dempsey et al., "Hepatitis C virus targets the T cell secretory machinery as a mechanism of immune evasion," *Hepatology*, vol. 53, no. 6, pp. 1846–1853, 2011.

[35] A. Villanueva, S. Toffanin, and J. M. Llovet, "Linking molecular classification of hepatocellular carcinoma and personalized medicine: preliminary steps," *Current Opinion in Oncology*, vol. 20, no. 4, pp. 444–453, 2008.

[36] S. Boyault, D. S. Rickman, A. De Reyniès et al., "Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets," *Hepatology*, vol. 45, no. 1, pp. 42–52, 2007.

[37] H. Huynh, T. T. Tuyen Nguyen, K.-H. Pierce Chow, P. H. Tan, K. C. Soo, and E. Tran, "Over-expression of the mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK in hepatocellular carcinoma: its role in tumor progression and apoptosis," *BMC Gastroenterology*, vol. 3, article 19, 2003.

[38] M. D. Desai, B. S. Saroya, and A. C. Lockhart, "Investigational therapies targeting the ErbB (EGFR, HER2, HER3, HER4) family in GI cancers," *Expert Opinion on Investigational Drugs*, vol. 22, no. 3, pp. 341–356, 2013.

[39] L. Goyal, M. D. Muzumdar, and A. X. Zhu, "Targeting the HGF/c-MET pathway in hepatocellular carcinoma," *Clinical Cancer Research*, vol. 19, no. 9, pp. 2310–2318, 2013.

[40] Y. Chao, C.-P. Li, G.-Y. Chau et al., "Prognostic significance of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin in patients with resectable hepatocellular carcinoma after surgery," *Annals of Surgical Oncology*, vol. 10, no. 4, pp. 355–362, 2003.

[41] R. T. P. Poon, J. W. Y. Ho, C. S. W. Tong, C. Lau, I. O. L. Ng, and S.-T. Fan, "Prognostic significance of serum vascular endothelial growth factor and endostatin in patients with hepatocellular carcinoma," *British Journal of Surgery*, vol. 91, no. 10, pp. 1354–1360, 2004.

[42] R. J. Epstein, "VEGF signaling inhibitors: more pro-apoptotic than anti-angiogenic," *Cancer and Metastasis Reviews*, vol. 26, no. 3-4, pp. 443–452, 2007.

[43] N. K. Saxena, D. Sharma, X. Ding et al., "Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells," *Cancer Research*, vol. 67, no. 6, pp. 2497–2507, 2007.

[44] J. M. Llovet and J. Bruix, "Molecular targeted therapies in hepatocellular carcinoma," *Hepatology*, vol. 48, no. 4, pp. 1312–1327, 2008.

[45] T. Ishiguro, M. Sugimoto, Y. Kinoshita et al., "Anti-glypican 3 antibody as a potential antitumor agent for human liver cancer," *Cancer Research*, vol. 68, no. 23, pp. 9832–9838, 2008.

[46] W. Piao, Y. Wang, Y. Adachi et al., "Insulin-like growth factor-I receptor blockade by a specific tyrosine kinase inhibitor for human gastrointestinal carcinomas," *Molecular Cancer Therapeutics*, vol. 7, no. 6, pp. 1483–1493, 2008.

[47] E. Unitt, A. Marshall, W. Gelson et al., "Tumour lymphocytic infiltrate and recurrence of hepatocellular carcinoma following liver transplantation," *Journal of Hepatology*, vol. 45, no. 2, pp. 246–253, 2006.

[48] T. Takayama, T. Sekine, M. Makuuchi et al., "Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial," *The Lancet*, vol. 356, no. 9232, pp. 802–807, 2000.

[49] R. Thimme, M. Neagu, T. Boettler et al., "Comprehensive analysis of the α-fetoprotein-specific CD8+ T cell responses in patients with hepatocellular carcinoma," *Hepatology*, vol. 48, no. 6, pp. 1821–1833, 2008.

[50] H. Komori, T. Nakatsura, S. Senju et al., "Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma," *Clinical Cancer Research*, vol. 12, no. 9, pp. 2689–2697, 2006.

[51] X.-Y. Shang, H.-S. Chen, H.-G. Zhang et al., "The spontaneous CD8+ T-cell response to HLA-A2-restricted NY-ESO-1b peptide in hepatocellular carcinoma patients," *Clinical Cancer Research*, vol. 10, no. 20, pp. 6946–6955, 2004.

[52] G. Bricard, H. Bouzourene, O. Martinet et al., "Naturally acquired MAGE-A10- and SSX-2-specific CD8+ T cell responses in patients with hepatocellular carcinoma," *Journal of Immunology*, vol. 174, no. 3, pp. 1709–1716, 2005.

[53] E. Mizukoshi, Y. Nakamoto, Y. Marukawa et al., "Cytotoxic T cell responses to human telomerase reverse transcriptase in patients with hepatocellular carcinoma," *Hepatology*, vol. 43, no. 6, pp. 1284–1294, 2006.

[54] E. Unitt, S. M. Rushbrook, A. Marshall et al., "Compromised lymphocytes infiltrate hepatocellular carcinoma: the role of T-regulatory cells," *Hepatology*, vol. 41, no. 4, pp. 722–730, 2005.

[55] B. Hoechst, L. A. Ormandy, M. Ballmaier et al., "A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)CD25(+)Foxp3(+) T cells," *Gastroenterology*, vol. 135, no. 1, pp. 234–243, 2008.

[56] K. Fujiwara, T. Higashi, K. Nouso et al., "Decreased expression of B7 costimulatory molecules and major histocompatibility complex class-I in human hepatocellular carcinoma," *Journal of Gastroenterology and Hepatology*, vol. 19, no. 10, pp. 1121–1127, 2004.

[57] K. Kurokohchi, M. Carrington, D. L. Mann et al., "Expression of HLA class I molecules and the transporter associated with antigen processing in hepatocellular carcinoma," *Hepatology*, vol. 23, no. 5, pp. 1181–1188, 1996.

[58] T. Tatsumi, T. Takehara, K. Katayama et al., "Expression of costimulatory molecules B7-1 (CD80) and B7-2 (CD86) on human hepatocellular carcinoma," *Hepatology*, vol. 25, no. 5, pp. 1108–1114, 1997.

[59] F. Shi, M. Shi, Z. Zeng et al., "PD-1 and PD-L1 upregulation promotes CD8+ T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients," *International Journal of Cancer*, vol. 128, no. 4, pp. 887–896, 2011.

[60] S. Behboudi, A. Alisa, S. Boswell, J. Anastassiou, A. A. Pathan, and R. Williams, "Expansion of anti-AFP Th1 and Tc1 responses in hepatocellular carcinoma occur in different stages of disease," *British Journal of Cancer*, vol. 102, no. 4, pp. 748–753, 2010.

[61] E. Breous and R. Thimme, "Potential of immunotherapy for hepatocellular carcinoma," *Journal of Hepatology*, vol. 54, no. 4, pp. 830–834, 2011.

[62] K. Teramoto, Y. Ohshio, T. Fujita, J. Hanaoka, and K. Kontani, "Simultaneous activation of T helper function can augment the potency of dendritic cell-based cancer immunotherapy," *Journal of Cancer Research and Clinical Oncology*, vol. 139, no. 5, pp. 861–870, 2013.

[63] J. M. O'Bryan, J. A. Potts, H. L. Bonkovsky, A. Mathew, and A. L. Rothman, "Extended interferon-alpha therapy accelerates telomere length loss in human peripheral blood T lymphocytes," *PLoS ONE*, vol. 6, no. 8, Article ID e20922, 2011.

[64] A. D. Pardee and L. H. Butterfield, "Immunotherapy of hepatocellular carcinoma: unique challenges and clinical opportunities," *Oncoimmunology*, vol. 1, no. 1, pp. 48–55, 2012.

[65] T. Stroffolini, A. Spadaro, V. Di Marco et al., "Current practice of chronic hepatitis B treatment in Southern Italy," *European Journal of Internal Medicine*, vol. 23, no. 5, pp. e124–e127, 2012.

[66] G. Bertino, A. Ardiri, P. M. Boemi et al., "Epoetin alpha improves the response to antiviral treatment in HCV-related chronic hepatitis," *European Journal of Clinical Pharmacology*, vol. 66, no. 10, pp. 1055–1063, 2010.

[67] F. Zhao, F. Korangy, and T. F. Greten, "Cellular immune suppressor mechanisms in patients with hepatocellular carcinoma," *Digestive Diseases*, vol. 30, no. 5, pp. 477–482, 2012.

[68] L. Cai, Z. Zhang, L. Zhou et al., "Functional impairment in circulating and intrahepatic NK cells and relative mechanism in hepatocellular carcinoma patients," *Clinical Immunology*, vol. 129, no. 3, pp. 428–437, 2008.

[69] M. El Ansary, S. Mogawer, S. A. Elhamid et al., "Immunotherapy by autologous dendritic cell vaccine in patients with advanced HCC," *Journal of Cancer Research and Clinical Oncology*, vol. 139, no. 1, pp. 39–48, 2013.

[70] B. Xie, D. H. Wang, and S. J. Spechler, "Sorafenib for treatment of hepatocellular carcinoma: a systematic review," *Digestive Diseases and Sciences*, vol. 57, no. 5, pp. 1122–1129, 2012.

[71] G. G. Di Costanzo, R. Tortora, L. Iodice et al., "Safety and effectiveness of sorafenib in patients with hepatocellular carcinoma in clinical practice," *Digestive and Liver Disease*, vol. 44, no. 9, pp. 788–792, 2012.

[72] A.-L. Cheng, Y.-K. Kang, Z. Chen et al., "Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial," *The Lancet Oncology*, vol. 10, no. 1, pp. 25–34, 2009.

[73] M. A. Wörns, M. Schuchmann, C. Düber, G. Otto, P. R. Galle, and A. Weinmann, "Sunitinib in patients with advanced hepatocellular carcinoma after progression under sorafenib treatment," *Oncology*, vol. 79, no. 1-2, pp. 85–92, 2010.

[74] A. X. Zhu, D. V. Sahani, E. di Tomaso et al., "Sunitinib monotherapy in patients with advanced heaptocellular carcinoma: insights from a multidisciplinary phase II study," *Journal of Clinical Oncology*, vol. 26, supplement, abstract 4521, 2008.

[75] D. Hoda, C. Catherine, J. Strosberg et al., "Phase II study of sunitinib malate in adult patients with metastatic or surgically unresectable hepatocellular carcinoma," *Gastrointestinal Cancers Symposium*, abstarct 267, 2008.

[76] H. Huynh, S. P. Choo, H. C. Toh et al., "Comparing the efficacy of sunitinib with sorafenib in xenograft models of human hepatocellular carcinoma: mechanistic explanation," *Current Cancer Drug Targets*, vol. 11, no. 8, pp. 944–953, 2011.

[77] C. M. Bagi, D. F. Gebhard, and C. J. Andresen, "Antitumor effect of vascular endothelial growth factor inhibitor sunitinib in preclinical models of hepatocellular carcinoma," *European Journal of Gastroenterology and Hepatology*, vol. 24, no. 5, pp. 563–574, 2012.

[78] R. Finn, "Emerging targeted strategies in advanced hepatocellular carcinoma," *Seminars in Liver Disease*, vol. 33, supplement 1, pp. S11–S19, 2013.

[79] H. C. Toh, P.-J. Chen, B. I. Carr et al., "Phase 2 trial of linifanib (ABT-869) in patients with unresectable or metastatic hepatocellular carcinoma," *Cancer*, vol. 119, no. 2, pp. 380–387, 2013.

[80] C. Cainap, S. Qin, W. T. Huang et al., "Phase III trial of linifanib versus sorafenib in patients with advanced hepatocellular carcinoma (HCC)," *Journal of Clinical Oncology*, vol. 31, supplement 4, abstract 249, 2013.

[81] E. Gherardi, W. Birchmeier, C. Birchmeier, and G. V. Woude, "Targeting MET in cancer: rationale and progress," *Nature Reviews Cancer*, vol. 12, no. 2, pp. 89–103, 2012.

[82] N. Munshi, S. Jeay, Y. Li et al., "ARQ 197, a novel and selective inhibitor of the human c-Met receptor tyrosine kinase with antitumor activity," *Molecular Cancer Therapeutics*, vol. 9, no. 6, pp. 1544–1553, 2010.

[83] S. Previdi, G. Abbadessa, F. Dalò, D. S. France, and M. Broggini, "Breast cancer-derived bone metastasis can be effectively reduced through specific c-MET inhibitor tivantinib (ARQ 197) and shRNA c-MET knockdown," *Molecular Cancer Therapeutics*, vol. 11, no. 1, pp. 214–223, 2012.

[84] A. Santoro, L. Rimassa, I. Borbath et al., "Tivantinib for second-line treatment of advanced hepatocellular carcinoma: a randomised, placebo-controlled phase 2 study," *The Lancet Oncology*, vol. 14, no. 1, pp. 55–63, 2013.

[85] A. Santoro, C. Porta, L. Rimassa et al., "Metiv-HCC: a phase III clinical trial evaluating tivantinib (ARQ 197), a MET inhibitor, versus placebo as second-line in patients (pts) with MET-high inoperable hepatocellular carcinoma (HCC)," *Journal of Clinical Oncology*, vol. 31, supplement, abstract TPS4159, 2013.

[86] H. Hurwitz, L. Fehrenbacher, W. Novotny et al., "Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer," *New England Journal of Medicine*, vol. 350, no. 23, pp. 2335–2342, 2004.

[87] R. Wang, N. Zhao, S. Li et al., "MicroRNA-195 suppresses angiogenesis and metastasis of hepatocellular carcinoma by inhibiting the expression of VEGF, VAV2, and CDC42," *Hepatology*, vol. 58, no. 2, pp. 642–653, 2013.

[88] T. Chou and R. S. Finn, "Brivanib: a review of development," *Future Oncology*, vol. 8, no. 9, pp. 1083–1090, 2012.

[89] R. S. Finn, "Overview and description of hepatocellular carcinoma," *Clinical Advances in Hematology and Oncology*, vol. 5, no. 4, pp. 4–7, 2013.

[90] R. S. Finn, Y.-K. Kang, M. Mulcahy et al., "Phase II, open-label study of brivanib as second-line therapy in patients with advanced hepatocellular carcinoma," *Clinical Cancer Research*, vol. 18, no. 7, pp. 2090–2098, 2012.

[91] J. M. Llovet, T. Decaens, J. L. Raoul et al., "Brivanib in patients with advanced hepatocellular carcinoma who were intolerant to sorafenib or for whom sorafenib failed: results from the randomized phase III BRISK-PS study," *Journal of Clinical Oncology*, vol. 31, no. 28, pp. 3509–3516, 2013.

[92] C. Verslype, A. L. Cohn, R. K. Kelley et al., "Activity of cabozantinib (XL184) in hepatocellular carcinoma: results from a Phase 2 randomized discontinuation trial (RDT)," *Journal of Clinical Oncology*, vol. 30, supplement, abstract 4007, 2012.

[93] A. X. Zhu, M. Kudo, M. Eric Assenat et al., "EVOLVE-1: phase 3 study of everolimus for advanced HCC that progressed during or after sorafenib," *Journal of Clinical Oncology*, vol. 32, supplement 3, abstract 172, 2014.

[94] J. J. Knox, R. Qin, and J. R. Strosberg, "A phase II trial of temsirolimus (TEM) and bevacizumab (BEV) in patients with advanced hepatocellular carcinoma (HCC)," *Journal of Clinical Oncology*, vol. 30, supplement, abstract 4099, 2012.

[95] J.-H. Zhong, L. Ma, L.-C. Wu et al., "Adoptive immunotherapy for postoperative hepatocellular carcinoma: a systematic review," *International Journal of Clinical Practice*, vol. 66, no. 1, pp. 21–27, 2012.

[96] W.-C. Lee, H.-C. Wang, C.-F. Hung, P.-F. Huang, C.-R. Lia, and M.-F. Chen, "Vaccination of advanced hepatocellular carcinoma patients with tumor lysate-pulsed dendritic cells: a clinical trial," *Journal of Immunotherapy*, vol. 28, no. 5, pp. 496–504, 2005.

[97] H. Kayashima, T. Toshima, S. Okano et al., "Intratumoral neoadjuvant immunotherapy using IL-12 and dendritic cells is an effective strategy to control recurrence of murine hepatocellular carcinoma in immunosuppressed mice," *Journal of Immunology*, vol. 185, no. 1, pp. 698–708, 2010.

[98] N. Schmidt, C. Neumann-Haefelin, and R. Thimme, "Cellular immune responses to hepatocellular carcinoma: lessons for immunotherapy," *Digestive Diseases*, vol. 30, no. 5, pp. 483–491, 2012.

[99] E. Mizukoshi, Y. Nakamoto, K. Arai et al., "Comparative analysis of various tumor-associated antigen-specific t-cell responses in patients with hepatocellular carcinoma," *Hepatology*, vol. 53, no. 4, pp. 1206–1216, 2011.

[100] K. Sakuishi, L. Apetoh, J. M. Sullivan, B. R. Blazar, V. K. Kuchroo, and A. C. Anderson, "Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity," *Journal of Experimental Medicine*, vol. 207, no. 10, pp. 2187–2194, 2010.

[101] A. D. Pardee, D. McCurry, S. Alber, P. Hu, A. L. Epstein, and W. J. Storkus, "A therapeutic OX40 agonist dynamically alters dendritic, endothelial, and T cell subsets within the established tumor microenvironment," *Cancer Research*, vol. 70, no. 22, pp. 9041–9052, 2010.

[102] K. M. Kee, C. H. Hung, J. H. Wang, and S. N. Lu, "Serial changes of clinical parameters in a patient with advanced hepatocellular carcinoma with portal vein thrombosis achieving complete response after treatment with sorafenib," *OncoTargets and Therapy*, vol. 7, pp. 829–834, 2014.

[103] A. X. Zhu, D. G. Duda, D. V. Sahani, and R. K. Jain, "HCC and angiogenesis: possible targets and future directions," *Nature Reviews Clinical Oncology*, vol. 8, no. 5, pp. 292–301, 2011.

[104] J. Heo, T. Reid, L. Ruo et al., "Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer," *Nature Medicine*, vol. 19, no. 3, pp. 329–336, 2013.

[105] W. Wang, L. Liu, H. Chen et al., "EASL- and mRECIST-evaluated responses to combination therapy predict survival in patients with hepatocellular carcinoma," *Clinical Cancer Research*, 2014.

[106] F. Y. Yao, "Conundrum of treatment for early-stage hepatocellular carcinoma: radiofrequency ablation instead of liver transplantation as the first-line treatment?" *Liver Transplantation*, vol. 20, no. 3, pp. 257–260, 2014.

[107] N. Mehta, J. L. Dodge, J. P. Roberts, R. Hirose, and F. Y. Yao, "Outcome after liver transplantation in patients with hepatocellular carcinoma with a low risk of dropout from the transplant waiting list," *Liver Transplantation*, 2014.

[108] G. K. Abou-Alfa, "Sorafenib use in hepatocellular carcinoma: more questions than answers," *Hepatology*, 2014.

[109] T. Arizumi, K. Ueshima, H. Takeda et al., "Comparison of systems for assessment of post-therapeutic response to sorafenib for hepatocellular carcinoma," *Journal of Gastroenterology*, 2014.

[110] G. Bertino, I. Di Carlo, A. Ardiri et al., "Systemic therapies in hepatocellular carcinoma: present and future," *Future Oncology*, vol. 9, no. 10, pp. 1533–1548, 2013.

[111] G. Bertino, S. Demma, N. Bertino, and A. Ardiri, "Management of hepatocellular carcinoma: an update at the start of 2014," *Journal of Gastrointestinal & Digestive System*, vol. 4, no. 2, pp. 1–7, 2014.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 832712, 9 pages
http://dx.doi.org/10.1155/2014/832712

Research Article

A Critical Role for ZDHHC2 in Metastasis and Recurrence in Human Hepatocellular Carcinoma

Chuanhui Peng,[1] Zhijun Zhang,[2] Jian Wu,[1,2] Zhen Lv,[1] Jie Tang,[2] Haiyang Xie,[1,2] Lin Zhou,[1,2] and Shusen Zheng[1,2]

[1] *Key Laboratory of Combined Multi-Organ Transplantation, Ministry of Public Health, Hangzhou, Zhejiang 310003, China*
[2] *Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310003, China*

Correspondence should be addressed to Lin Zhou; linzhou19@163.com and Shusen Zheng; shusenzheng@zju.edu.cn

Received 28 March 2014; Accepted 22 May 2014; Published 9 June 2014

Academic Editor: Youmin Wu

It has been demonstrated that loss of heterozygosity (LOH) was frequently observed on chromosomes 8p22-p23 in hepatocellular carcinoma (HCC) and was associated with metastasis and prognosis of HCC. However, putative genes functioning on this chromosomal region remain unknown. In this study, we evaluated LOH status of four genes on 8p22-p23 (MCPH1, TUSC3, KIAA1456, and ZDHHC2). LOH on ZDHHC2 was associated with early metastatic recurrence of HCC following liver transplantation and was correlated with tumor size and portal vein tumor thrombi. Furthermore, our results indicate that ZDHHC2 expression was frequently decreased in HCC. Overexpression of ZDHHC2 could inhibit proliferation, migration, and invasion of HCC cell line Bel-7402 *in vitro*. These results suggest an important role for ZDHHC2 as a tumor suppressor in metastasis and recurrence of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common human malignancies worldwide, with a high rate of incidence and mortality [1, 2]. Over the past decades, many efforts have been made to improve the overall survival rate of HCC, of which both liver transplantation (LT) and surgical resection are considered potentially curative treatments for early-stage HCC. However, the long-term survival of HCC patients after surgery still remains poor due to the frequent metastasis and recurrence. Therefore, it is essential to explore the molecular pathogenesis of HCC metastasis and recurrence and detect novel molecular targets in HCC.

Loss of heterozygosity (LOH) refers to the loss of one of the two alleles at one or more loci in a heterozygote [3]. LOH in a chromosomal fragment implies the presence of putative tumor suppressor genes (TSGs) and can be used as an indirect way to identify TSGs [4]. Inactivation of TSGs was thought to be correlated with the metastasis and recurrence of primary cancer [5, 6]. Previous reports have revealed that frequent allelic losses on chromosomes 1p, 4q, 6q, 8p, 9p, 10q, 11p, 13q, 14q, 16q, and 17p are commonly observed in HCC patients [3, 4, 7–10]. Notably, loss of 8p is one of the most frequent chromosomal alterations in a variety of cancers including HCC [11–16]. Several studies have demonstrated that deletions of allele on 8p22-p23 are associated with metastasis and prognosis of HCC [7, 11, 17]. This suggests that 8p22-p23 might harbor one or more TSGs that are important in the progression, especially in the metastasis and recurrence of HCC. Until now, only few critical TSGs have been found on 8p22-p23, and it is necessary to investigate the LOH status of candidate genes in order to identify TSGs within this region. Additionally, studies about the roles of LOH in progression and prognosis of HCC in patients with LT are limited in number.

In this study, we selected the four most noteworthy genes on 8p22-p23 (MCPH1, TUSC3, KIAA1456, and ZDHHC2) to reveal the potential relationships between LOH of these genes

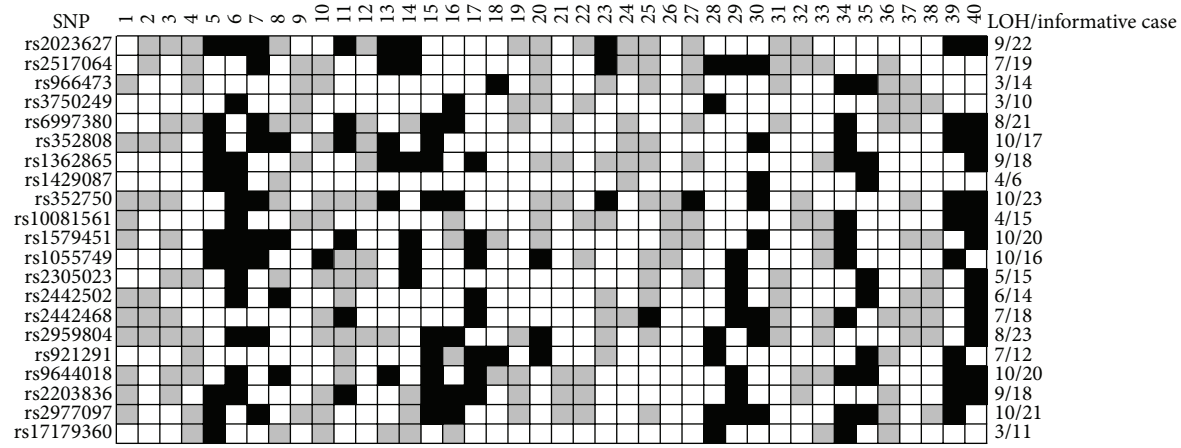


Figure 1: Status and frequency of LOH on each SNP in 40 HCC cases. Black box: LOH; gray box: retention; white box: noninformative case.

and the clinical characteristics in HCC after LT. We found that the LOH on ZDHHC2 was associated with early metastatic recurrence of HCC following LT and was correlated with tumor size and portal vein tumor thrombi (PVTT). Further study provides the evidence that ZDHHC2 has important role as a tumor suppressor in metastasis and recurrence of HCC.

2. Materials and Methods

2.1. Clinical Specimens. This study involved three independent cohorts of HCC patients. All 3 cohorts were from the First Affiliated Hospital, Zhejiang University School of Medicine, Zhejiang, China. Cohort 1 was a total of 40 randomly selected HCC patients with LT performed during 2006 and 2009; cohort 2 and cohort 3 were, respectively, a total of 55 and 23 HCC patients following partial hepatectomy performed during 2010 and 2011. This study was approved by the Ethical Review Committee of the First Affiliated Hospital, Zhejiang University School of Medicine, and informed consent was obtained according to the Declaration of Helsinki.

2.2. Sample Collection. Primary HCC and adjacent normal liver tissues were obtained during the operation. All tissue samples were immediately cut into small pieces, snap-frozen in liquid nitrogen, and stored at −80°C. Diagnosis for all samples was confirmed by histopathological examination. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions.

2.3. SNP-Based LOH Analysis. Five SNPs (rs2023627, rs2517064, rs966473, rs3750249, and rs6997380) that resided in ZDHHC2, six SNPs (rs352808, rs1362865, rs1429087, rs352750, rs10081561, and rs1579451) in TUSC3, six SNPs (rs1055749, rs2305023, rs2442502, rs2442468, rs2959804, and rs921291) in MCPH1, and four SNPs (rs9644018, rs2203836, rs2977097, and rs17179360) in KIAA1456 were selected to determine LOH. Sequences of these SNPs were obtained from the Human SNP Database. The methods for multiplex polymerase chain reaction (PCR) and LOH analysis were described previously [3], and SNPs were amplified under the same PCR conditions as microsatellite markers. Information for each SNP on these candidates is shown in Figure 1. LOH was defined as the value of LOH index within a range between 0.7 and 1.5 [18]. LOH on a single gene was determined to be positive when one or more SNPs on each gene detected LOH.

2.4. qRT-PCR. Total RNA from HCC samples was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions; then cDNA was synthesized. qPCR reactions were performed by the ABI7500 system (Applied Biosystems, CA) and SYBR green dye (TaKaRa Biotechnology, Dalian, China). GAPDH was used as an internal control. The primers' sequences used in this study were listed in Supplementary Table 1 available online at http://dx.doi.org/10.1155/2014/832712. Every sample was tested in duplicate.

2.5. Immunohistochemistry. Immunohistochemistry (IHC) of paraffin-embedded tissue sections was performed using the antibody ZDHHC2 (AP5592a, Abgent, CA, USA) as we have previously described [19]. Evaluation and semiquantitative estimation of IHC results were performed independently by two pathologists without knowledge of the clinicopathological outcomes. Intensity was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining). The abundance of positive cells was graded from 0 to 4 (0, <5% positive cells; 1, 5–25%; 2, 26–50%; 3, 51–75%; 4, >75%). The composite score obtained by multiplying the two values was analyzed.

2.6. Cell Culture. Normal liver cell line L-02; liver cancer cell lines Hep3B, HuH-7, and Bel-7402; and the metastasis-capable human HCC cell lines MHCC97L and HCCLM3 were purchased from American Type Culture Collection (Manassas, VA), Shanghai Institute of Cell Biology (Shanghai, China), and Liver Cancer Institute of Fudan University

(Shanghai, China). All of the cell lines were maintained in the recommended culture condition and incubated at 37°C humidified environment containing 5% CO_2.

2.7. Overexpression of ZDHHC2. The plasmid of the coding sequence (CDS) length ZDHHC2 with EGFP-tag and the negative control plasmid only containing EGFP-tag were completed by the Genechem Company (Shanghai, China). A PCR-amplified CDS-length ZDHHC2 fragment was inserted into the XhoI/KpnI sites of GV230 vector (Genechem, Shanghai, China), using the primers forward, 5′-TCCGCTCGAGATGGCGCCCTCGGGCC-3′ and reverse, 5′-ATGGGGTACCGTAGTCTCATTTTCCATGGTTAA-TG-3′. Transfection of the vectors was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

2.8. Western Blotting Analysis. Protein extraction and western blotting analysis were performed as previously described [20]. The following antibodies were used: anti-ZDHHC2, SantaCruz (sc-292338); anti-b-actin, Sigma-Aldrich (A1978); anti-GFP-Tag, Abmart (M20004).

2.9. Cell Proliferation Assay. Cell growth was determined by Cell Counting Kit-8 (CCK-8) cell proliferation assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions as previously described [21].

2.10. Transwell Migration and Invasion Assay. For invasion assay, 48 hours after transfection, $5 \times 10E4$ Bel-7402 cells in serum-free RPMI1640 were seeded into the upper chambers of each well (24-well insert, 8-mm pore size, Millipore, Billerica, MA, USA) coated with Matrigel (BD Bioscience, San Jose, CA, USA). For migration assay, the upper chambers were not coated with Matrigel, and cells were seeded after 24-hour transfection. RPMI1640 containing 10% FBS was placed in the lower chambers as a chemoattractant. After 24 hours of incubation, cells on the upper membrane surface were wiped off, and the cells that invaded across the Matrigel membrane were fixed with 100% methanol and stained with 0.2% crystal violet. The number of invasive cells was then counted (five randomly chosen high-power fields for each membrane) under a microscope.

2.11. Statistical Analysis. Comparisons between LOH and clinicopathological data were analyzed using chi-square test or Fisher exact test. Cumulative recurrence-free survival was assessed by Kaplan-Meier method and univariate analysis by log-rank test. Variables were brought into multivariate analysis when there was statistical significance in univariate analysis. Multivariate analysis was assessed using Cox proportional hazard model to identify variables that were independent predictors of clinical outcome. Independent Student's t-test was used to analyze the differences between two groups. Data are presented as the mean±SD, unless otherwise indicated. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with the use of SPSS version 16.0 software (SPSS, Chicago, IL).

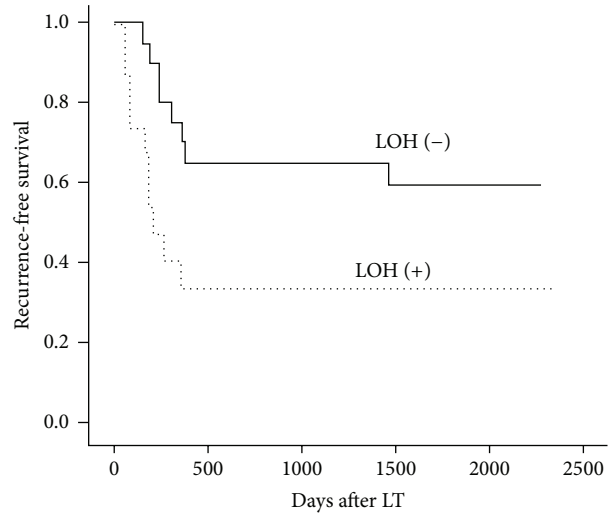


Figure 2: Kaplan-Meier analysis and log-rank test of the recurrence-free survival according to the LOH status of ZDHHC2 in 38 informative cases.

3. Results

3.1. LOH of ZDHHC2 Associates with Recurrence of Hepatocellular Carcinoma following Liver Transplantation. DNA from forty individuals with HCC who had received a liver transplants was analyzed for twenty-one SNPs of these four specific genes. Their mean heterozygosity was 87.5%. The heterozygosity on ZDHHC2, MCPH1, TUSC3, and KIAA1456 was correspondingly 95% (38 of 40), 92.5% (37 of 40), 85% (34 of 40), and 77.5% (31 of 40). Distribution of LOH on each SNP is shown in Figure 1. The LOH frequencies at all selected genes were so high, with a mean value of 50.3%. The frequencies of LOH on ZDHHC2, MCPH1, TUSC3, and KIAA1456 were 45% (17 of 38), 54% (20 of 37), 50% (17 of 34), and 52% (16 of 31), respectively.

To study the association of LOH on each gene with clinicopathological characteristics and to explore its potential biological role in HCC initiation, development, and progression, we compared frequencies of LOH based on clinicopathological findings including age, preoperative serum AFP level, tumor number, tumor size, PVTT and histopathologic grading. All of the results between clinicopathological features and LOH in HCC were shown in Table 1.

To determine the association between gene LOH status and prognostic data, we performed univariate and multivariate survival analysis. As a result, we found that the 1-year cumulative recurrence-free survival in HCC patients with LOH on ZDHHC2 was significantly lower than that with heterozygosity retention ($P = 0.022$, Figure 2). However, there were no significant differences between recurrence-free survival and LOHon MCPH1 ($P = 0.320$), TUSC3 ($P = 0.546$), and KIAA1456 ($P = 0.564$). Furthermore,

Table 1: Clinicopathological correlation with LOH in HCC cases (cohort 1).

Parameters	Genes	LOH/informative case		P value
Age		≤50 year	>50 year	
	ZDHHC2	12/17	5/21	0.004
	TUSC3	10/16	7/18	0.169
	MCPH1	11/17	9/20	0.231
	KIAA1456	10/16	6/15	0.210
Preoperative AFP level		≤400 ng/mL	>400 ng/mL	
	ZDHHC2	4/15	13/23	0.070
	TUSC3	5/12	12/22	0.473
	MCPH1	6/14	14/23	0.286
	KIAA1456	5/11	11/20	0.611
Tumor number		Single	Multiple	
	ZDHHC2	4/15	13/23	0.070
	TUSC3	5/12	12/22	0.473
	MCPH1	4/14	16/23	0.015
	KIAA1456	4/11	12/20	0.208
Tumor size		≤5 cm	>5 cm	
	ZDHHC2	6/23	11/15	0.004
	TUSC3	9/22	8/12	0.151
	MCPH1	9/22	11/15	0.052
	KIAA1456	8/18	8/13	0.347
PVTT		Absent	Present	
	ZDHHC2	11/31	6/7	0.031
	TUSC3	14/29	3/5	1.000
	MCPH1	14/30	6/7	0.097
	KIAA1456	11/24	5/7	0.394
Histopathologic grading		Well + moderately	Poorly	
	ZDHHC2	5/16	12/22	0.154
	TUSC3	3/11	14/23	0.067
	MCPH1	9/16	11/21	0.815
	KIAA1456	5/14	11/17	0.108

AFP: alpha-fetoprotein; PVTT: portal vein tumor thrombi.

based on the univariate analysis, we investigate the relationship between 1-year cumulative recurrence-free survival and clinicopathological factors. Taken together, significant associations were found between HCC recurrence and the following variables: LOH on ZDHHC2, preoperative AFP level >400 ng/mL, tumor size >5 cm, and presence of PVTT (Table 2). Factors which were found to have prognostic value including the clinicopathologic characteristics and gene LOH status in the univariate analysis were added into the corresponding multivariate Cox model of survival, and only PVTT was identified as independent prognostic factor for recurrence-free survival in HCC patients after LT (Table 3).

3.2. Decreased Expression of ZDHHC2 Is Observed in HCC Tissue Samples and HCC Cell Lines. To investigate the expression of ZDHHC2 in tumor tissues and peritumor tissues from HCC samples, we conducted qRT-PCR in cohort 2 of 55 HCC samples and IHC in cohort 3 of 23 HCC samples. We found that mRNA level of ZDHHC2 expression is significantly lower in tumor tissues than peritumor tissues from the same patients ($P = 0.003$) (Figure 3(a)). We analyzed the correlation between ZDHHC2 mRNA expression and clinical parameters including age, preoperative serum AFP level, tumor number, tumor size, PVTT, and histopathologic grading. In accord with the correlation of LOH of ZDHHC2 and clinical parameters, lower expression of ZDHHC2 was associated with the tumor size and the presence of PVTT (Supplementary Table 2).

Furthermore, by multiplying the values of staining intensity and relative abundance of positive cells, we found that the mean staining score of ZDHHC2 in tumor tissues is significantly lower than those in peritumor tissues ($P = 0.015$) (Figure 3(b)). The representative pictures of tumors and peritumors are shown in Figures 3(c) and 3(d).

Moreover, ZDHHC2 expression level in HCC cell lines was detected by western blotting. Compared to normal liver cell line L-02, ZDHHC2 expression levels in HCC cell lines (Hep3B, HuH-7, Bel-7402, MHCC97L, and HCCLM3) were significantly lower (Figure 4).

3.3. Overexpression of ZDHHC2 Inhibits Cell Proliferation and Invasion in BEL-7402 In Vitro. To determine whether

Table 2: Univariate analysis of HCC recurrence.

Variables	*n*	1-year cumulative recurrence rate (%)	*P* value
LOH on ZDHHC2			
Positive	17	66.7 ± 12.2	0.022
Negative	11	30.0 ± 10.2	
Preoperative AFP level			
≤400 ng/mL	15	15.4 ± 10.0	0.005
>400 ng/mL	25	62.5 ± 9.9	
Tumor size			
≤5 cm	25	27.3 ± 9.5	0.001
>5 cm	15	73.3 ± 11.4	
PVTT			
Absent	33	35.5 ± 8.6	<0.001
Present	7	100	

AFP: alpha-fetoprotein; PVTT: portal vein tumor thrombi.

Table 3: Multivariate analysis of HCC recurrence.

Variables	Hazard ratio	95% confidence interval	*P* value
LOH on ZDHHC2	1.263	0.405–3.935	0.687
AFP >400 ng/mL	4.156	0.864–19.994	0.076
Tumor size >5 cm	1.615	0.430–6.063	0.478
PVTT (+)	3.468	1.004–11.982	0.049

AFP: alpha-fetoprotein; PVTT: portal vein tumor thrombi.

reduction of ZDHHC2 affects biological function in HCC and overexpression of ZDHHC2 could be used as potential consideration to treat HCC, further experimental studies were then performed. Overexpression vector and negative control vector are transiently transfected into Bel-7402 HCC cell line, then downstream analysis was performed. Transient transfection was successfully performed as shown in western blotting analysis (Figure 5). ZDHHC2 overexpression lasts stably at 24-, 48-, and 72-hour time points.

CCK-8 cell proliferation assay and transwell migration and invasion assay were conducted in Bel-7402 without any treatment (BLANK), transiently transfected with negative control vector (NC), and transiently transfected with overexpression vector (ZDHHC2). The results showed that overexpression of ZDHHC2 significantly impaired the proliferation (Figure 6(a)) and migratory and invasive capacity of Bel-7402 cell (Figures 6(b) and 6(c)) compared to both BLANK group and NC group.

4. Discussion

HCC is a frequent and highly aggressive malignancy worldwide with very poor prognosis. Recently, LT has been recognized as a potential curative treatment for early-stage HCC patients. However, the clinical outcome remains challenging because of a high incidence of tumor recurrence after LT [22]. Recurrence is principally attributable to the presence of microscopic extrahepatic metastatic foci before LT [23], which is also known as metastastic recurrence. A better understanding of the underlying molecular mechanisms governing cancer metastastic recurrence will be a great help for predicting prognosis.

Numerous studies have revealed a high-LOH frequency on 8p22-p23 in HCC [7, 9, 17, 24]. Our previous report has also supported [3]. This implies that one or several TSGs may lie within this region. Therefore, the genes on 8p22-p23 are widely investigated and several new genes have been identified as TSGs in recent years, such as CUB and Sushi multiple domains 1 (CSMD1) [9], deleted in liver cancer 1 (DLC1) [25] and mitochondrial tumour suppressor 1 (MTUS1) [26].

In the current study, we confirmed that LOH was a prevalent event on 8p22-p23 in HCC, with frequencies of LOHs on four specific genes (MCPH1, KIAA1456, TUSC3, and ZDHHC2) ranging from 45% to 54%. Interestingly, LOH on ZDHHC2 was clearly associated with early metastastic recurrence of HCC following LT, although it had the lowest LOH frequency of these four genes. In all 38 informative cases, patients with LOH had an increasing risk of early metastastic recurrence post-LT, which directly linked to patients' long-term survival. Multivariate analyses revealed that LOH on ZDHHC2 could predict the risk of HCC early recurrence together with AFP level, tumor size, and PVTT but was not an independent prognostic factor. In addition, our data showed that LOH status of ZDHHC2 was correlated with some clinicopathological parameters, including tumor size and PVTT. These data suggested that LOH on ZDHHC2 may serve as a molecular event in advanced HCC, as tumor size >5 cm and PVTT were representatives of advancement of HCC.

ZDHHC2 was originally described as REAM (reduced expression in metastasis), because its silence was associated with increased metastatic potential of cancer cells [27]. It was reported that reduced ZDHHC2 expression is observed in gastric adenocarcinoma patients and associated with lymph node metastasis and independently predicts an unfavorable prognosis [28]. However, the expression level of ZDHHC2 in HCC remains to be explored. Our study provides the evidence that ZDHHC2 expression was frequently decreased in HCC. Overexpression of ZDHHC2 could inhibit proliferation, migration, and invasion of HCC cell line Bel-7402 *in vitro*. These results suggest an important role for ZDHHC2 as a tumor suppressor in metastasis and recurrence of HCC.

DHHC2, encoded by ZDHHC2, is one member of a family of more than 20 palmitoyl acyltransferases (PATs) characterized by an Asp-His-His-Cys (DHHC) motif [29]. Protein palmitoylation, mediated by PATs, can affect proteins in many ways, including regulating membrane attachment, subcellular trafficking, and membrane microlocalisation [29]. Many DHHC2-substrate interactions have been identified so far, including PSD95, SNAP25, SNAP23, CKAP4, CD9, and CD151 [29]. The decrease or absence of DHHC2 expression could influence the palmitoylation of these substrates, and whatever role palmitoylation had in signaling downstream from that event would be disrupted. Of these substrates, CKAP4, CD9, and CD151 have been associated with initiation

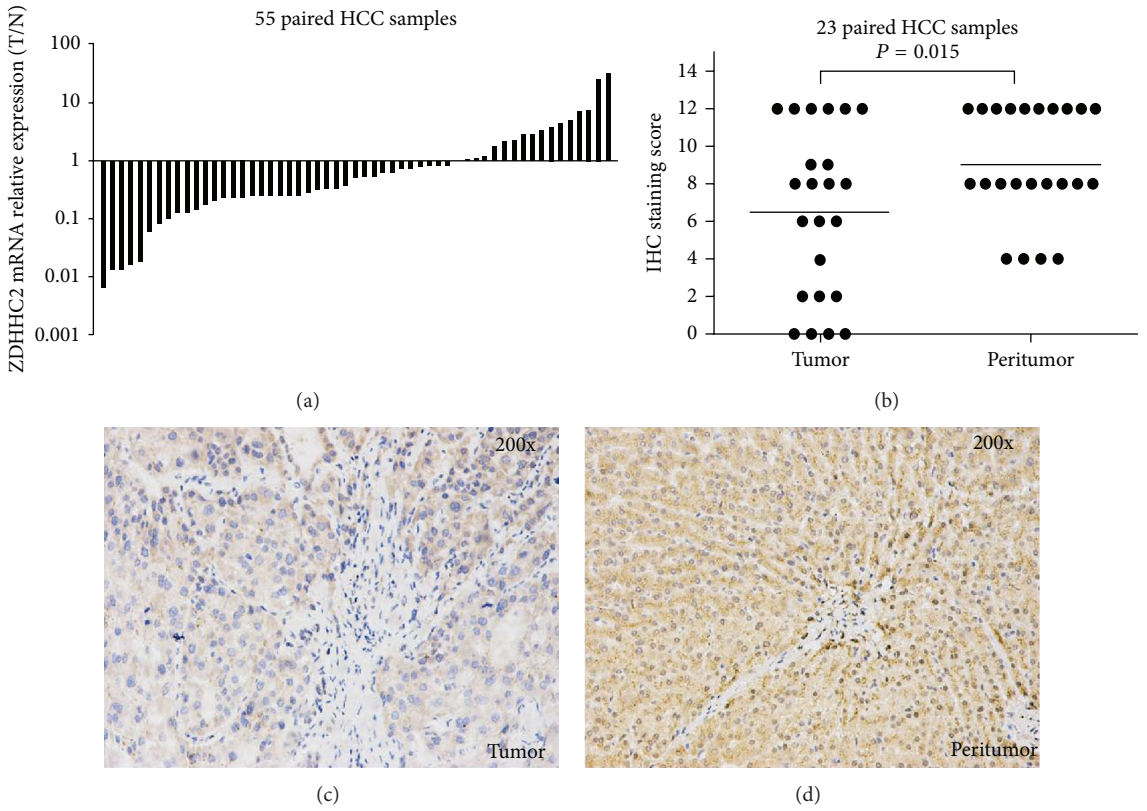


Figure 3: Frequently decreased expression of ZDHHC2 in human HCC tissues. (a) In cohort 2 (55 paired HCC tissue samples), the expression levels of ZDHHC2 mRNA are significantly lower in tumor tissues than peritumor tissues from the same patients ($P = 0.003$). (b) Relative IHC staining of ZDHHC2 expression in paired HCC tissue samples (cohort 3, $n = 23$). The ZDHHC2 expression level was significantly downregulated in tumors compared with the corresponding adjacent nontumor liver tissues ($P = 0.015$). (c) and (d) are preventative pictures, respectively (200x).

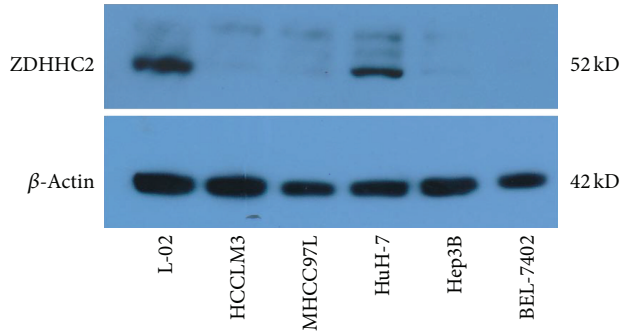


Figure 4: ZDHHC2 expression levels in HCC cell lines were detected by western blot. Compared to normal liver cell line L-02, ZDHHC2 expression level in HCC cell lines was significantly lower.

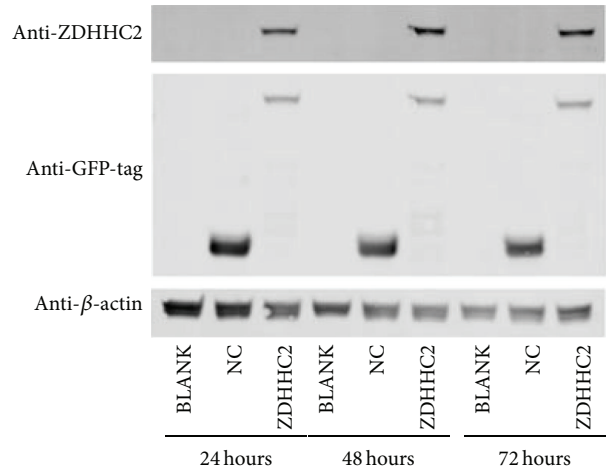


Figure 5: Plasmids containing ZDHHC2-EGFP or EGFP were transfected into Bel-7402. The transfection was monitored by western blotting analysis at the 24-, 48-, and 72-hour time points. Bel-7402 without any treatment (BLANK), transiently transfected with negative control vector (NC), and transiently transfected with overexpression vector (ZDHHC2).

and progression of cancer. Decreased expression of DHHC2 leads to reduced palmitoylation of CKAP4, making it no longer traffick efficiently to the cell surface where it act as a receptor for antiproliferative factor (APF) [30, 31]. Therefore, the ability of APF to halt cell proliferation and suppress the expression of genes involved in tumorigenesis is inhibited. Moreover, DHHC2 could stimulate palmitoylation of tetraspanins CD9 and CD151 [32]. CD9 is a potential tumor suppressor [33], while CD151 is supposed to promote tumor metastasis [34]. Our data together with these previous results support the notion that DHHC2 and its substrates may play an important role in the process of development, metastasis, and recurrence in various cancers including HCC. Despite

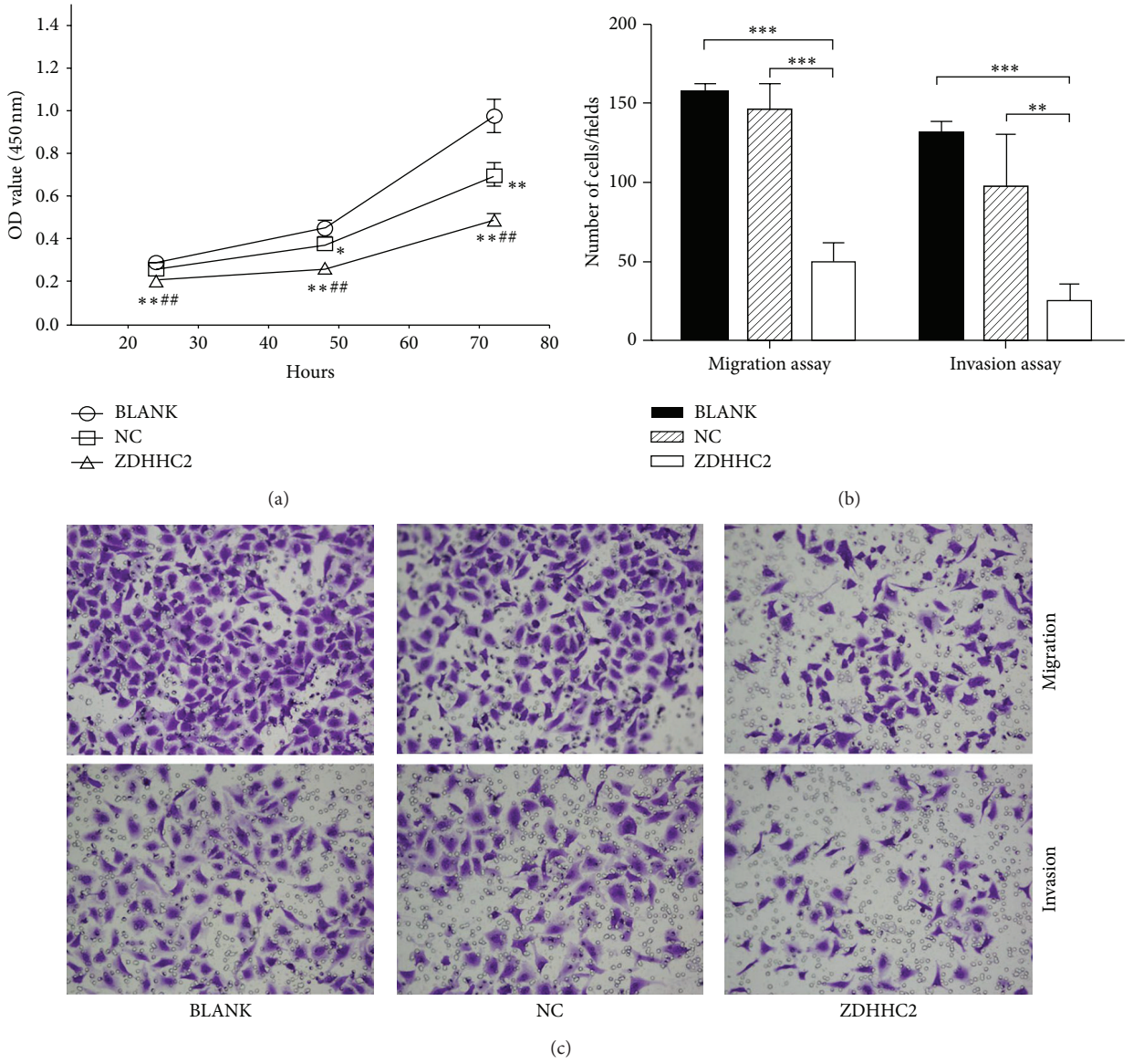


Figure 6: CCK-8 cell proliferation assay and transwell migration and invasion assay were conducted in Bel-7402 without any treatment (BLANK), transiently transfected with negative control vector (NC), and transiently transfected with overexpression vector (ZDHHC2). (a) Overexpression of ZDHHC2 significantly impaired Bel-7402 proliferation ($n = 3$, $^{*}P < 0.05$ compared to BLANK group, $^{**}P < 0.01$ compared to BLANK group, and $^{\#\#}P < 0.01$ compared to NC group). (b) Overexpression of ZDHHC2 inhibits the migration and invasion of BEL-7402 cell ($n = 5$, $^{**}P < 0.01$, and $^{***}P < 0.001$) compared to both BLANK group and NC group. Representative pictures are shown in (c).

several substrates have been identified, it is possible that more functional substrates remain unknown. Accordingly, our further study should focus on searching novel substrates and investigating the biological function of DHHC2 and its substrates in HCC, which may well provide novel and important targets for pharmacological intervention in the progression of HCC.

5. Conclusions

LOH was frequent on ZDHHC2, MCPH1, TUSC3, and KIAA1456 in human HCC. Of these, LOH on ZDHHC2 might contribute to early metastatic recurrence of HCC after LT. Reduced expression of ZDHHC2 was detected in HCC and associates with biological function of HCC such as proliferation, migration, and invasion. These results suggest an important role for ZDHHC2 as a tumor suppressor in metastasis and recurrence of HCC.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Chuanhui Peng and Zhijun Zhang contributed equally to this work.

Acknowledgments

This study was supported by National S&T Major Project (no. 2012ZX10002-017), NSFC for Innovative Research Group (no. 81121002), National High Technology Research and Development Program 863 of China (no. 2012AA021002), and Special Fund for Health Research in the Public Welfare (201302009). The authors thank Penghong Song, Kezhen Shen, Xiaowen Feng, Wenfeng Song, Guoqiang Cao, Leiming Chen, and Rong Su for their valuable technical assistance.

References

[1] H. B. El-Serag and K. L. Rudolph, "Hepatocellular carcinoma: epidemiology and molecular carcinogenesis," *Gastroenterology*, vol. 132, no. 7, pp. 2557–2576, 2007.

[2] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[3] L. Zhou, W. Zhou, L. Wu, X. Yu, C. Xing, and S. Zheng, "The association of frequent allelic loss on 17p13.1 with early metastastic recurrence of hepatocellular carcinoma after liver transplantation," *Journal of Surgical Oncology*, vol. 102, no. 7, pp. 802–808, 2010.

[4] S. Li, H. Wang, J. Li et al., "Genome-wide analyses on loss of heterozygosity in hepatocellular carcinoma in Southern China," *Journal of Hepatology*, vol. 34, no. 6, pp. 840–849, 2001.

[5] A. Schwendel, H. Langreck, M. Reichel et al., "Primary small-cell lung carcinomas and their metastases are characterized by a recurrent pattern of genetic alterations," *International Journal of Cancer*, vol. 74, no. 1, pp. 86–93, 1997.

[6] U. Bockmühl, S. Petersen, S. Schmidt et al., "Patterns of chromosomal alterations in metastasizing and nonmetastasizing primary head and neck carcinomas," *Cancer Research*, vol. 57, no. 23, pp. 5213–5216, 1997.

[7] T. Lu and H. Hano, "Identification of minimal regions of deletion at 8p23.1-22 associated with metastasis of hepatocellular carcinoma," *Liver International*, vol. 27, no. 6, pp. 782–790, 2007.

[8] T. Okuno, M. Ueda, T. Tsuruyama et al., "Loss of heterozygosity on 10q23 is involved in metastatic recurrence of hepatocellular carcinoma," *Cancer Science*, vol. 100, no. 3, pp. 520–528, 2009.

[9] Y. Midorikawa, S. Yamamoto, S. Tsuji et al., "Allelic imbalances and homozygous deletion on 8p23.2 for stepwise progression of hepatocarcinogenesis," *Hepatology*, vol. 49, no. 2, pp. 513–522, 2009.

[10] Y. W. Chou, K. Chung, L. Jeng, T. Chen, and Y. Liaw, "Frequent allelic loss on chromosomes 4q and 16q associated with human hepatocellular carcinoma in Taiwan," *Cancer Letters*, vol. 123, no. 1, pp. 1–6, 1998.

[11] M. Emi, Y. Fujiwara, H. Ohata et al., "Allelic loss at chromosome band 8p21.3-p22 is associated with progression of hepatocellular carcinoma," *Genes Chromosomes and Cancer*, vol. 7, no. 3, pp. 152–157, 1993.

[12] Y. Fujiwara, H. Ohata, M. Emi et al., "A 3-Mb physical map of the chromosome region 8p21.3-p22, including a 600-kb region commonly deleted in human hepatocellular carcinoma, colorectal cancer, and non-small cell lung cancer," *Genes Chromosomes and Cancer*, vol. 10, no. 1, pp. 7–14, 1994.

[13] Y. Fujiwara, M. Emi, H. Ohata et al., "Evidence for the presence of two tumor suppressor genes on chromosome 8p for colorectal carcinoma," *Cancer Research*, vol. 53, no. 5, pp. 1172–1174, 1993.

[14] R. Anbazhagan, H. Fujii, and E. Gabrielson, "Allelic loss of chromosomal arm 8p in breast cancer progression," *The American Journal of Pathology*, vol. 152, no. 3, pp. 815–819, 1998.

[15] G. S. Bova, B. S. Carter, M. J. G. Bussemakers et al., "Homozygous deletion and frequent allelic loss of chromosome 8p22 loci in human prostate cancer," *Cancer Research*, vol. 53, no. 17, pp. 3869–3873, 1993.

[16] M. A. Knowles, M. E. Shaw, and A. J. Proctor, "Deletion mapping of chromosome 8 in cancers of the urinary bladder using restriction fragment length polymorphisms and microsatellite polymorphisms," *Oncogene*, vol. 8, no. 5, pp. 1357–1364, 1993.

[17] J. Pang, L. Qin, N. Ren et al., "Loss of heterozygosity at D8S298 is a predictor for long-term survival of patients with tumor-node-metastasis stage I of hepatocellular carcinoma," *Clinical Cancer Research*, vol. 13, no. 24, pp. 7363–7369, 2007.

[18] N. Funel, M. Morelli, E. Giovannetti et al., "Loss of heterozygosity status of d9s105 marker is associated with downregulation of Krüppel-like factor 4 expression in pancreatic ductal adenocarcinoma and pancreatic intraepithelial lesions," *Pancreatology*, vol. 11, no. 1, pp. 30–42, 2011.

[19] F. Zhang, L. Wu, L. Zhou et al., "Predictive value of expression and promoter hypermethylation of XAF1 in hepatitis B virus-associated hepatocellular carcinoma treated with transplantation," *Annals of Surgical Oncology*, vol. 15, no. 12, pp. 3494–3502, 2008.

[20] J. Cheng, L. Zhou, Q. Xie et al., "The impact of miR-34a on protein output in hepatocellular carcinoma HepG2 cells," *Proteomics*, vol. 10, no. 9, pp. 1557–1572, 2010.

[21] L. Wu, Z. Yang, L. Zhou et al., "Identification of histone deacetylase 3 as a biomarker for tumor recurrence following liver transplantation in HBV-associated hepatocellular carcinoma," *PLoS ONE*, vol. 5, no. 12, Article ID e14460, 2010.

[22] S. Zheng, X. Xu, J. Wu et al., "Liver transplantation for hepatocellular carcinoma: Hangzhou experiences," *Transplantation*, vol. 85, no. 12, pp. 1726–1732, 2008.

[23] M. A. Zimmerman, R. M. Ghobrial, M. J. Tong et al., "Recurrence of hepatocellular carcinoma following liver transplantation: a review of preoperative and postoperative prognostic indicators," *Archives of Surgery*, vol. 143, no. 2, pp. 182–188, 2008.

[24] T. Lu, H. Hano, C. Meng, K. Nagatsuma, S. Chiba, and M. Ikegami, "Frequent loss of heterozygosity in two distinct regions, 8p23.1 and 8p22, in hepatocellular carcinoma," *World Journal of Gastroenterology*, vol. 13, no. 7, pp. 1090–1097, 2007.

[25] T. J. Seng, J. S. W. Low, H. Li et al., "The major 8p22 tumor suppressor DLC1 is frequently silenced by methylation in both endemic and sporadic nasopharyngeal, esophageal, and cervical carcinomas, and inhibits tumor cell colony formation," *Oncogene*, vol. 26, no. 6, pp. 934–944, 2007.

[26] S. Rodrigues-Ferreira, A. Di Tommasso, A. Dimitrov et al., "8p22 MTUS1 gene product ATIP3 is a novel anti-mitotic protein underexpressed in invasive breast carcinoma of poor prognosis," *PLoS ONE*, vol. 4, no. 10, Article ID e7239, 2009.

[27] T. Oyama, Y. Miyoshi, K. Koyama et al., "Isolation of a novel gene on 8p21. 3-22 whose expression is reduced significantly

in human colorectal cancers with liver metastasis," *Genes, Chromosomes and Cancer*, vol. 29, no. 1, pp. 9–15, 2000.

[28] S. Yan, J. Tang, C. Huang et al., "Reduced expression of ZDHHC2 is associated with lymph node metastasis and poor prognosis in gastric adenocarcinoma," *PLoS ONE*, vol. 8, no. 2, Article ID e56366, 2013.

[29] J. Greaves and L. H. Chamberlain, "DHHC palmitoyl transferases: substrate interactions and (patho)physiology," *Trends in Biochemical Sciences*, vol. 36, no. 5, pp. 245–253, 2011.

[30] J. Zhang, S. L. Planey, C. Ceballos, S. M. Stevens Jr., S. K. Keay, and D. A. Zacharias, "Identification of CKAP4/p63 as a major substrate of the palmitoyl acyltransferase DHHC2, a putative tumor suppressor, using a novel proteomics method," *Molecular and Cellular Proteomics*, vol. 7, no. 7, pp. 1378–1388, 2008.

[31] S. L. Planey, S. K. Keay, C. Zhang, and D. A. Zacharias, "Palmitoylation of cytoskeleton associated protein 4 by DHHC2 regulates antiproliferative factor-mediated signaling," *Molecular Biology of the Cell*, vol. 20, no. 5, pp. 1454–1463, 2009.

[32] C. Sharma, X. H. Yang, and M. E. Hemler, "DHHC2 affects palmitoylation, stability, and functions of tetraspanins CD9 and CD151," *Molecular Biology of the Cell*, vol. 19, no. 8, pp. 3415–3425, 2008.

[33] M. Miyake, H. Inufusa, M. Adachi et al., "Suppression of pulmonary metastasis using adenovirally motility related protein-1 (MRP-1/CD9) gene delivery," *Oncogene*, vol. 19, no. 46, pp. 5221–5226, 2000.

[34] M. Zöller, "Gastrointestinal tumors: metastasis and tetraspanins," *Zeitschrift fur Gastroenterologie*, vol. 44, no. 7, pp. 573–586, 2006.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 732516, 13 pages
http://dx.doi.org/10.1155/2014/732516

Research Article

Baicalein Induces Apoptosis and Autophagy via Endoplasmic Reticulum Stress in Hepatocellular Carcinoma Cells

Zhongxia Wang,[1] Chunping Jiang,[1,2] Weibo Chen,[1] Guang Zhang,[3] Dongjun Luo,[3] Yin Cao,[1] Junhua Wu,[4] Yitao Ding,[1,3] and Baorui Liu[5,6]

[1] *Department of Hepatobiliary Surgery, the Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, China*
[2] *Department of Hepatobiliary Surgery, Nanjing Drum Tower Hospital Clinical College of Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing, Jiangsu 210008, China*
[3] *Department of Hepatobiliary Surgery, Nanjing Drum Tower Hospital Clinical College of Nanjing Medical University, Nanjing, Jiangsu 210008, China*
[4] *School of Medicine, Nanjing University, Nanjing, Jiangsu 210093, China*
[5] *The Comprehensive Cancer Center, the Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, China*
[6] *The Comprehensive Cancer Center, Nanjing Drum Tower Hospital Clinical College of Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing, Jiangsu 210008, China*

Correspondence should be addressed to Yitao Ding; yitao_ding@163.com and Baorui Liu; baoruiliu@nju.edu.cn

Received 27 March 2014; Accepted 5 May 2014; Published 3 June 2014

Academic Editor: Jingmin Zhao

Background. Hepatocellular carcinoma (HCC) remains a disastrous disease and the treatment for HCC is rather limited. Separation and identification of active compounds from traditionally used herbs in HCC treatment may shed light on novel therapeutic drugs for HCC. *Methods*. Cell viability and colony forming assay were conducted to determine anti-HCC activity. Morphology of cells and activity of caspases were analyzed. Antiapoptotic Bcl-2 family proteins and JNK were also examined. Levels of unfolded protein response (UPR) markers were determined and intracellular calcium was assayed. Small interfering RNAs (siRNAs) were used to investigate the role of UPR and autophagy in baicalein-induced cell death. *Results*. Among four studied flavonoids, only baicalein exhibited satisfactory inhibition of viability and colony formation of HCC cells within water-soluble concentration. Baicalein induced apoptosis via endoplasmic reticulum (ER) stress, possibly by downregulating prosurvival Bcl-2 family, increasing intracellular calcium, and activating JNK. CHOP was the executor of cell death during baicalein-induced ER stress while eIF2α and IRE1α played protective roles. Protective autophagy was also triggered by baicalein in HCC cells. *Conclusion*. Baicalein exhibits prominent anti-HCC activity. This flavonoid induces apoptosis and protective autophagy via ER stress. Combination of baicalein and autophagy inhibitors may represent a promising therapy against HCC.

1. Introduction

Hepatocellular carcinoma (HCC) represents a major health problem worldwide. It is the fifth most common cancer and ranks 3rd among the causes of cancer-related death [1]. Treatment of HCC largely relies on surgical resection, liver transplantation, and radiofrequency ablation, which are potentially curative interventions. However, a majority of HCC patients were diagnosed at advanced stage, especially in less-developed countries. For late-stage HCC, radical therapies are not suitable [2]. Options of treatment at this situation are even more limited. There is still no effective systemic chemotherapy available for HCC, which is notoriously known as a highly resistant cancer to most of the drugs [3]. Although transarterial chemoembolization (TACE) and orally available targeted drug sorafenib are proven to increase survival in selected candidates, the prognosis of advanced-stage HCC patients remains poor [4].

HCC often develops on the background of viral hepatitis, nonalcoholic fatty liver disease, alcoholic cirrhosis, and other sorts of chronic liver injury which ultimately transform hepatocytes to malignancies through oxidative stress, inflammation, and accumulation of mutations during injury-repair cycles [2, 4, 5]. Such circumstances may put endoplasmic reticulum (ER) under stress [6, 7]. To cope with ER stress, cells evoke an adaptive mechanism named unfolded protein response (UPR). Three ER transmembrane receptors, protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6), initiate UPR through a signaling network. When UPR fails to rebuild homeostasis, programmed cell death could be induced to eliminate injured cells [8]. Along with UPR, autophagy could be triggered. The activation of autophagy flux reflects a possible compensatory reaction to relieve the burden of unfolded proteins and damaged organelles by autophagic degradation [9]. However, autophagy may either protect stressed cells or promote cell death via autophagic pathways. The fate of cells under ER stress might result from the balance between UPR and autophagy [10]. Growing evidence indicates the role of ER stress and autophagy in hepatocarcinogenesis [11, 12]. On the other hand, activation of ER stress and modification of autophagy activity may shed light on novel potential therapeutic approaches against HCC [13–15].

The root of *Scutellaria baicalensis* Georgi (Huang-qin in Chinese) has been broadly used in remedies for hepatitis, cirrhosis, jaundice, and HCC in traditional Chinese, Japanese, and Korean medicine [16]. Current analysis of active constituents of this herbal medicine revealed that flavonoids such as baicalein, baicalin, wogonin, and wogonoside are responsible for its liver protective activity [17]. To date, emerging studies suggest these flavonoids exhibit anti-HCC effects. Induction of apoptosis and cell cycle arrest and inhibition of migration and invasion by active compounds in *Scutellaria baicalensis* Georgi have been reported [16–22]. Detailed mechanisms of the inhibitory effects of flavonoids from *Scutellaria baicalensis* Georgi remain elusive. Possible molecular mechanisms include 12-lipoxygenase (12-LOX) [19], PI3K/Akt [18, 20], MEK/ERK [22, 23], and NF-κB [24] transduction pathways. In this present study, we further investigated the potential inhibitory activity of HCC cells by four major flavonoid components of *Scutellaria baicalensis* Georgi: baicalein, baicalin, wogonin, and wogonoside. This study also revealed the roles of ER stress and autophagy in baicalein-induced HCC cell apoptosis.

2. Materials and Methods

2.1. Reagents. Baicalein (purity 98%), baicalin (purity 95%), wogonin (purity > 98%), wogonoside (purity > 95%), and tunicamycin were obtained from Sigma-Aldrich (St. Louis, MO). Cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) and Fluo-3 AM were from Beyotime Institute of Biotechnology (Nantong, China). Antiphospho-PERK (Thr-981) rabbit polyclonal antibody (sc-32577) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies were obtained from Cell Signaling Technology (Beverly, MA).

2.2. Cell Culture. Human HCC cell lines SMMC-7721 and Bel-7402 were purchased from Cell Bank of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. SMMC-7721 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (10% FBS, Gibco, Gaithersburg, MD). Bel-7402 cells were maintained in RPMI-1640 medium (Gibco, Gaithersburg, MD) containing 10% FBS. All cell lines were maintained at 37°C in a humidified atmosphere with 5% CO_2.

2.3. Cell Viability Evaluation. CCK-8 assay was used to evaluate relative cell viability. Briefly, 5×10^3 cells growing on 96-well plate were treated with anticipated concentration of indicated flavonoids for 24 h or 48 h in triplicate. Control group was treated with dilution vehicle. After the desired time of treatment, medium with flavonoids was removed and 100 uL CCK-8 working solution diluted with fresh medium was added into each well. Cells were then incubated for another 4 h and optical density (OD) was measured at 450 nm using a VERSAmax microtiter plate reader (Molecular Devices Corporation, Sunnyvale, CA). Relative cell viability was calculated with the following formula: relative cell viability (%) = OD (treatment group)/OD (control group) × 100%.

2.4. Colony Forming Assay. 300–500 cells were suspended in medium containing 10% FBS and plated in 6-well plates. After the attachment of cells for 24 h, they were treated with the indicated dose of flavonoids. After 24 h of treatment, fresh complete culture medium was changed and cell colonies were allowed to grow for 10 days. Colonies were then fixed with 3% paraformaldehyde and stained with 0.1% crystal violet for 30 min. Stained cell colonies were washed with phosphate buffered saline (PBS) for three times and dried. Images were obtained by a digital camera and colonies were counted using ImageJ software (U.S. National Institutes of Health, Bethesda, MD).

2.5. Western Blotting. Cell lysates were prepared by using radioimmune precipitation assay (RIPA) lysis buffer (Beyotime, Nantong, China) supplemented with a cocktail of protease inhibitors (Roche, Basel, Switzerland). Total protein concentration was determined by BCA reagent following the manufacturer's instruction (Thermo Scientific, Rockford, IL). Equal amounts of soluble proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After being transferred to 0.45 μm polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), proteins were detected by incubation with primary antibodies followed by HRP-conjugated secondary antibodies. Enhanced chemiluminescence (ECL) reagent (Millipore, Bedford, MA) was applied to the membranes and specific protein bands were visualized by FluorChem FC2 Imaging System (Alpha Innotech, San Leandro, CA).

2.6. Fluorescence Microscopy Analysis. To determine the morphology of nuclei after drug treatment, cells were treated with or without the indicated concentration of baicalein for 24 h. Cells were then fixed with 3% paraformaldehyde and stained with 10 μg/mL DAPI for 15 min. Images were captured with an Olympus BX53 fluorescence microscope (Olympus, Tokyo, Japan).

2.7. Measurement of Intracellular Calcium Concentration. Cells were treated with the indicated concentration of baicalein for 24 h before analysis. After the treatment, HCC cells were incubated with 5 μM Fluo-3 AM calcium probe for 1 h. Medium containing Fluo-3 AM was then replaced by fresh medium and the cells were placed at 37°C for another 30 min to allow sufficient conversion of Fluo-3 AM into fluorescent Fluo-3. Cells were then detached by trypsin digestion and washed before detection of Fluo-3 on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) following the manufacturer's instructions. Data were analyzed using FlowJo software (Treestar, Inc., San Carlos, CA).

2.8. Small Interfering RNA (siRNA) Transfection. siRNAs against human eIF2α, CHOP, IRE1α, Beclin 1, and Atg5 were synthesized by GenePharma (Shanghai, China). The sequences of siRNAs against eIf2α, CHOP, and IRE1α were from a previously published study by Shi et al. [25]. The sequences of other siRNAs were as follows: Atg5, GGGAAGCAGAACCAUACUATT; Beclin 1, CAGTTTG-GCACAATCAATA. For transfection, SMMC-7721 cells were plated in 6-well plate and allowed to grow to 70% confluence. Transfection was conducted using Lipofectamine RNAiMAX reagent (Life Technologies, Carlsbad, CA) following the manufacturer's guidance. A scrambled siRNA was transfected as negative control.

2.9. Statistical Analysis. Numeric data were expressed as mean ± standard deviation (SD). Difference between groups was analyzed by one-way analysis of variance with Bonferroni's multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Baicalein Inhibits Proliferation of HCC Cells within Water-Soluble Concentrations. We firstly undertook a study to preliminarily evaluate anti-HCC effects of four major flavonoids, baicalein, baicalin, wogonin, and wogonoside, from *Scutellaria baicalensis* Georgi. The structures of the compounds are shown in Figure 1(a). Two human HCC cell lines, SMMC-7721 and Bel-7402, were used for screening. The concentrations causing 50% inhibition of cell viability (IC_{50}s) were listed in Table 1. After 24 h treatment, both baicalein and wogonin caused significant proliferation inhibition on HCC cells. In contrast, baicalin showed little activity against HCC cells with calculated IC_{50}s markedly higher than baicalein in both cells. The effect of wogonoside on HCC cells was negligible. The proliferation of both SMMC-7721 and Bel-7402 cells remained uninterrupted even at 200 μM concentration of wogonoside. We next prolonged the duration of drug treatment to further observe potential late effects of the tested flavonoids. Of note, the inhibitory effect of baicalein at 48 h increased dramatically whereas the IC_{50} values of wogonin only slightly dropped. At the same time, the IC_{50} of baicalin against Bel-7402 cells decreased to 169.35 μM though the value for SMMC-7721 remains relatively high. Wogonoside showed no activity on both of the HCC cell lines even at 48 h.

Table 1: IC_{50} values of baicalein, baicalin, wogonin, and wogonoside.

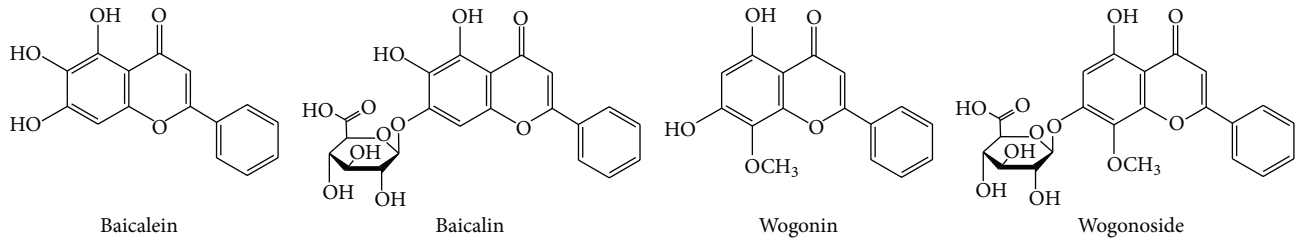
IC_{50} (μM)	SMMC-7721		Bel-7402	
	24 h	48 h	24 h	48 h
Baicalein	94.84	19.89	134.81	59.52
Baicalin	1246.10	837.24	400.39	169.35
Wogonin	53.39	42.71	77.13	49.65
Wogonoside	N/I	N/I	N/I	N/I

IC_{50}: concentration at which cells were inhibited by 50%; N/I: no inhibition.

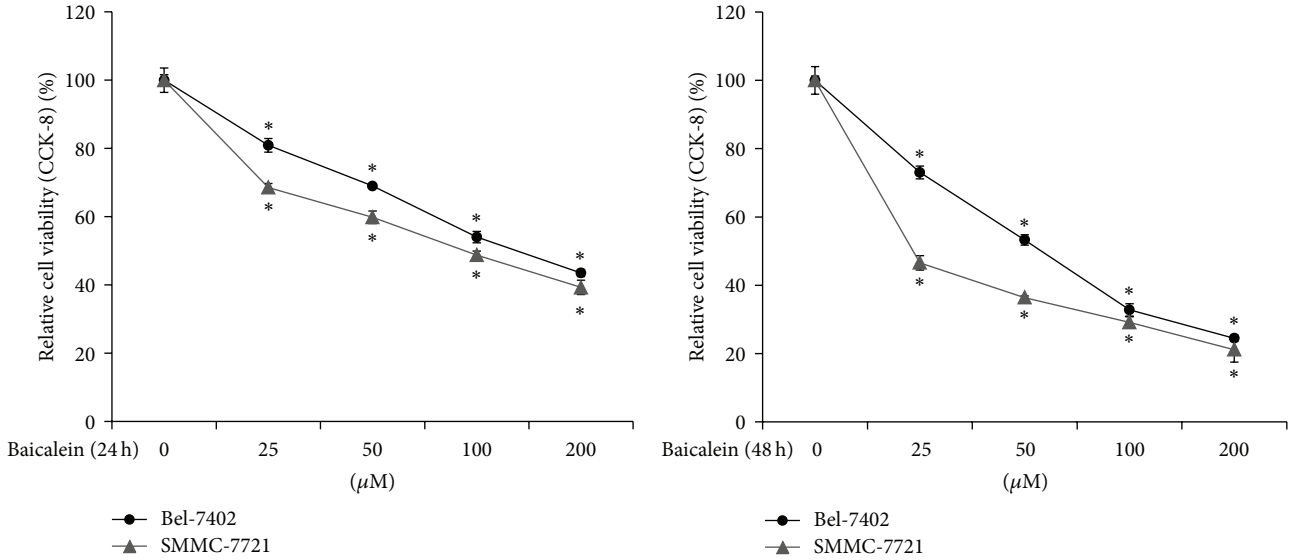
In summary, our preliminary evaluation revealed that baicalein exhibited significant inhibition of proliferation of HCC cells in a time- and dose-dependent manner (Figure 1(b)). However, its glycoside baicalin showed only weak activity towards liver cancer cells (Figure 1(c)). On the other hand, although wogonin notably decreased the viability of HCC cells, its poor water solubility prevented us from further investigating this activity since this compound easily crystalized at lower concentration, especially when contrasted with the satisfactory solubility of baicalein within the wide testing concentration range. Even when treated with 200 μM wogonoside for 48 h, proliferation of the tested cells remained intact, suggesting wogonoside had no inhibitory activity on HCC cells.

3.2. Baicalein Prevents HCC Cells from Forming Colonies. To study the anti-HCC effect of baicalein, we conducted colony forming assay to observe if baicalein interferes with the ability of single cell to form growing colony, which represents an important character of cancer cells' ability to attach, survive, and proliferate. As shown in Figure 2(a), baicalein treatment dose-dependently suppressed the formation of HCC cell colonies in both SMMC-7721 and Bel-7402 cells. Similar to the results of cell viability assay, baicalin exhibited only a weak activity at higher doses against Bel-7402 cells. Measurements of colony number and colony size indicated that baicalein reduced both the amount and size of colonies in a dose-dependent manner. Interestingly, baicalin showed inhibition of foci size of Bel-7402 without an obvious decline of colony amount while its activity against SMMC-7721 cell colony formation remained minimal (Figures 2(b) and 2(c)).

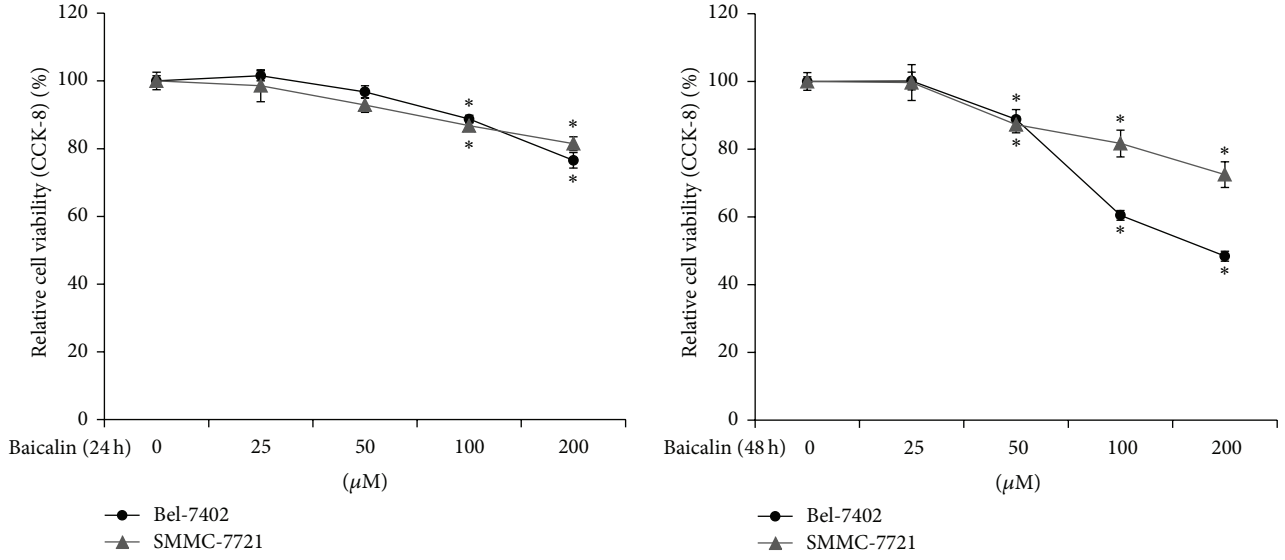
3.3. Baicalein Induces Apoptosis in HCC Cells. We next investigated the type of cell death underlying the inhibition of HCC cells mediated by baicalein. Following the treatment of baicalein, the appearance of HCC cells dramatically changed.



(a)



(b)



(c)

Figure 1: Baicalein inhibits proliferation of HCC cells. (a) Structures of the flavonoids used: baicalein, baicalin, wogonin, and wogonoside. (b) Human HCC cell lines Bel-7402 and SMMC-7721 were treated with 0, 25, 50, 100, and 200 μM of baicalein for 24 h (upper panel) or 48 h (down panel). Relative cell viability was determined by CCK-8 assay. (c) Bel-7402 and SMMC-7721 cells were treated with 0, 25, 50, 100, and 200 μM of baicalin for 24 h (upper panel) or 48 h (down panel). Relative cell viability was determined by CCK-8 assay. Data were expressed as mean ± SD. $^{*}P < 0.05$, compared with control group.

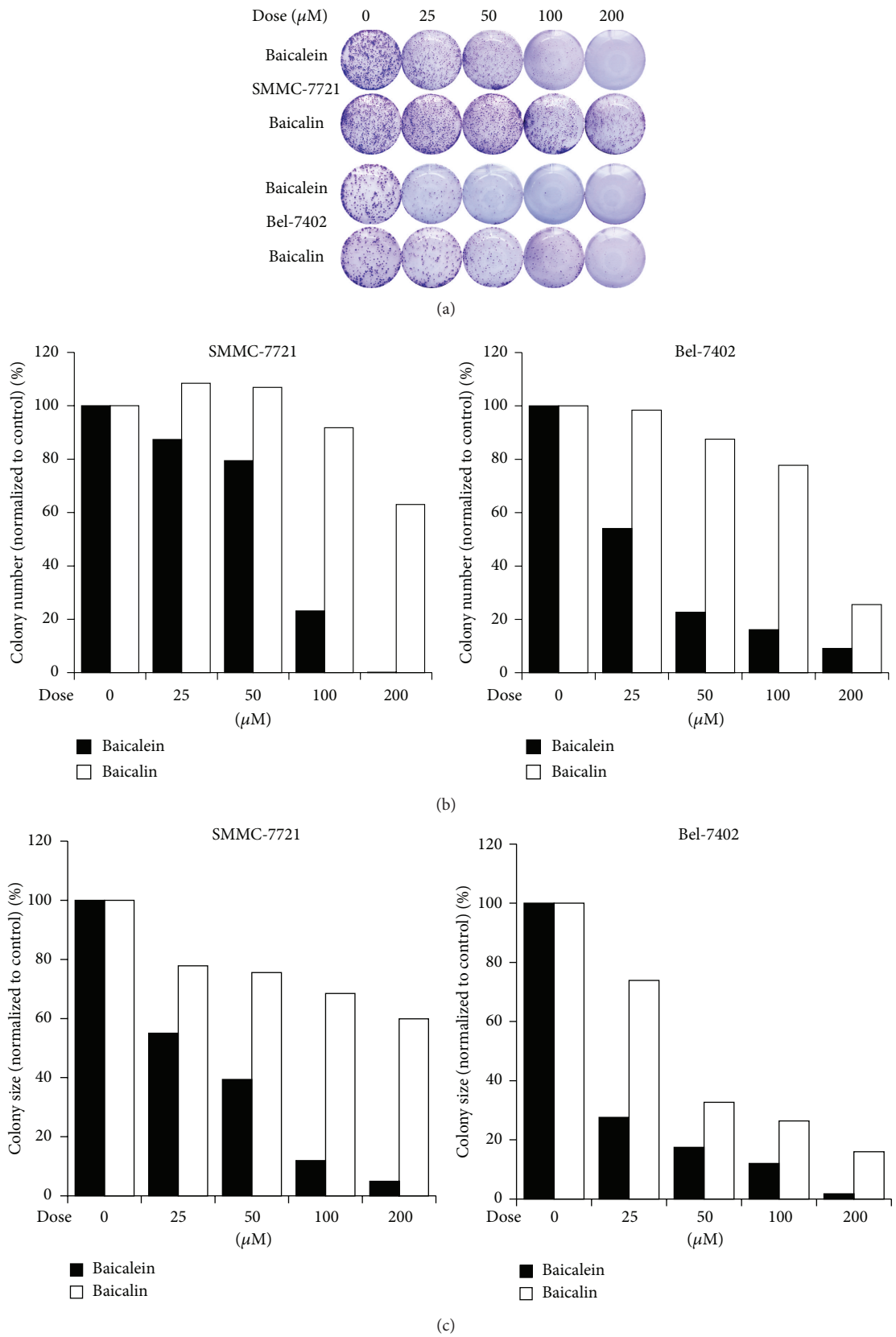


Figure 2: Baicalein inhibits colony formation of HCC cells. (a) SMMC-7721 and Bel-7402 cells were treated with the indicated dose of baicalein or baicalin. Cell colonies were visualized by crystal violet staining. (b) The amount of cell colonies formed after treatment of either baicalein or baicalin. Data were normalized to control and expressed as percentage. (c) The size of cell colonies after treatment of the indicated dose of baicalein or baicalin. Data were normalized to control and expressed as percentage.

As shown in Figure 3(a), cells in control group were in a typical polygonal or spindle-like intact appearance whereas baicalein-treated cells showed cell shrinkage, rounding, and blebbing and finally detached and floated in culture medium, which were representative morphological changes of apoptosis. To determine if cell death induced by baicalein was mediated by apoptosis, we examined the activity of caspase pathway by western blotting. The results indicated that baicalein caused marked cleavage of caspase-9, caspase-3, and PARP dose- and time-dependently. The induction of PARP cleavage happened as early as 12 h posttreatment (Figures 3(b) and 3(c)). The morphology of nuclei also showed typical appearances of apoptosis such as pyknosis and karyorrhexis (Figure 3(d)). Taken together, these results demonstrated that baicalein promoted HCC cell death through inducing apoptosis.

3.4. Baicalein Induces ER Stress and Activates UPR Pathways. During baicalein-induced apoptosis, cellular vacuolization was observed using contrast microscopy in dying cells while morphologically normal cells were free of this phenomenon (Figure 4(a)). Previous study indicates that these cytoplasmic vacuoles may be dilated ER lumens under stress [26]. We therefore conducted western blotting to determine whether baicalein-treated cells were under ER stress. As shown in Figures 4(b) and 4(c), PERK and IRE1α, receptors responsible for UPR signaling, were significantly activated dose- and time-dependently. Accordingly, the levels of several UPR downstream molecules such as CHOP and phosphorylated eIF2α were also upregulated at as early as 6 h and 12 h after baicalein treatment. As a responsive feedback, the expression of chaperone protein BiP was also enhanced. The expression patterns of these UPR-related proteins in baicalein-treated cells were consistent with cells treated by a well-characterized ER stress inducer, tunicamycin. Intracellular calcium homeostasis is among the functions of ER and aberrant calcium distribution may represent a typical manifestation of ER stress. Flow cytometry was employed to study intracellular calcium concentration using Fluo-3 AM calcium-sensitive fluorescence probe. Our results revealed that baicalein-induced prominent elevation of cytoplasmic calcium level (Figure 4(d)). The median fluorescence intensity of calcium probe escalated in a dose-dependent manner and reached as high as 3–5 times over vehicle control cells (Figure 4(e)). These results suggested that baicalein triggered ER stress in HCC cells and activated UPR signaling pathways, which may be closely related to apoptosis induced by this flavonoid.

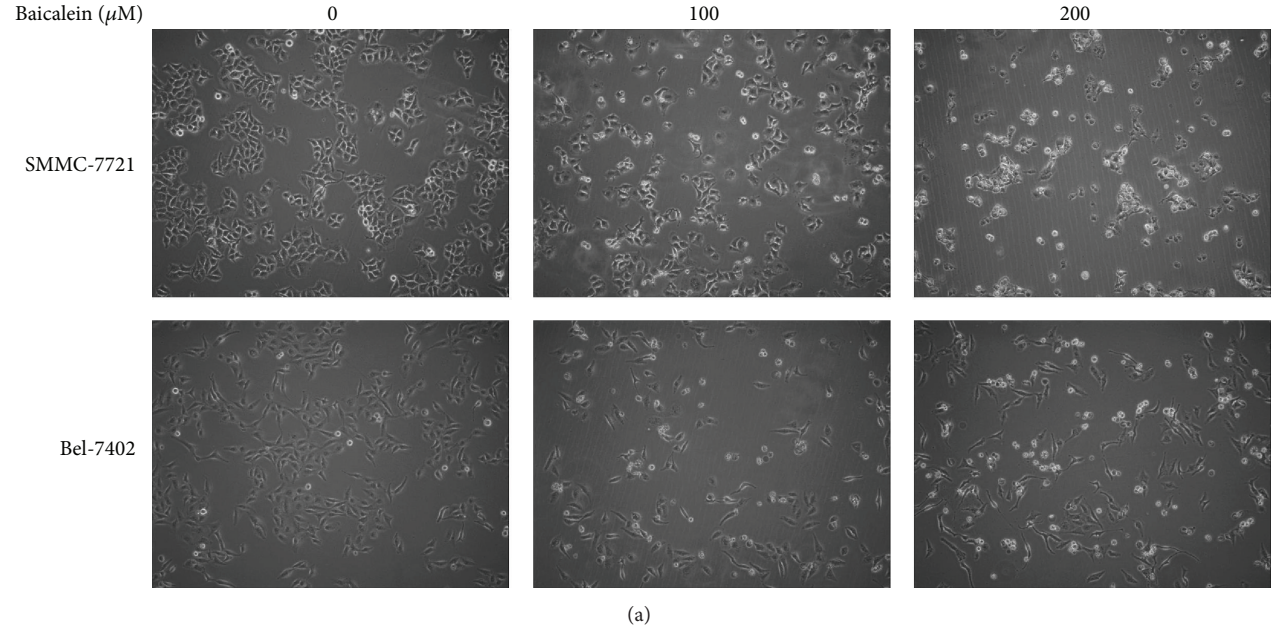
3.5. Baicalein Suppresses the Expression of Antiapoptotic Bcl-2 Family Proteins and Activates JNK. It is reported that antiapoptotic Bcl-2 family proteins are downregulated during ER stress and JNK is activated to turn the balance towards apoptosis [10]. To test if this regulation also occurred when HCC cells were treated with baicalein, we studied the levels of Bcl-2, Bcl-xL, and Mcl-1, which are typical antiapoptotic Bcl-2 family members. As shown in Figure 5(a), baicalein suppressed the expression of these antiapoptotic regulators in both HCC cell lines. Meanwhile, phosphorylation of JNK was also detected in a dose-dependent manner, indicating that JNK pathway was activated after baicalein treatment (Figure 5(b)).

3.6. CHOP Induction Is Required for ER Stress-Mediated Apoptosis While eIF2α and IRE1α Play Protective Roles. To further explore the roles of UPR signaling pathways in baicalein-induced apoptosis, we used siRNA-mediated gene knockdown to suppress the expression of UPR transducing molecules. Transfection of CHOP-targeting siRNA significantly attenuated the induction of CHOP after baicalein treatment. Interestingly, the suppression of CHOP markedly reduced cell apoptosis as indicated by reduced amount of cleaved PARP (Figure 6(a)). siRNA knockdown significantly reduced the level of eIF2α and almost totally abolished the phosphorylation of this protein. Interestingly, inhibition of eIF2α activation dramatically increased apoptosis (Figure 6(b)). Similar to eIF2α, siRNA-mediated silencing of IRE1α also blocked the activation of this pathway and exacerbated cell death by baicalein. Although IRE1α was thought to activate JNK pathway to facilitate apoptosis, our results demonstrated that knockdown of IRE1α did not inhibit baicalein-induced JNK activation (Figure 6(c)).

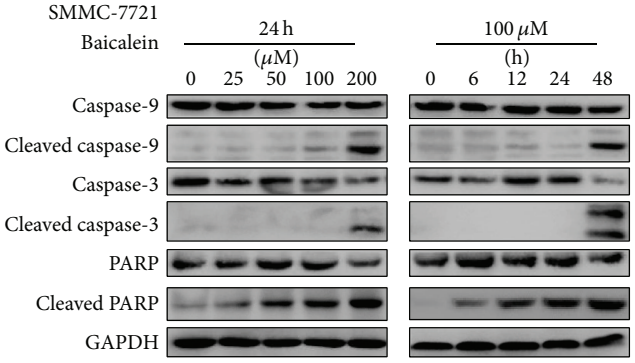
3.7. Protective Autophagy Is Induced by Baicalein. We next investigated if baicalein induces autophagy, which is a frequently observed response coupling ER stress, in HCC cells. By western blotting, the conversion of LC-3I into LC-3II, a classic marker of autophagy activity, was determined. As shown in Figure 7(a), the amount of intracellular LC3-II was intriguingly increased in both tested cells, indicating possible upregulation of autophagy flux. To determine the role of baicalein-induced autophagy in cell death, we inhibited the expression of important regulators of autophagy pathway by siRNA. Our results showed that knockdown of Atg5 and Beclin 1 significantly aggravated apoptosis in baicalein-treated HCC cells (Figures 7(b) and 7(c)).

4. Discussion

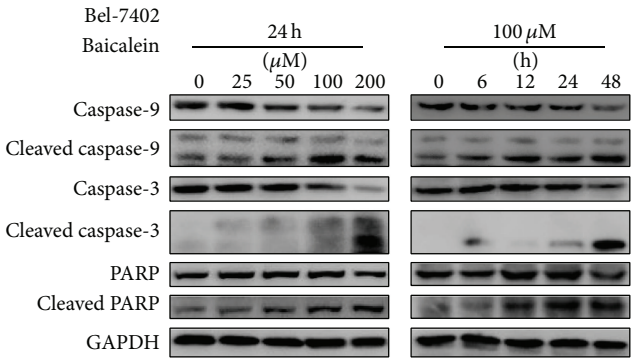
In spite of recent advances in therapeutic strategies, HCC remains a disastrous disease for the majority of patients [27]. Surgical resection and liver transplantation are first-line treatments for HCC [4]. However, recurrence after surgery represents a tough problem and the prognosis of patients with recurrent disease is pessimistic [28]. For patients with advanced-stage HCC and without opportunity to receive curative therapy, effective treatment is even more limited [29]. HCC is well known for its resistance to chemotherapy. Systemic chemotherapy using traditional cytotoxic drugs has little effect on HCC patients; left small molecular targeted drug sorafenib is the only medication with evidence to improve prognosis of advanced-stage HCC [30, 31]. The absence of ideal therapy for HCC largely contributes to the current dilemma of HCC treatment. Therefore, much effort has been expended to discover novel molecular targets and potential effective drugs for HCC [32–34]. For thousands of years, herbal medicine had been widely used to treat



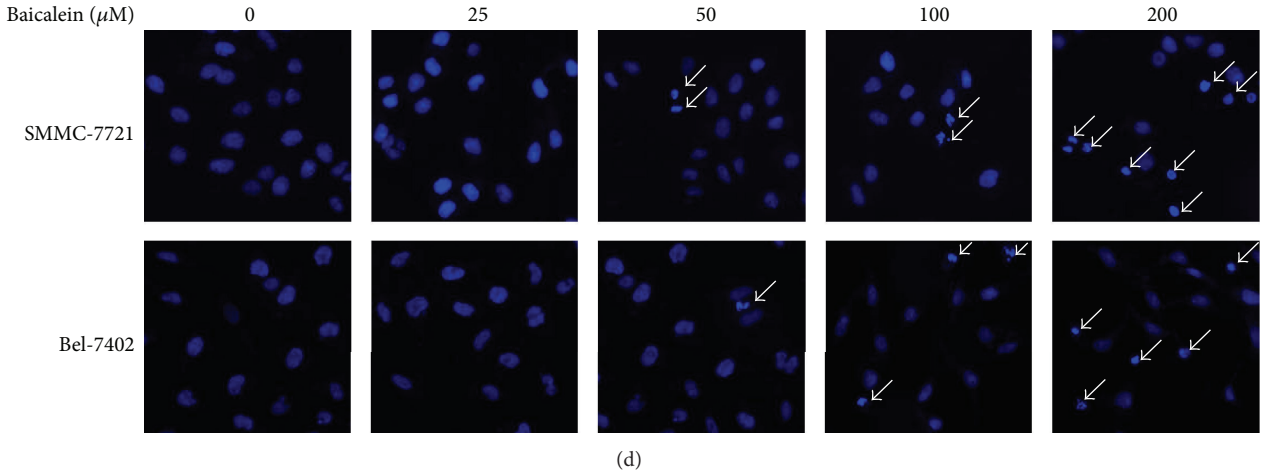
(a)



(b)



(c)



(d)

Figure 3: Baicalein induces apoptosis in HCC cells. (a) Morphology of SMMC-7721 and Bel-7402 cells under contrast microscopy (40x) after treating with 0, 100, or 200 μM of Baicalein for 24 h. (b and c) The protein levels of full length and cleaved form of caspase-9, caspase-3, and PARP in SMMC-7721 (b) and Bel-7402 (c) cells were determined by western blotting following the treatment of the indicated dose of baicalein for the indicated time. GAPDH served as a loading control. (d) Morphology of nuclei after treatment of the indicated dose of baicalein for 24 h. Pyknosis and karyorrhexis were pointed by white arrow.

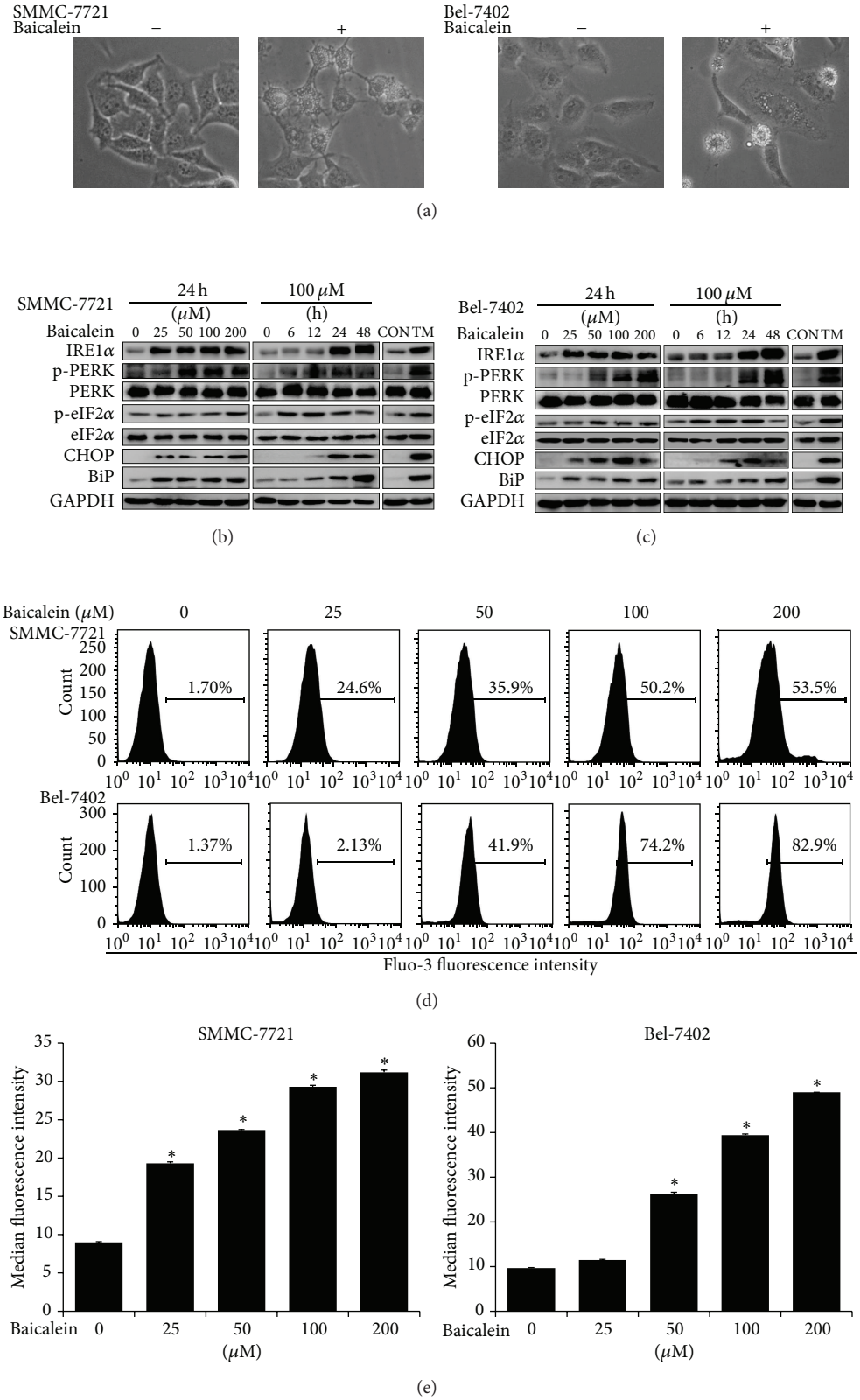


Figure 4: Baicalein induces ER stress. (a) Morphology change of HCC cells after the treatment of 100 μM Baicalein (100x). (b and c) Levels of UPR proteins in SMMC-7721 (b) and Bel-7402 (c) cells were determined by western blotting after the treatment of the indicated dose of baicalein for the indicated time. Tunicamycin (TM, 5 μg/mL) treatment for 6 h was used as positive control of ER stress induction. CON: control cells without drug treatment. GAPDH served as a loading control. (d) Intracellular calcium level of HCC cells was analyzed by flow cytometry. Cells were treated with the indicated concentration of baicalein for 24 h. (e) Median fluorescence intensity of calcium probe in HCC cells after treatment of the indicated dose of baicalein for 24 h. $^*P < 0.05$, compared with control group.

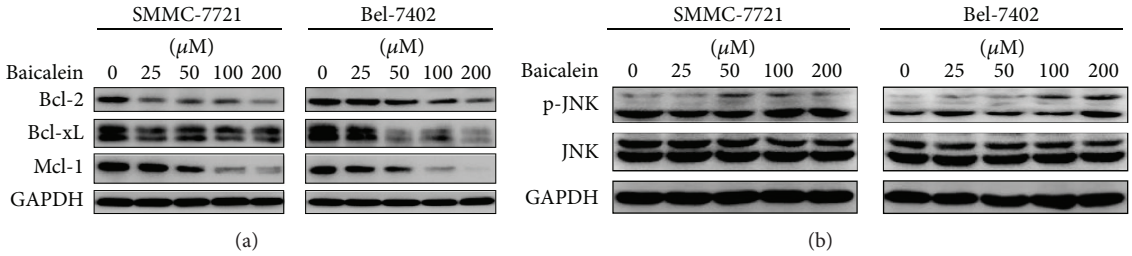


Figure 5: Baicalein suppresses the expression of antiapoptotic Bcl-2 family proteins and activates JNK pathway. (a) SMMC-7721 and Bel-7402 cells were treated with the indicated dose of baicalein for 24 h. Levels of Bcl-2, Bcl-xL, and Mcl-1 were determined by western blotting. (b) Phosphorylated JNK and total JNK were analyzed by western blotting after cells were treated with the indicated dose of baicalein. GAPDH served as a loading control.

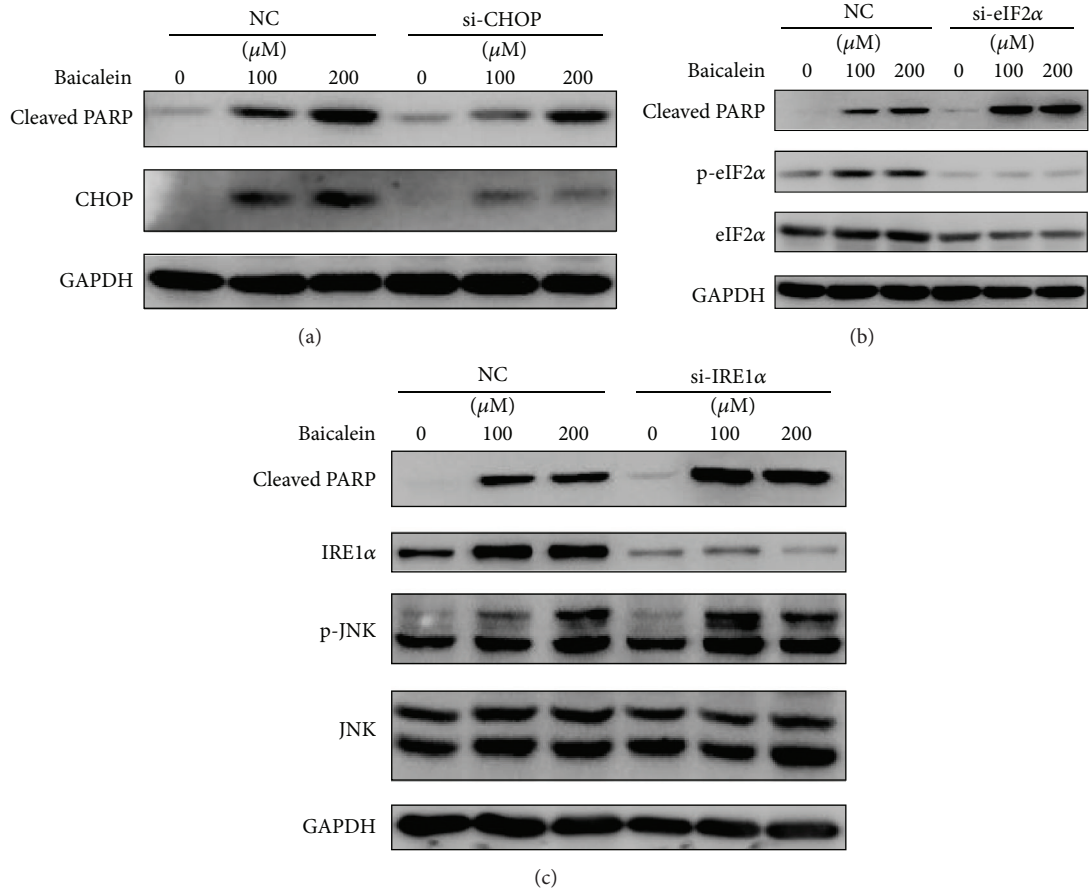


Figure 6: Diverse roles of UPR proteins in baicalein-induced apoptosis.(a) SMMC-7721 cells were transfected with scrambled RNA (NC) or CHOP-targeting siRNA (si-CHOP) for 48 h and treated with 0, 100, and 200 μM baicalein for 24 h. Protein levels of cleaved PARP and CHOP were determined by western blotting. (b) SMMC-7721 cells were transfected with scrambled RNA (NC) or eIF2α-targeting siRNA (si-eIF2α) and then treated with 0, 100, and 200 μM baicalein for 24 h. Protein levels of cleaved PARP phosphorylated eIF2α and eIF2α were determined. (c) After being transfected with scrambled RNA (NC) or IRE1α-targeting siRNA (si-IRE1α), SMMC-7721 cells were treated with the indicated dose of baicalein for 24 h and subjected to western blotting to analyze the level of cleaved PARP, IRE1α, phosphorylated JNK, and total JNK. GAPDH served as a loading control.

liver diseases in China, Japan, Korea, and other districts around the world [35]. Separation and identification of active compounds from herbal medicine may provide potential drugs for HCC and help improve the prognosis of this deadly disease.

Huang-qin, the root of *Scutellaria baicalensis* Georgi, has been a major component of many traditional remedies for liver disorders, including HCC [17, 21, 36–38]. Modern sciences suggest that flavonoids in Huang-qin may be responsible for therapeutic effects of this herbal medicine [39]. In

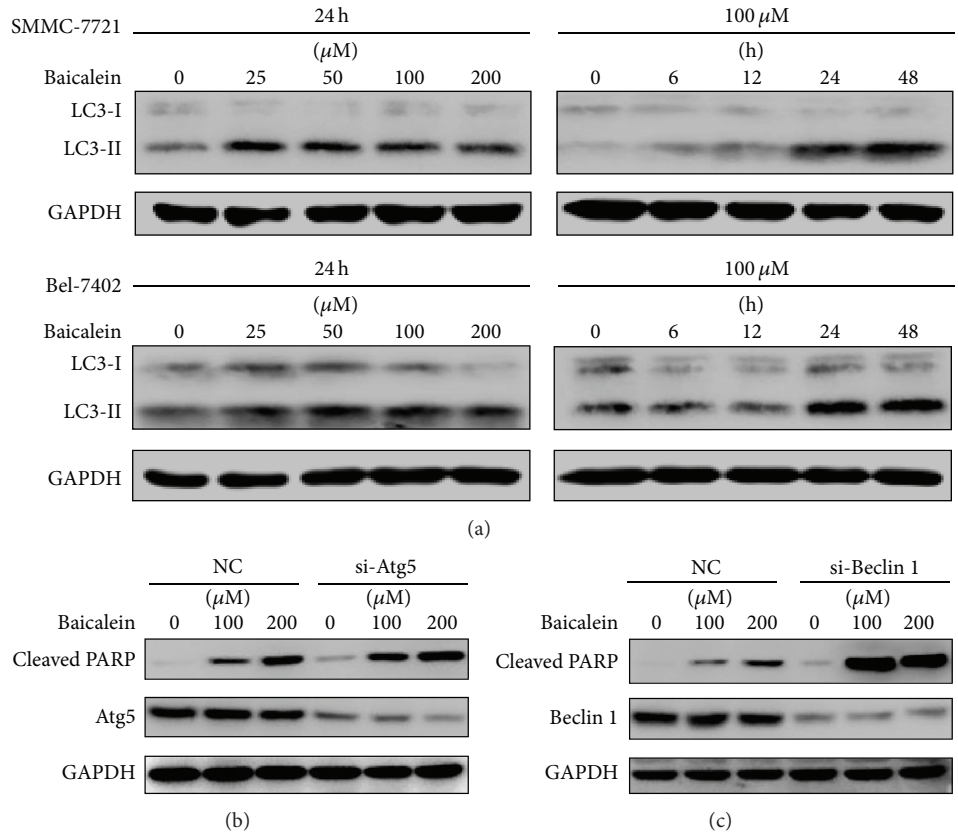


Figure 7: Baicalein induces protective autophagy. (a) HCC cells were treated with the indicated dose of baicalein for the indicated time and the level of LC-3 was determined. (b) SMMC-7721 cells were transfected with scrambled RNA (NC) or Atg5-targeting siRNA (si-Atg5) for 48 h and then treated with 0, 100, and 200 μM baicalein for another 24 h. Cleaved PARP and Atg5 were analyzed by western blotting. (c) SMMC-7721 cells were transfected with scrambled RNA (NC) or Beclin 1-targeting siRNA (si-Beclin 1) for 48 h and incubated with the indicated concentration of baicalein for 24 h. Cleaved PARP and Beclin 1 were analyzed by western blotting. GAPDH served as a loading control.

this study, we analyzed the inhibitory activity of four common flavonoids from Huang-qin (baicalein, baicalin, wogonin, and wogonoside) and found that baicalein showed potent inhibition of HCC cells within water-soluble concentration. This flavonoid also attenuated the ability of single HCC cell to form growing colony, which is an important character of cancer cells' ability to survive, attach, and proliferate to form tumors. Our results support several previous studies which reported the activity of baicalein against HCC cells [16–19, 22–24, 38, 40, 41]. This inhibition is of great importance because previous papers have provided evidence that baicalein preferentially kills HCC cells and leaves normal liver cells intact, demonstrating a selective anti-HCC activity [18, 23, 24].

However, the mechanisms of baicalein's anti-HCC activity remain elusive till now. Recent studies have shed light on potential molecular pathways involved in the activity of baicalein against HCC. Chang et al. revealed that baicalein induces cell cycle arrest and apoptosis in HCC cells [16]. Their later study indicated that apoptosis induced by baicalein may be attributed to mitochondrial dysfunction [17]. Mitochondria-dependent caspase pathway as well as AIF and Endo G pathways is also found to contribute to the induction of apoptosis by baicalein [41]. Our results also proved that cell death caused by baicalein is caspase-mediated apoptosis, supported by typical apoptotic morphology and change of nuclei appearance.

As for the role of signaling pathways in baicalein-induced HCC inhibition, Liang et al. recently revealed that MEK/ERK plays an important role both *in vitro* and *in vivo*. Baicalein inhibits MEK1 and subsequently reduces the activation of ERK1/2, leading to apoptosis and tumor growth arrest in mice bearing liver cancer [23]. Suppression of this pathway may also lead to attenuated cell migration and invasion by blocking multiple proteases degrading extracellular matrix [22]. The antitumor effect of baicalein may also be attributed to the deactivation of PI3K/Akt pathways. A recent study from Zheng et al. demonstrated that baicalein inhibited Akt and promoted the degradation of β-catenin and cyclin D1 independent of GSK-3β. This result is also confirmed in animal model [18]. Besides the abovementioned pathways, NF-κB may also be responsible for the anticancer activity of baicalein [24].

Our present study provides additional mechanism explaining baicalein-induced HCC cell death. When observing the morphology of HCC cells undergoing apoptosis, we

found an interesting phenomenon that baicalein treatment induced cellular vacuolization in HCC cell lines. This leads us to hypothesize that the vacuoles may be enlarged ERs under stress [25]. The following investigation revealed that baicalein treatment significantly activated UPR receptors PERK and IRE1α. As a result, downstream signal transduction molecules such as eIF2α and CHOP were also phosphorylated and induced, respectively. BiP, an ER chaperone which helps in protein folding and inhibits UPR in resting state, was also markedly upregulated, implying a feedback response towards baicalein-induced ER stress [42]. ER acts as a significant intracellular calcium pool and regulates calcium homeostasis. Calcium mobilization from ER into cytosol represents an emblematical event in response to various stimuli and has been implicated in the regulation of ER stress and UPR [25, 43]. Using a sensitive fluorescent probe, we found that intracellular calcium level was dramatically elevated following baicalein treatment. Taken together, our results suggest that baicalein induces ER stress in HCC cells and activates UPR.

UPR is a highly conserved cellular response aimed at reducing the burden of unfolded protein and restoring ER homeostasis. Multiple signaling pathways participate in UPR and functions diversely. Upon activation, PERK phosphorylates and activates eIF2α. As a translational regulator, eIF2α leads to a general translation block to reduce protein load in ER, thus preventing cells from overstress [44]. A set of genes including CHOP may escape this block and are translated with priority [45]. When UPR fails to relieve continuing pressure brought by ER stress, CHOP is found to mediate cell death and eliminate injured cells. CHOP signaling increases protein synthesis and exacerbates ER stress as well as downregulating antiapoptotic Bcl-2 family genes, which tip the balance towards cell apoptosis [10, 43]. IRE1α signaling pathway may also play an important role in ER stress-related apoptosis via potentiating PERK signaling and upregulating CHOP [46]. It is also reported to initiate cell death by activating JNK pathway [47]. In contrast, there is also evidence supporting a prosurvival role of IRE1α [48, 49]. Elevated intracellular calcium level may also contribute to apoptosis of cells under ER stress [50]. Our results indicated that prosurvival Bcl-2 family proteins, Bcl-2, Bcl-xL, and Mcl-1, were downregulated during baicalein-induced ER stress. Meanwhile, JNK was activated. Intracellular calcium level also escalated as mentioned above. As consequences of ER stress brought by baicalein, downregulation of antiapoptotic factors, increase of calcium concentration, and activation of proapoptotic JNK pathway may cooperate to execute apoptosis in HCC cells. In siRNA knockdown assays, as hypothesized, suppression of executor protein CHOP protected cells from apoptosis. However, interference of eIF2α potentiated baicalein-induced apoptosis, which could be explained by this protein's role of "burden reliever" in ER stress. Interestingly, our results suggested that inhibition of IRE1α also promoted HCC cell apoptosis. Knockdown of IRE1α did not alleviate the activation of JNK, indicating that IRE1α may not be responsible for regulating the activity of JNK pathway in baicalein-induced ER stress. In summary, CHOP is the major executor of ER stress-related apoptosis after treatment of baicalein, while eIF2α and IRE1α serve as protective factors.

In addition to the roles of UPR molecules in ER stress-related apoptosis, accumulating evidence suggests that autophagy may also closely interact with ER stress to determine cell fate [9, 10]. Autophagy may either protect cells from destruction or act as an inducer of cell death [25]. In this study, we observed a significant increase of conversion from LC-3I to LC-3II, which represents an important event during activation of autophagy. Inhibition of autophagy activity by siRNA-mediated gene knockdown of key regulators of autophagy, Atg5 and Beclin 1, revealed that autophagy induced by baicalein may be protective for cells against the pressure of ER stress. This may implicate a possible strategy to enhance the anti-HCC activity of baicalein by synchronously inhibiting autophagy.

In conclusion, to the best of our knowledge, our study for the first time provided evidence that baicalein induces apoptosis and autophagy via ER stress in HCC cells. Baicalein may represent a potential therapeutic drug with promising inhibitory activity against HCC. A combination of baicalein with inhibitors of autophagy may further enhance its anti-HCC effect.

Conflict of Interests

The authors declared no conflict of interests.

Authors' Contribution

Zhongxia Wang and Chunping Jiang contributed equally to this study.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. NSFC30801417); the Natural Science Foundation of Jiangsu Province (no. BK2009010); the Doctoral Fund of the Ministry of Education of China (no. RFDP200802841004); Key Project supported by Medical Science and Technology Development Foundation, Nanjing Department of Health (no. ZKX12030); and the Scientific Research Foundation of Graduate School of Nanjing University (no. 2013CL14).

References

[1] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA: Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[2] H. B. El-Serag, "Hepatocellular carcinoma," *The New England Journal of Medicine*, vol. 365, no. 12, pp. 1118–1127, 2011.

[3] J. M. Luk, X. Wang, P. Liu et al., "Traditional Chinese herbal medicines for treatment of liver fibrosis and cancer: from laboratory discovery to clinical evaluation," *Liver International*, vol. 27, no. 7, pp. 879–890, 2007.

[4] A. Forner, J. M. Llovet, and J. Bruix, "Hepatocellular carcinoma," *The Lancet*, vol. 379, no. 9822, pp. 1245–1255, 2012.

[5] G. Ramakrishna, A. Rastogi, N. Trehanpati et al., "From cirrhosis to hepatocellular carcinoma: new molecular insights on inflammation and cellular senescence," *Liver Cancer*, vol. 2, pp. 367–383, 2013.

[6] L. Dara, C. Ji, and N. Kaplowitz, "The contribution of endoplasmic reticulum stress to liver diseases," *Hepatology*, vol. 53, no. 5, pp. 1752–1763, 2011.

[7] H. Malhi and R. J. Kaufman, "Endoplasmic reticulum stress in liver disease," *Journal of Hepatology*, vol. 54, no. 4, pp. 795–809, 2011.

[8] C. Xu, B. Bailly-Maitre, and J. C. Reed, "Endoplasmic reticulum stress: cell life and death decisions," *Journal of Clinical Investigation*, vol. 115, no. 10, pp. 2656–2664, 2005.

[9] J. Su, L. Zhou, X. Kong et al., "Endoplasmic reticulum is at the crossroads of autophagy, inflammation, and apoptosis signaling pathways and participates in the pathogenesis of diabetes mellitus," *Journal of Diabetes Research*, vol. 2013, Article ID 193461, 6 pages, 2013.

[10] L. Moretti, Y. I. Cha, K. J. Niermann, and B. Lu, "Switch between apoptosis and autophagy: radiation-induced endoplasmic reticulum stress?" *Cell Cycle*, vol. 6, no. 7, pp. 793–798, 2007.

[11] M. Moenner, O. Pluquet, M. Bouchecareilh, and E. Chevet, "Integrated endoplasmic reticulum stress responses in cancer," *Cancer Research*, vol. 67, no. 22, pp. 10631–10634, 2007.

[12] M. Shuda, N. Kondoh, N. Imazeki et al., "Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis," *Journal of Hepatology*, vol. 38, no. 5, pp. 605–614, 2003.

[13] I. Kim, W. Xu, and J. C. Reed, "Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities," *Nature Reviews Drug Discovery*, vol. 7, no. 12, pp. 1013–1030, 2008.

[14] A. Moeini, H. Cornella, and A. Villanueva, "Emerging signaling pathways in hepatocellular carcinoma," *Liver Cancer*, vol. 1, pp. 83–93, 2012.

[15] P. di Fazio, M. Ocker, and R. Montalbano, "New drugs, old fashioned ways: ER stress induced cell death," *Current Pharmaceutical Biotechnology*, vol. 13, no. 11, pp. 2228–2234, 2012.

[16] W.-H. Chang, C.-H. Chen, and F.-J. Lu, "Different effects of baicalein, baicalin and wogonin on mitochondrial function, glutathione content and cell cycle progression in human hepatoma cell lines," *Planta Medica*, vol. 68, no. 2, pp. 128–132, 2002.

[17] W.-H. Chang, C.-H. Chen, R.-J. Gau et al., "Effect of baicalein on apoptosis of the human Hep G2 cell line was induced by mitochondrial dysfunction," *Planta Medica*, vol. 68, no. 4, pp. 302–306, 2002.

[18] Y.-H. Zheng, L.-H. Yin, T. H. M. Grahn, A.-F. Ye, Y.-R. Zhao, and Q.-Y. Zhang, "Anticancer effects of baicalein on hepatocellular carcinoma cells," *Phytotherapy Research*, 2014.

[19] X.-M. Xu, G.-J. Yuan, J.-J. Deng et al., "Inhibition of 12-lipoxygenase reduces proliferation and induces apoptosis of hepatocellular carcinoma cells in vitro and in vivo," *Hepatobiliary and Pancreatic Diseases International*, vol. 11, no. 2, pp. 193–202, 2012.

[20] M. Xu, N. Lu, H. Zhang et al., "Wogonin induced cytotoxicity in human hepatocellular carcinoma cells by activation of unfolded protein response and inactivation of AKT," *Hepatology Research*, vol. 43, no. 8, pp. 890–905, 2013.

[21] C. C. Chou, S. L. Pan, C. M. Teng, and J. H. Guh, "Pharmacological evaluation of several major ingredients of Chinese herbal medicines in human hepatoma Hep3B cells," *European Journal of Pharmaceutical Sciences*, vol. 19, no. 5, pp. 403–412, 2003.

[22] K. Chen, S. Zhang, Y. Ji et al., "Baicalein inhibits the invasion and metastatic capabilities of hepatocellular carcinoma cells via down-regulation of the ERK pathway," *PLoS ONE*, vol. 8, no. 9, Article ID e72927, 2013.

[23] R.-R. Liang, S. Zhang, J.-A. N. Qi et al., "Preferential inhibition of hepatocellular carcinoma by the flavonoid Baicalein through blocking MEK-ERK signaling," *International Journal of Oncology*, vol. 41, no. 3, pp. 969–978, 2012.

[24] Y.-W. Chiu, T.-H. Lin, W.-S. Huang et al., "Baicalein inhibits the migration and invasive properties of human hepatoma cells," *Toxicology and Applied Pharmacology*, vol. 255, no. 3, pp. 316–326, 2011.

[25] Y.-H. Shi, Z.-B. Ding, J. Zhou et al., "Targeting autophagy enhances sorafenib lethality for hepatocellular carcinoma via ER stress-related apoptosis," *Autophagy*, vol. 7, no. 10, pp. 1159–1172, 2011.

[26] W.-X. Ding, H.-M. Ni, W. Gao et al., "Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival," *Journal of Biological Chemistry*, vol. 282, no. 7, pp. 4702–4710, 2007.

[27] Z. Wang, Y. Cao, C. Jiang, G. Yang, J. Wu, and Y. Ding, "Lack of association of two common polymorphisms rs2910164 and rs11614913 with susceptibility to hepatocellular carcinoma: a meta-analysis," *PLoS ONE*, vol. 7, no. 6, Article ID e40039, 2012.

[28] J. M. Llovet, M. Schwartz, and V. Mazzaferro, "Resection and liver transplantation for hepatocellular carcinoma," *Seminars in Liver Disease*, vol. 25, no. 2, pp. 181–200, 2005.

[29] Y. C. Shen, Z. Z. Lin, C. H. Hsu et al., "Clinical trials in hepatocellular carcinoma: an update," *Liver Cancer*, vol. 2, pp. 345–364, 2013.

[30] J. Shindoh, A. Kaseb, and J. N. Vauthey, "Surgical strategy for liver cancers in the era of effective chemotherapy," *Liver Cancer*, vol. 2, pp. 47–54, 2013.

[31] V. di Marco, F. de Vita, J. Koskinas, D. Semela, P. Toniutto, and C. Verslype, "Sorafenib: from literature to clinical practice," *Annals of Oncology*, vol. 24, supplement 2, pp. ii30–ii37, 2013.

[32] G. Zhang, Z. Wang, W. Luo, H. Jiao, J. Wu, and C. Jiang, "Expression of potential cancer stem cell marker ABCG2 is associated with malignant behaviors of hepatocellular carcinoma," *Gastroenterology Research and Practice*, vol. 2013, Article ID 782581, 12 pages, 2013.

[33] Z. Wang, J. Wu, G. Zhang, Y. Cao, C. Jiang, and Y. Ding, "Associations of miR-499 and miR-34b/c polymorphisms with susceptibility to hepatocellular carcinoma: an evidence-based evaluation," *Gastroenterology Research and Practice*, vol. 2013, Article ID 719202, 8 pages, 2013.

[34] R. Finn, "Emerging targeted strategies in advanced hepatocellular carcinoma," *Seminars in Liver Disease*, vol. 33, supplement 1, pp. S11–S19, 2013.

[35] Y. Gong, "Identifying the targets for treatment of liver fibrosis and hepatocellular carcinoma from both Western medicine and Chinese medicine," *Chinese Journal of Integrative Medicine*, vol. 18, no. 4, pp. 245–249, 2012.

[36] Y. Chen and J. Zhu, "Anti-HBV effect of individual traditional Chinese herbal medicine in vitro and in vivo: an analytic review," *Journal of Viral Hepatitis*, vol. 20, no. 7, pp. 445–452, 2013.

[37] K. Okita, Q. Li, T. Murakamio, and M. Takahashi, "Anti-growth effects with components of Sho-saiko-to (TJ-9) on cultured human hepatoma cells," *European Journal of Cancer Prevention*, vol. 2, no. 2, pp. 169–175, 1993.

[38] Y. Motoo and N. Sawabu, "Antitumor effects of saikosaponins, baicalin and baicalein on human hepatoma cell lines," *Cancer Letters*, vol. 86, no. 1, pp. 91–95, 1994.

[39] C. Yu, F. Qu, Y. Mao et al., "Different extraction pretreatments significantly change the flavonoid contents of *Scutellaria baicalensis*," *Pharmaceutical Biology*, vol. 51, no. 10, pp. 1228–1235, 2013.

[40] Y. Matsuzaki, N. Kurokawa, S. Terai, Y. Matsumura, N. Kobayashi, and K. Okita, "Cell death induced by baicalein in human hepatocellular carcinoma cell lines," *Japanese Journal of Cancer Research*, vol. 87, no. 2, pp. 170–177, 1996.

[41] H.-M. Kuo, H.-C. Tsai, Y.-L. Lin et al., "Mitochondrial-dependent caspase activation pathway is involved in baicalein-induced apoptosis in human hepatoma J5 cells," *International Journal of Oncology*, vol. 35, no. 4, pp. 717–724, 2009.

[42] Y. Kimata and K. Kohno, "Endoplasmic reticulum stress-sensing mechanisms in yeast and mammalian cells," *Current Opinion in Cell Biology*, vol. 23, no. 2, pp. 135–142, 2011.

[43] S. E. Logue, P. Cleary, S. Saveljeva, and A. Samali, "New directions in ER stress-induced cell death," *Apoptosis*, vol. 18, no. 5, pp. 537–546, 2013.

[44] H. P. Harding, Y. Zhang, and D. Ron, "Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase," *Nature*, vol. 397, pp. 271–274, 1999.

[45] H. P. Harding, I. Novoa, Y. Zhang et al., "Regulated translation initiation controls stress-induced gene expression in mammalian cells," *Molecular Cell*, vol. 6, no. 5, pp. 1099–1108, 2000.

[46] X.-Z. Wang, H. P. Harding, Y. Zhang, E. M. Jolicoeur, M. Kuroda, and D. Ron, "Cloning of mammalian Ire1 reveals diversity in the ER stress responses," *EMBO Journal*, vol. 17, no. 19, pp. 5708–5717, 1998.

[47] Y.-R. Chen, C. F. Meyer, and T.-H. Tan, "Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in γ radiation-induced apoptosis," *Journal of Biological Chemistry*, vol. 271, no. 2, pp. 631–634, 1996.

[48] F. J. Guo, Z. Xiong, X. Lu, M. Ye, X. Han, and R. Jiang, "ATF6 upregulates XBP1S and inhibits ER stress-mediated apoptosis in osteoarthritis cartilage," *Cellular Signalling*, vol. 26, no. 2, pp. 332–342, 2014.

[49] K. H. Tay, Q. Luan, A. Croft et al., "Sustained IRE1 and ATF6 signaling is important for survival of melanoma cells undergoing ER stress," *Cellular Signalling*, vol. 26, pp. 287–294, 2014.

[50] J. Groenendyk and M. Michalak, "Endoplasmic reticulum quality control and apoptosis," *Acta Biochimica Polonica*, vol. 52, no. 2, pp. 381–395, 2005.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 516518, 9 pages
http://dx.doi.org/10.1155/2014/516518

Research Article

BAG3 and HIF-1α Coexpression Detected by Immunohistochemistry Correlated with Prognosis in Hepatocellular Carcinoma after Liver Transplantation

Heng Xiao,[1,2] Rongliang Tong,[1,2] Shaobing Cheng,[1,2] Zhen Lv,[1] Chaofeng Ding,[1] Chengli Du,[1] Haiyang Xie,[2] Lin Zhou,[2] Jian Wu,[2] and Shusen Zheng[1,2]

[1] *Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China*
[2] *Key Lab of Combined Multi-Organ Transplantation, Ministry of Public Health, Hangzhou 310003, China*

Correspondence should be addressed to Jian Wu; drwujian@hotmail.com and Shusen Zheng; shusenzheng@zju.edu.cn

Received 28 February 2014; Revised 16 April 2014; Accepted 18 April 2014; Published 8 May 2014

Academic Editor: Jingmin Zhao

Objective. The objective is to determine the effects of BAG3 and HIF-1α expression on the prognosis of HCC patients after liver transplantation. *Methods*. Samples from 31 patients with HCC receiving liver transplantation were collected for this study. The immunohistochemistry was used to detect the expression of BAG3 and HIF-1α of HCC samples. *Results*. According to the immunohistochemistry results, BAG3 and HIF-1α staining were significantly associated with tumor TNM stage ($P = 0.004$, $P = 0.012$). A significant association between high BAG3/HIF-1α levels and a shorter overall survival was detected, so as the combined BAG3 and HIF-1α analysis. *Conclusion*. The results suggested that the expression level of BAG3 and HIF-1α is efficient prognostic parameters in patients with HCC after liver transplantation.

1. Introduction

Hepatocellular carcinoma (HCC), a highly vascular tumor, is the third leading cause of cancer death worldwide and the second in China [1, 2]. Because existing therapies are insufficient for the high frequency of tumor recurrence after liver transplantation, the prognosis of HCC patients remains pessimistic. So it is important to establish the identity of new targets for therapeutic approach that will improve the prognosis of HCC patients after liver transplantation.

BAG3 can interact with different partners through a BAG domain, a WW domain, and a proline-rich repeat [3]. As a multifaceted protein, BAG3 regulates many biological processes and impacts the progression of tumor in different ways. The expression of BAG3 was reported that related to survival, apoptosis, motility and adhesion, angiogenesis, and epithelial-mesenchymal transition of human neoplastic cells [3–9]. In most studies, BAG3 is demonstrated as a protein favoring tumor progression.

In our previous research, a decreased expression of HIF-1α was observed in knockdown of BAG3 by western blot [10]. As an important regulator in hypoxia adaptation, HIF-1α regulates proliferation, apoptosis, metastasis, inflammation, and angiogenesis in tumors [11, 12]. In a report of Dai et al., HIF-1α was found to affect the inflammation and angiogenesis of HCC. The expression level of HIF-1α was also observed associated with the development and prognosis of HCC in that study [13]. The value of HIF-1α in predicting prognosis of HCC was supported by some researchers [14–16]. However, there are also evidences that object to it [17, 18].

In the present work we have evaluated the expression of BAG3 and HIF-1α in HCC tissue and analyzed the prognosis of HCC after liver transplantation and we hope to find some

Table 1: Relationship between BAG3/HIF-1α expression and clinicopathologic features.

Variable	BAG3 density		P value	HIF-1α density		P value
	Low-BAG3	High-BAG3		Low-HIF-1α	High-HIF-1α	
In general						
Tumor tissue	14	17		13	18	
Sex						
Male	12	16	0.425	12	16	0.624
Female	2	1		1	2	
Age (years)						
≦50	8	9	0.551	9	8	0.158
>50	6	8		4	10	
Tumor size (cm)						
≦5	9	4	**0.027***	7	6	0.22
>5	5	13		6	12	
AFP (ng/mL)						
≦400	8	12	0.343	8	12	0.343
>400	6	5		5	6	
HBsAg						
Positive	12	14	0.597	12	14	0.285
Negative	2	3		1	4	
Anti-HCV						
Positive	1	1	0.708	1	1	0.671
Negative	13	16		12	17	
Vascular invasion						
Yes	6	12	0.117	6	12	0.22
No	8	5		7	6	
TNM stage						
I-II	10	3	**0.004***	9	4	**0.012***
III-IV	4	14		4	14	
Tumor differentiation						
I-II	2	3	0.597	2	3	0.659
III-IV	12	14		11	15	
TACE or RFA using before LT						
Yes	5	7	0.525	4	8	0.347
No	9	10		9	10	
Rapamycin using after LT						
Yes	2	4	0.429	2	4	0.501
No	12	13		11	14	

AFP: α-fetoprotein. TACE: transcatheter arterial chemoembolization. RFA: radiofrequency ablation.
* $P < 0.05$.

new molecular targets that will improve the prognosis of HCC patients after liver transplantation.

2. Materials and Methods

2.1. Cell Culture. Eight human HCC cell lines (HepG2, Huh-7, Bel-7402, SK-Hep-1, SMMC-7721, MHCC-97L, MHCC-97H, and MHCC-LM3) and one immortalized liver cell lines (L-02) were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences, and were cultivated as described by the suppliers. Cell lines treatment was as follows: hypoxia (O_2 of 1%) in a hypoxia chamber for 72 hours or add $CoCl_2$ (300 μmol/L) to the medium to induce hypoxia condition.

2.2. Western Blot and RT-PCR. Western blot and RT-PCR were performed as described previously [10].

2.3. Study Subjects. Samples from 40 patients with HCC receiving liver transplantation at our hospital (First Affiliated Hospital, Zhejiang University School of Medicine, Zhejiang, China) between 2005 and 2010 were collected for this study. Letters of consent were obtained from all patients, and the experimental protocols were approved by the local ethics

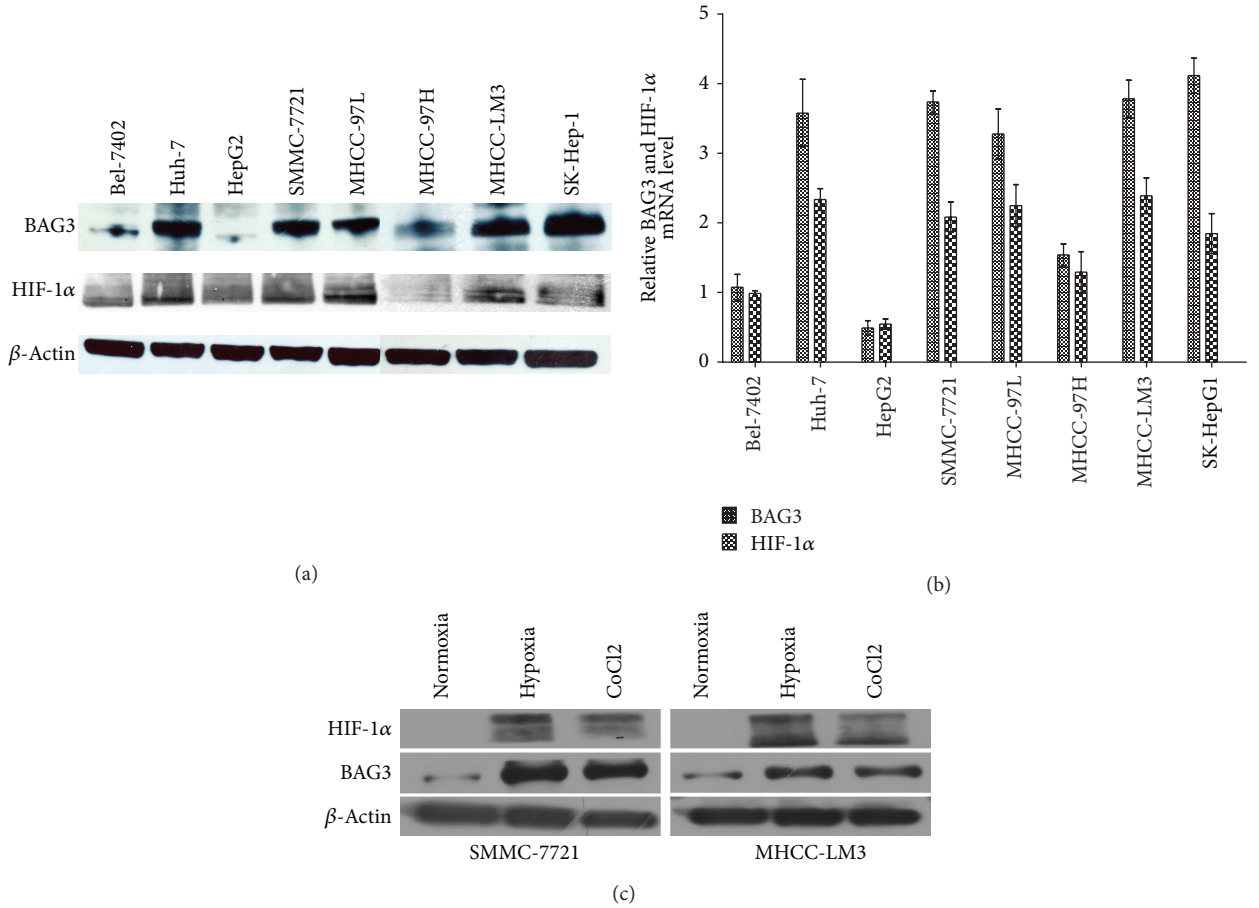


Figure 1: (a) BAG3 and HIF-1α expressions were evaluated in the indicated cell lines by western blot. (b) BAG3 and HIF-1α expressions were evaluated in the indicated cell lines by RT-PCR. (c) BAG3 and HIF-1α expressions of HCC cell lines under hypoxia were measured using western blot.

committee. Patient charts were reviewed to obtain clinical data about age, gender, tumor size, AFP, HBsAg, vascular invasion, TNM stage (AJCC), tumor differentiation, TACE and RFA usage before LT, Rapamycin usage after LT, time of recurrence, and death or time of last followup. Patient survival was calculated from the day of surgery until death, in months. However, 5 patients had cholangiocarcinoma, and 4 patients lost to followup. So we just analyzed 31 patients at last.

2.4. Immunohistochemistry. The paraffin-embedded tissue as that used for the HE-stained section was chosen for immunohistochemistry. They were cut at 3 um, deparaffinized in xylene, and rehydrated in a series of graded alcohol dilutions. Heat epitope retrieval was done for 20 minutes in target-retrieval solution at pH 7.5. Sections were incubated with a rabbit monoclonal antibody to human BAG3 (cat. number: 2783-1, Epitomics-an abcam company, Cambridge, MA, USA; dilution 1/1000) at dilution of 1 : 100 and with a rabbit monoclonal antibody to human HIF-1α (cat. number: 2015-1, Epitomics-an abcam company, Cambridge, MA, USA; dilution 1/1000) at dilution of 1 : 200 overnight at 4C°. Slides were then incubated with HRP at room temperature for 30 minutes and were visualized using DAB as chromogen for 5–10 minutes.

Sections were scored semiquantitatively as follows [19]: (negative), 0% immunoreactive cells; + ≦5% immunoreactive cells; ++ >5–50% immunoreactive cells; +++ ≧50 immunoreactive cells. For statistical purposes, cases with scores 0 and + were considered low expression and those with scores ++ and +++ were considered high expression.

2.5. Statistical Analysis. The data were performed using SPSS version 17.0. The chi-square test or Fisher's exact test was used to evaluate any potential association between the BAG3/HIF-1α expression and the clinicopathologic parameters. Overall survival and tumor-free survival rates were calculated with the Kaplan-Meier method, and the statistical difference between survival curves was determined with the log-rank test. Statistical significance was accepted if $P < 0.05$.

3. Results

3.1. Clinicopathologic Characteristics of Patients Included in the Study. The study included tumors from 31 patients (28 males and 3 females). Patients' characteristics are shown in Table 1.

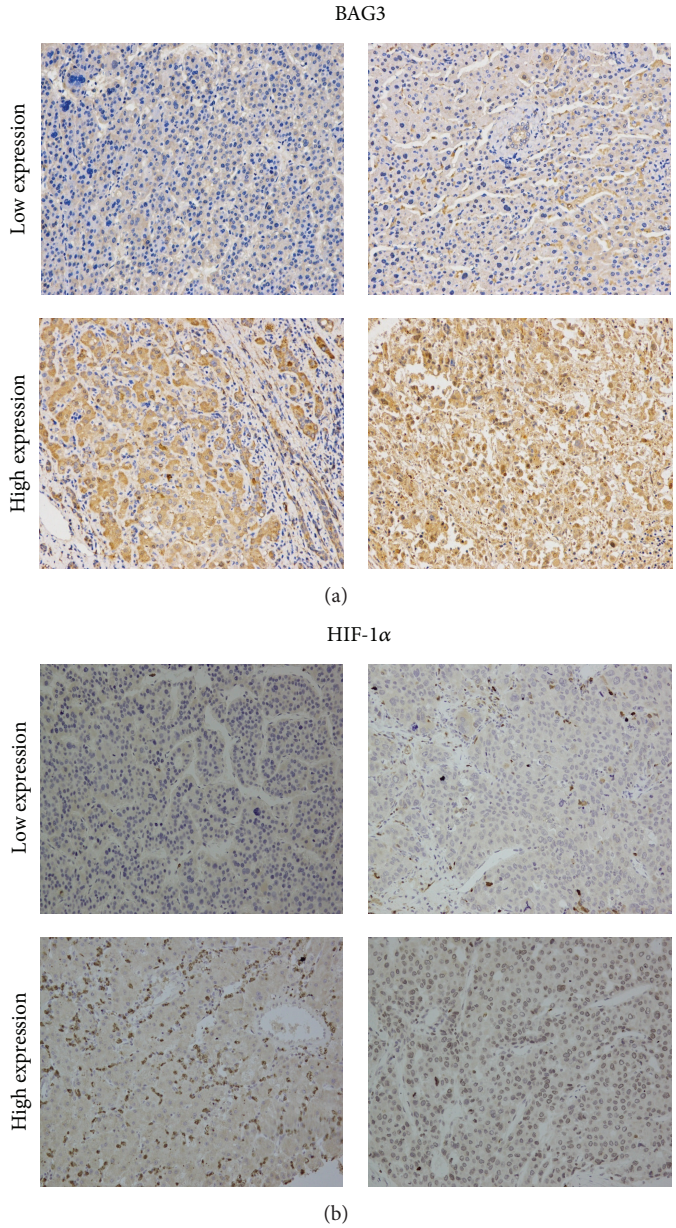


Figure 2: HCC samples were immunostained with BAG3 and HIF-1α antibody (×200). (a) Protein expression of BAG3 (up, low-expression; down, high-expression). (b) Protein expression of HIF-1α (up, low-expression; down, high-expression).

BAG3 staining was significantly associated with tumor size ($P = 0.027$) and tumor TNM stage ($P = 0.004$). Like BAG3, HIF-1α staining was significantly associated with tumor TNM stage ($P = 0.012$), but not with tumor size ($P = 0.22$). High expression of BAG3 and HIF-1α assessed was found as follows: BAG3 in 17 (54.8%) cases and HIF-1α in 18 (58.1%) cases.

3.2. BAG3 and HIF-1α Expression in the HCC Cell Lines. To prove the significance of the above clinical data, we examined the BAG3 and HIF-1α expression in the eight HCC cell lines. We found that HIF-1α showed the same change with the level of BAG3 expression (Figures 1(a) and 1(b)). To determine the relationship between BAG3 and HIF-1α, SMMC-7721 and MHCC-LM3 were cultured under the hypoxia condition. As shown in Figure 1(c), both BAG3 and HIF-1α expressions were increased under hypoxia condition.

3.3. Survival Analysis in Patients with HCC after Liver Transplantation. A low and high staining reaction of BAG3 in patients with HCC is shown in Figure 2(a). The 5-year overall survival rate for patients with low expression of BAG3 and for patients with high expression of BAG3 was 52.4% and 23.5% ($P = 0.021$), respectively. The 5-year tumor-free survival rate for patients with low expression of BAG3 and for patients with

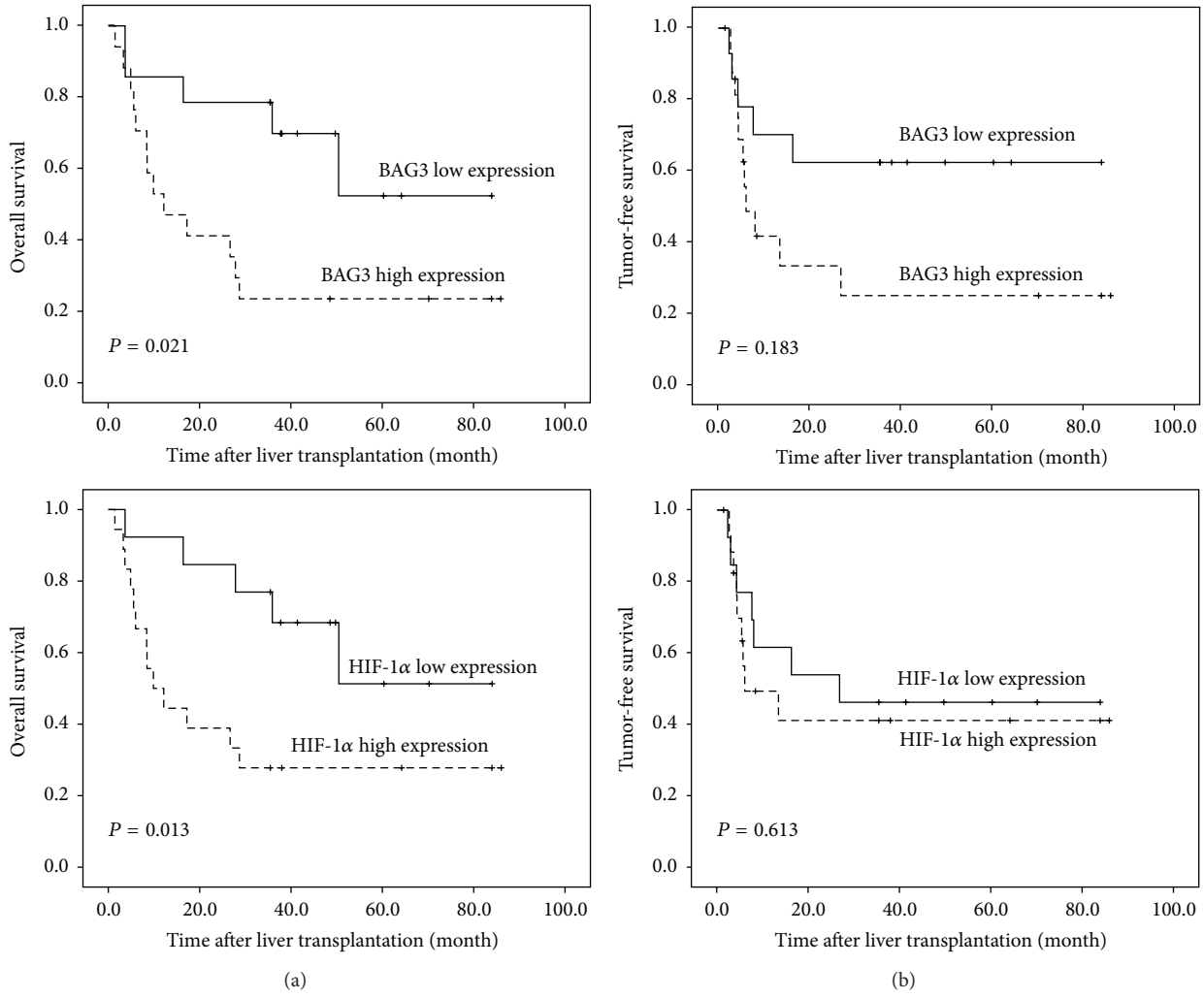


Figure 3: (a) BAG3 and HIF-1α overall survival rate. (b) BAG3 and HIF-1α tumor-free survival rate.

Table 2: Relationship between BAG3 expression and survival rate.

Survival measurement	BAG3 density		P value
	low-BAG3	high-BAG3	
1-year overall survival (%)	85.7 ± 9.4	52.9 ± 12.1	**0.021***
5-year overall survival (%)	52.4 ± 17.9	23.5 ± 10.3	
1-year tumor-free survival (%)	70.1 ± 12.6	41.7 ± 12.7	0.183
5-year tumor-free survival (%)	62.3 ± 13.4	25.0 ± 11.9	

$^*P < 0.05$.

Table 3: Relationship between HIF-1α expression and survival rate.

Survival measurement	HIF-1α density		P value
	low-HIF-1α	high-HIF-1α	
1-year overall survival (%)	92.3 ± 7.4	50.0 ± 11.8	**0.013***
5-year overall survival (%)	51.3 ± 17.8	27.8 ± 10.6	
1-year tumor-free survival (%)	61.5 ± 13.5	49.3 ± 12.8	0.613
5-year tumor-free survival (%)	46.2 ± 13.8	41.1 ± 13.0	

$^*P < 0.05$.

high expression of BAG3 was 62.3% and 25.0% ($P = 0.183$), respectively (Table 2).

A staining reaction of HIF-1α in patients with HCC is shown in Figure 2(b) like BAG3 (51.3% versus 27.8%, $P = 0.013$), but not tumor-free survival rate ($P = 0.613$) (Table 3).

A Kaplan-Meier curve regarding the association between BAG3 and HIF-1α staining and overall and tumor-free survival is shown in Figure 3.

3.4. Combined BAG3 and HIF-1α Analysis. The frequency of BAG3 and HIF-1α staining in HCC is shown in Table 4. A significant correlation was observed between BAG3 and HIF-1α staining ($P = 0.009$). Tissue analysis revealed a correlation of BAG3 with HIF-1α ($r = 0.815$, $P = 0.000$), and HIF-1α showed the same change with the level of BAG3 expression in most of the tumor tissue (Figures 4(a) and 4(b)). Tumors were divided into two groups according to the BAG3 and HIF-1α expression (Figure 4(c)). Group A ($n = 13$) tumors had both BAG3 and HIF-1α low expression levels;

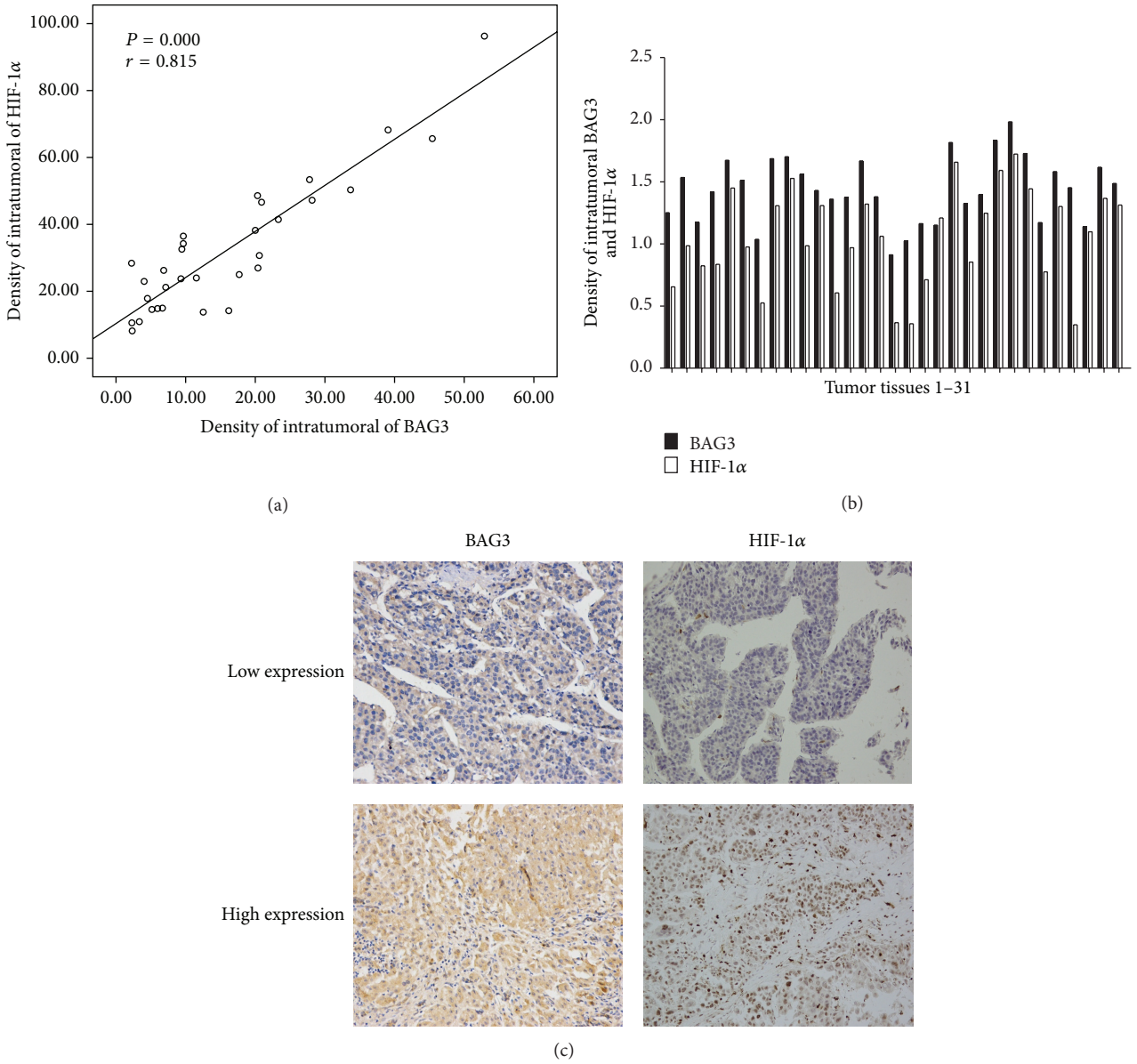


Figure 4: (a) Correlation between BAG3 expression and HIF-1α level was examined in tumor tissue derived from 31 patients. (b) Protein levels of BAG3 and HIF-1α were determined in 31 HCC samples. (c) Representative serial sections (one sample) of HCC immunostained for BAG3 and HIF-1α (×200).

group B ($n = 12$) had both BAG3 and HIF-1α high expression levels. Patients in group A had either worse overall survival or shorter tumor-free survival rate than group B (Figure 5) ($P = 0.007$, $P = 0.185$, resp.). Consistently, the 1-year and 5-year overall survival and tumor-free survival rate after liver transplantation were better for group A than group B (Table 5).

Table 4: Correlation of BAG3 and HIF-1α in HCC.

HIF-1α	BAG3			
	Negative	+	++	+++
Negative	0	4	0	0
+	0	6	2	1
++	0	4	0	4
+++	0	0	1	9

Kendall's correlation coefficient, $P = 0.009$.

4. Discussion

BAG3 was found highly expressed, compared with normal human cells or tissue, in many solid tumors, such as HCC [10], nonsmall cell lung cancer [8], thyroid carcinoma [9], pancreatic cancer [20], glioblastoma [21], and colorectal carcinomas [22]. In most studies, BAG3 is demonstrated as a protein favoring tumor progression. However, there are also

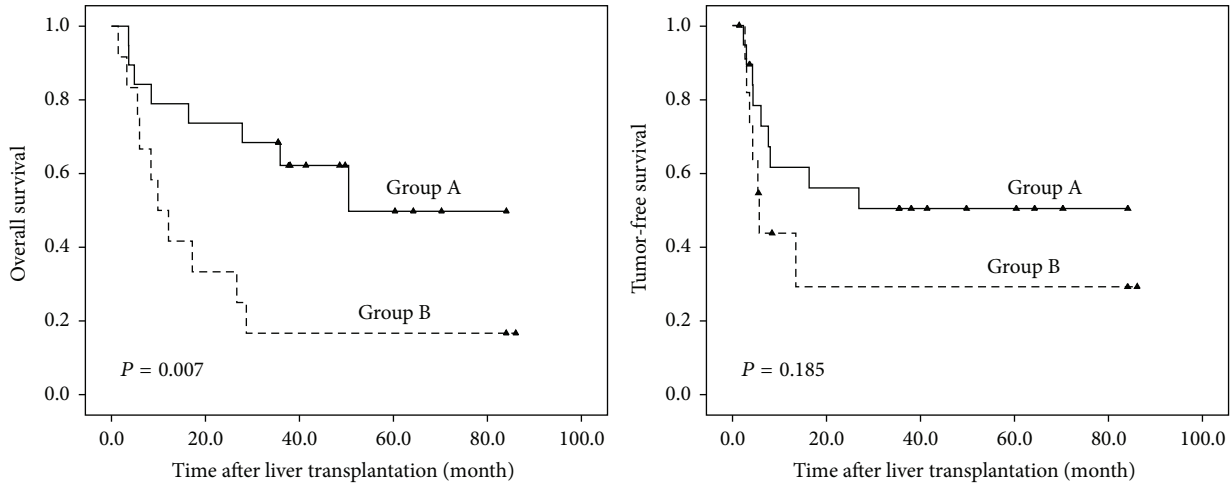


Figure 5: Overall survival and tumor-free survival rate of patients with HCC after liver transplantation in relation to coexpression of BAG3 and HIF-1α. Group A tumors had both BAG3 and HIF-1α low expression levels; group B had both BAG3 and HIF-1α high expression levels.

Table 5: Relationship between HIF-1α and BAG3 coexpression and survival rate.

Survival measurement	BAG3/HIF-1α density		P value
	low-BAG3/HIF-1α	high-BAG3/HIF-1α	
1-year overall survival (%)	90.0 ± 9.5	42.9 ± 13.2	**0.007***
5-year overall survival (%)	45.7 ± 21.2	14.3 ± 9.4	
1-year tumor-free survival (%)	60.0 ± 15.5	35.9 ± 13.9	0.185
5-year tumor-free survival (%)	50.0 ± 15.8	23.9 ± 13.5	

$^*P < 0.05$.

some opposite evidences. In a recently study, De-Hui Kong et al. observed a role of BAG3 in preventing antiapoptotic effect [23]. Li et al. reported that BAG3 could stabilize JunD mRNA to promote growth inhibition mediated by serum starvation [24]. In addition, a previous research also supported the antiproliferative function of BAG3 in human promyelocytic leukemia HL-60 cells [25]. However, a high expression of BAG3 is not equal to poor prognosis. With the results of an immunohistochemical study of prostate carcinoma, the expression level of BAG3 was observed to progressively increase from low- to well-differentiated carcinoma. That study also demonstrated that a low expression level of BAG3 predicted a poor prognosis of patients with prostate carcinoma [19]. As a conclusion, the role of BAG3 in tumors is not that clear.

A recent research by us focused on the role of BAG3 in HCC and demonstrated that BAG3 promoted epithelial–mesenchymal transition, tumor growth, invasiveness, and angiogenesis of HCC [10]. In that study, we also found that the HIF-1α signaling pathway might be included in the mechanisms of BAG3 in regulating the metastasis and angiogenesis of HCC cells. However, the conjecture has not been proved yet.

With the observations above, we evaluated the expression of BAG3 and HIF-1α in HCC cell lines by western blot and RT-PCR; we found that HIF-1α showed the same change with the level of BAG3 expression. Meanwhile, we detected the BAG3 and HIF-1α expression in HCC tissue by immunohistochemistry and tried to observe their role in predicting the prognosis of patients who were diagnosed as HCC and received liver transplantation. The high expression of BAG3 and the high expression of HIF-1α are both demonstrated relating to later TNM stage and poor overall survival. Compared with HIF-1α, BAG3 shows a better correlation with tumor size. In addition, our study is the first to demonstrate the value of BAG3 and HIF-1α in predicting the prognosis of HCC in the field of liver transplantation.

In this study, we also observed the positive correlation between the expression of BAG3 and HIF-1α in HCC. And when HCC cell lines were cultured under hypoxia condition, we found that both HIF-1α and BAG3 protein levels were significantly increased. Consistent with this result, a decreased expression of HIF-1α was observed in knockdown of BAG3 by western blot in our previous research [10]. Furthermore, HIF-1α was reported that could bind to and transcriptional upregulate HSP70-2 (also known as heat shock 70 kDa protein 1B), and BAG3 protein is a cochaperone that interacts with the ATPase domain of Hsp 70 [3, 26]. All of these evidences indicated that the mechanisms of BAG3 in impacting the progression of tumor may involve the HIF-1α signaling pathway. However, further studies and more evidences are necessary to make the mechanism clear.

The limitations of our study are listed as follows. Firstly, a significant correlation between tumor-free survival and

the expression level of BAG3 and/or HIF-1α is not observed. This may be a result of the low number of patients. Second, an effective statistical analysis of the patients with BAG3 high-/HIF-1α low- or BAG3 low-/HIF-1α high-expression in tumor is not available. The lacking of patients could be the reason for both. Furthermore, our study is retrospective. And the interrelationship between BAG3 and HIF-1α needs further studies.

5. Conclusions

Our study is the first to demonstrate that the expression level of BAG3 and HIF-1α is efficient prognostic parameters in patients with HCC after liver transplantation.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Heng Xiao and Rongliang Tong contributed equally to this work.

Acknowledgments

This research was funded by Grants from the National High Technology Research and Development Program 863 of China (no. 2012AA021002), the Special Fund for Health Research in the Public Welfare (201302009), and Zhejiang Provincial Natural Science Foundation for Young Distinguished Scholars (no. R2110125).

References

[1] J. He, D. Gu, X. Wu et al., "Major causes of death among men and women in China," *The New England Journal of Medicine*, vol. 353, no. 11, pp. 1124–1134, 2005.

[2] R. W. C. Pang, J. W. Joh, P. J. Johnson, M. Monden, T. M. Pawlik, and R. T. P. Poon, "Biology of hepatocellular carcinoma," *Annals of Surgical Oncology*, vol. 15, no. 4, pp. 962–971, 2008.

[3] A. Rosati, V. Graziano, V. de Laurenzi, M. Pascale, and M. C. Turco, "BAG3: a multifaceted protein that regulates major cell pathways," *Cell Death and Disease*, vol. 2, no. 4, article e141, 2011.

[4] M. Suzuki, M. Iwasaki, A. Sugio et al., "BAG3 (BCL2-associated athanogene 3) interacts with MMP-2 to positively regulate invasion by ovarian carcinoma cells," *Cancer Letters*, vol. 303, no. 1, pp. 65–71, 2011.

[5] M. Iwasaki, S. Homma, A. Hishiya, S. J. Dolezal, J. C. Reed, and S. Takayama, "BAG3 regulates motility and adhesion of epithelial cancer cells," *Cancer Research*, vol. 67, no. 21, pp. 10252–10259, 2007.

[6] A. Falco, M. Festa, A. Basile et al., "BAG3 controls angiogenesis through regulation of ERK phosphorylation," *Oncogene*, vol. 31, no. 50, pp. 5153–5161, 2012.

[7] N. Li, Z. X. Du, Z. H. Zong et al., "PKCδ-mediated phosphorylation of BAG3 at Ser187 site induces epithelial-mesenchymal transition and enhances invasiveness in thyroid cancer FRO cells," *Oncogene*, vol. 32, no. 38, pp. 4539–4548, 2013.

[8] Y. Zhang, J.-H. Wang, Q. Lu, and Y.-J. Wang, "Bag3 promotes resistance to apoptosis through Bcl-2 family members in non-small cell lung cancer," *Oncology Reports*, vol. 27, no. 1, pp. 109–113, 2012.

[9] G. Chiappetta, M. Ammirante, A. Basile et al., "The antiapoptotic protein BAG3 is expressed in thyroid carcinomas and modulates apoptosis mediated by tumor necrosis factor-related apoptosis-inducing ligand," *Journal of Clinical Endocrinology and Metabolism*, vol. 92, no. 3, pp. 1159–1163, 2007.

[10] H. Xiao, S. Cheng, R. Tong et al., "BAG3 regulates epithelial-mesenchymal transition and angiogenesis in human hepatocellular carcinoma," *Laboratory Investigation*, vol. 94, no. 3, pp. 252–261, 2013.

[11] K. Hirota, "Hypoxia-inducible factor 1, a master transcription factor of cellular hypoxic gene expression," *Journal of Anesthesia*, vol. 16, no. 2, pp. 150–159, 2002.

[12] M. W. Dewhirst, "Relationships between cycling hypoxia, HIF-1, angiogenesis and oxidative stress," *Radiation Research*, vol. 172, no. 6, pp. 653–665, 2009.

[13] C.-X. Dai, Q. Gao, S.-J. Qiu et al., "Hypoxia-inducible factor-1 alpha, in association with inflammation, angiogenesis and MYC, is a critical prognostic factor in patients with HCC after surgery," *BMC Cancer*, vol. 9, article 418, 2009.

[14] S. Y. Cui, J. Y. Huang, Y. T. Chen et al., "The role of Aurora A in hypoxia-inducible factor 1alpha-promoting malignant phenotypes of hepatocelluar carcinoma," *Cell Cycle*, vol. 12, no. 17, pp. 2849–2866, 2013.

[15] Z.-L. Xiang, Z.-C. Zeng, J. Fan et al., "The expression of HIF-1α in primary hepatocellular carcinoma and its correlation with radiotherapy response and clinical outcome," *Molecular Biology Reports*, vol. 39, no. 2, pp. 2021–2029, 2012.

[16] L. Zhang, G. Huang, X. Li et al., "Hypoxia induces epithelial-mesenchymal transition via activation of SNAI1 by hypoxia-inducible factor-1alpha in hepatocellular carcinoma," *BMC Cancer*, vol. 13, no. 1, article 108, 2013.

[17] L. Ding, X.-P. Chen, and H.-P. Wang, "Expression and clinical significance of HIF-1a protein in hepatocellular carcinoma tissues," *Zhonghua Gan Zang Bing Za Zhi*, vol. 12, no. 11, pp. 656–659, 2004.

[18] G.-W. Huang, L.-Y. Yang, and W.-Q. Lu, "Expression of hypoxia-inducible factor 1α and vascular endothelial growth factor in hepatocellular carcinoma: impact on neovascularization and survival," *World Journal of Gastroenterology*, vol. 11, no. 11, pp. 1705–1708, 2005.

[19] S. Staibano, M. Mascolo, M. Di Benedetto et al., "BAG3 protein delocalisation in prostate carcinoma," *Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 31, no. 5, pp. 461–469, 2010.

[20] Q. Liao, F. Ozawa, H. Friess et al., "The anti-apoptotic protein BAG-3 is overexpressed in pancreatic cancer and induced by heat stress in pancreatic cancer cell lines," *The FEBS Letters*, vol. 503, no. 2-3, pp. 151–157, 2001.

[21] M. Festa, L. Del Valle, K. Khalili et al., "BAG3 protein is overexpressed in human glioblastoma and is a potential target for therapy," *The American Journal of Pathology*, vol. 178, no. 6, pp. 2504–2512, 2011.

[22] X. Yang, Z. Tian, W. F. Gou et al., "Bag-3 expression is involved in pathogenesis and progression of colorectal carcinomas," *Histology and Histopathology*, vol. 28, no. 9, pp. 1147–1156, 2013.

[23] D.-H. Kong, Q. Zhang, X. Meng et al., "BAG3 sensitizes cancer cells exposed to DNA damaging agents via direct interaction

with GRP78," *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research*, vol. 1833, no. 12, pp. 3245–3253, 2013.

[24] C. Li, S. Li, D. H. Kong et al., "BAG3 is upregulated by c-Jun and stabilizes JunD," *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research*, vol. 1833, no. 12, pp. 3346–3354, 2013.

[25] Y.-J. Seo, M.-H. Jeon, J.-H. Lee et al., "Bis induces growth inhibition and differentiation of HL-60 cells via up-regulation of p27," *Experimental and Molecular Medicine*, vol. 37, no. 6, pp. 624–630, 2005.

[26] W.-J. Huang, L.-M. Xia, F. Zhu et al., "Transcriptional upregulation of HSP70-2 by HIF-1 in cancer cells in response to hypoxia," *International Journal of Cancer*, vol. 124, no. 2, pp. 298–305, 2009.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 468605, 8 pages
http://dx.doi.org/10.1155/2014/468605

Research Article

Association between Two Common Polymorphisms and Risk of Hepatocellular Carcinoma: Evidence from an Updated Meta-Analysis

Zhaoming Wang,[1,2] Lei Zhang,[1] Xuesong Shi,[1] Huayu Xu,[3] Ting Wang,[4] and Jianmin Bian[1]

[1] *Department of General Surgery, The Affiliated Nanjing First Hospital with Nanjing Medical University, No. 68 Changle Road, Nanjing 210006, China*
[2] *Department of General Surgery, The First People's Hospital of Changzhou, Changzhou 213000, China*
[3] *The First Clinical Medical College, Nanjing Medical University, Nanjing 210000, China*
[4] *School of Public Health, Nanjing Medical University, Nanjing 210000, China*

Correspondence should be addressed to Jianmin Bian; jmbian0324@gmail.com

Received 23 February 2014; Accepted 27 March 2014; Published 17 April 2014

Academic Editor: Jingmin Zhao

Background. Recent studies suggested that two common polymorphisms, miR-146a G>C and miR-196a2 C>T, may be associated with individual susceptibility to hepatocellular carcinoma (HCC). However, the results remain conflicting rather than conclusive. *Object*. The aim of this study was to assess the association between miR-146a G>C and miR-196a2 C>T polymorphisms and the risk of HCC. *Methods*. A meta-analysis of 17 studies (10938 cases and 11967 controls) was performed. Odds ratios and 95% confidence intervals were used to evaluate the strength of the association. *Results*. For miR-146a G>C, the variant genotypes were associated with a decreased risk of HCC (CC versus GG: OR = 0.780 and 95% CI 0.700–0.869; GC/CC versus GG: OR = 0.865 and 95% CI 0.787–0.952; CC versus GC/GG: OR = 0.835 and 95% CI 0.774–0.901). For miR-196a2 C>T, significant association was also observed (TT versus CC: OR = 0.783, 95% CI: 0.649–0.943, and $P = 0.010$; CT versus CC: OR = 0.831, 95% CI 0.714–0.967, and $P = 0.017$; CT/TT versus CC: OR = 0.817, 95% CI 0.703–0.949, and $P = 0.008$). *Conclusion*. The two common polymorphisms miR-146a G>C and miR-196a2 C>T were associated with decreased HCC susceptibility, especially in Asian population.

1. Introduction

Liver cancer is the sixth most common cancer in the world, with 782,000 new cases diagnosed in 2012. Hepatocellular carcinoma (HCC) is its dominant histological type and accounts for 70–85% of primary liver cancer [1]. Because of the high fatality rates, its incidence approximately equals the mortality rate and nearly 53% of all liver cancer deaths worldwide were in China [2, 3]. Chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections are the major cause of HCC, but only a fraction of infected patients develop HCC during their lifetime [4, 5]. Recent studies have demonstrated that genetic alterations may be involved in the development and prognosis of HCC [6, 7].

MicroRNAs (miRNAs) are a large family of short noncoding and evolutionarily conserved RNAs (about 21–23 nucleotides) that function as negative gene regulators [8]. They exert their regulatory effects by binding to the 3′ untranslated region of target messenger RNAs (mRNAs) imperfectly, repressing target gene expression at a posttranscriptional level and inducing mRNA degradation eventually. These small molecules have been shown to play an important role in malignancy by targeting various tumor suppressors and oncogenes, taking part in cancer stem cell biology, angiogenesis, and epithelial-mesenchymal transition [9–12]. Single nucleotide polymorphism (SNP) is the most common genetic variation. SNPs in miRNA may affect the expression and function of mature miRNA and thereby influence individual susceptibility to cancer [13–15]. SNPs miR-146a G>C (rs2910164) and miR-196a2 C>T (rs11614913) are two of the most popular miRNA polymorphisms and have been shown to relate to tumorigenesis in several studies [16–20].

To date, several studies have investigated the association between the two polymorphisms miR-146a G>C and miR-196a2 C>T and hepatocellular carcinoma susceptibility. However, the results remain inconsistent rather than conclusive. In order to estimate the overall risk of miR-146a G>C and miR-196a2 C>T polymorphisms associated with hepatocellular carcinoma and to quantify the potential between study heterogeneity, we carried out a meta-analysis on all eligible case-control studies with a total of 10938 hepatocellular carcinoma cases and 11967 controls.

2. Materials and Methods

2.1. Identification and Eligibility of Relevant Studies. We searched the electronic literature from PubMed, EMBASE, Cochrane Central Register of Controlled Trials, ScienceDirect, Chinese National Knowledge Infrastructure (CNKI) databases, and Wanfang databases for all relevant reports (the last search update was February 10, 2014), using the search terms "miR-196a2 or microrna 196a2 or rs11614913 or miR-146a or microrna 146a or rs2910164," "polymorphism or variant or SNP," and "hepatocellular carcinoma or liver cancer or HCC." Publication country and publication language were not restricted in our search. In addition, studies were identified by a manual search of the reference lists of original studies. Of the studies with the same or overlapping data published by the same investigators, the most recent or complete articles with the largest sample sizes were included. In our meta-analysis, studies had to meet the following criteria: (a) evaluated the correlation between SNPs miR-146a rs2910164 and/or miR-196a2 rs11614913 and susceptibility to hepatocellular carcinoma, (b) contained available genotype frequency for both cases and controls, and (c) used a case-control design. Studies were mainly excluded for the following reasons: (a) no control population, (b) duplicating the previous publication, and (c) not for human cancer research.

2.2. Data Extraction. Two of the authors (Zhaoming Wang and Lei Zhang) extracted data independently complying with the inclusion criteria after the concealment of titles, authors, journals, supporting organizations, and funds to avoid investigators' bias. In the present study, the following variables were collected for each study: the first author's last name, year of publication, country of origin, ethnicity, source of controls (population- or hospital-based controls), genotyping method, and sample sizes of genotyped cases and controls. In the cases of conflicting evaluation, the two investigators checked the data and agreement was reached after a discussion. If disagreement still existed, senior investigator Jianmin Bian was invited to the discussion.

2.3. Statistical Analysis. For the control group of each study, the Hardy-Weinberg equilibrium (HWE) was calculated using a goodness-of-fit chi-square test. Odds ratios (ORs) with 95% confidence intervals (CIs) were used to assess the strength of association between the miR-146a rs2910164 and miR-196a2 rs11614913 polymorphism and susceptibility to HCC. The pooled ORs were performed for allele frequency comparison (miR-146a G>C: C versus G, and miR-196a2 C>T: T versus C), codominant model (miR-146a G>C: GC versus GG, CC versus GG, miR-196a2 C>T: CT versus CC, and TT versus CC), dominant model (miR-146a G>C: GC/CC versus GG, and miR-196a2 C>T: CT/TT versus CC), and recessive model (miR-146a G>C: CC versus GC/GG, and miR-196a2 C>T: TT versus CT/CC), respectively. The significance of pooled ORs was determined by Z-test and $P < 0.05$ was considered as statistically significant. The heterogeneity between the studies was assessed by Cochran's Q-test [21]. If the studies were shown to be homogeneous with a $P > 0.10$ for the Q test, the summary of OR estimate of each study was calculated using a fixed-effects model (the Mantel-Haenszel method) [22]. Otherwise, the random-effects model (the DerSimonian and Laird method) was used [23]. Sensitivity analyses were also performed to assess the stability of the results by deleting a single study in the meta-analysis each time to reflect the influence of the individual data set to the summary OR. To test the publication bias, both Funnel plots and Egger's linear regression tests were used [24]. All analyses were performed with Stata software (version 10.0; StataCorp LP, College Station, TX), using two-sided P values.

3. Results

3.1. Characteristics of Studies. There were 382 published articles relevant to the search terms (Figure 1). By choosing additional filters, 307 of these papers were excluded (243 not for hepatocellular carcinoma research, 45 not for polymorphism, and 19 not for human studies). 43 of these studies were excluded by screening the titles and abstracts. Only 32 articles were left for full text review, and among them another 15 were excluded. Finally, a total of 17 eligible studies involving 5689 cases and 6790 controls for miR-146a G>C and 10 studies involving 5249 cases and 5177 controls for miR-196a2 C>T were included in this meta-analysis [25–41]. The characteristics of the selected studies are summarized in Table 1. For miR-146a G>C, there were 12 studies on Asian population (11 Chinese and 1 Korean) and 1 study on Caucasian population (Turkish). As for miR-196a2 C>T, 9 studies were carried out on Asians (8 Chinese and 1 Korean) and one study on Caucasian (Turkish). Hepatocellular carcinomas were confirmed histologically or pathologically in most studies. All of the controls were matched with respect to ethnicity. Among them, 16 studies were population based while one was hospital based. Several genotyping methods were used in the studies, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), primer introduced restriction analysis-PCR (PIRA-PCR), PCR-ligase detection reaction (PCR-LDR), allele specific-PCR (AS-PCR), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), and MassArray. The distribution of genotypes in the controls was in agreement with HWE in all studies.

3.2. Quantitative Synthesis. As shown in Table 2, the miR-146a G>C polymorphism was significantly associated with a decreased risk of hepatocellular carcinoma in the following

Table 1: Characteristics of literatures included in the meta-analysis on hepatocellular carcinoma.

Author	Year	Country	Ethnicity	Source of controls	Genotyping method	Cases/controls	MicroRNA polymorphism	Allele frequency G/C or C/T	HWE
Xu et al. [36]	2008	China	Asian	Population	PCR-RFLP	479/504	miR-146a G>C	0.36/0.64	0.119
Xu [37]	2010	China	Asian	Population	PCR-RFLP	500/522	miR-146a G>C	0.39/0.61	0.296
						492/495	miR-196a2 C>T	0.46/0.54	0.621
Qi et al. [33]	2010	China	Asian	Population	PCR-LDR	361/391	miR-196a2 C>T	0.49/0.51	0.869
Li et al. [31]	2010	China	Asian	Hospital	PCR-RFLP	310/222	miR-196a2 C>T	0.42/0.58	0.402
Wang [34]	2011	China	Asian	Population	MALDI-TOF	1116/1869	miR-146a G>C	0.39/0.61	0.115
Akkiz et al. [25]	2011	Turkey	Caucasian	Population	PCR-RFLP	222/222	miR-146a G>C	0.80/0.20	0.384
Akkiz et al. [26]	2011	Turkey	Caucasian	Population	PCR-RFLP	185/185	miR-196a2 C>T	0.55/0.45	0.492
Zhang et al. [39]	2011	China	Asian	Population	PIRA-PCR	925/840	miR-146a G>C	0.41/0.59	0.149
						934/837	miR-196a2 C>T	0.47/0.53	0.972
Yu [38]	2012	China	Asian	Population	PCR-RFLP	100/100	miR-146a G>C	0.44/0.56	0.506
Li [32]	2012	China	Asian	Population	AS-PCR	560/560	miR-146a G>C	0.42/0.58	0.196
						560/560	miR-196a2 C>T	0.39/0.61	0.056
Kim et al. [30]	2012	Korea	Asian	Population	PCR-RFLP	159/201	miR-146a G>C	0.38/0.62	0.190
						159/201	miR-196a2 C>T	0.49/0.51	0.356
Xiang et al. [35]	2012	China	Asian	Population	PCR-RFLP	100/100	miR-146a G>C	0.44/0.56	0.506
Zhou et al. [41]	2012	China	Asian	Population	PCR-RFLP	186/483	miR-146a G>C	0.41/0.59	0.056
Huang et al. [29]	2013	China	Asian	Population	MALDI-TOF	110/110	miR-146a G>C	0.32/0.68	0.122
Zhang et al. [40]	2013	China	Asian	Population	MassArray	997/998	miR-146a G>C	0.39/0.61	0.911
						996/995	miR-196a2 C>T	0.42/0.58	0.245
Han et al. [27]	2013	China	Asian	Population	RT-PCR	1017/1009	miR-196a2 C>T	0.46/0.54	0.310
Hao et al. [28]	2013	China	Asian	Population	PCR-RFLP	235/281	miR-146a G>C	0.38/0.62	0.056
						235/281	miR-196a2 C>T	0.52/0.48	0.051

HWE: Hardy-Weinberg equilibrium; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism; PIRA: primer introduced restriction analysis; LDR: ligase detection reaction; AS: allele specific; MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight.

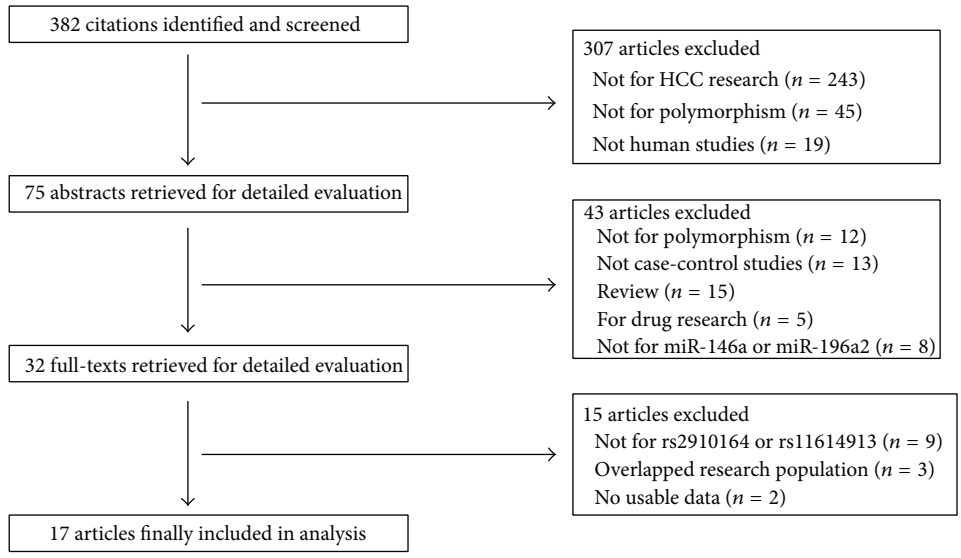


Figure 1: Articles identified with criteria for inclusion and exclusion.

Table 2: Meta-analysis of the miR-146a G>C polymorphism associated with hepatocellular carcinoma.

Comparisons	OR	95% CI	P	P_h
Overall				
C versus G	0.883	0.839–0.930	0.000	0.230
CC versus GG	0.780	0.700–0.869	0.000	0.167
GC versus GG	0.920	0.832–1.017	0.104	0.199
GC/CC versus GG	0.865	0.787–0.952	0.003	0.125
CC versus GC/GG	0.835	0.774–0.901	0.000	0.545
Asian				
C versus G	0.878	0.834–0.925	0.000	0.255
CC versus GG	0.777	0.697–0.867	0.000	0.129
GC versus GG	0.905	0.816–1.004	0.060	0.216
GC/CC versus GG	0.850	0.771–0.9373	0.001	0.159
CC versus GC/GG	0.834	0.771–0.901	0.000	0.461

P_h: P value of Q test for heterogeneity test; OR odds: odds ratio; CI: confidence interval.

models: C versus G: OR = 0.883, 95% CI 0.839–0.930, and $P = 0.000$; CC versus GG: OR = 0.780, 95% CI 0.700–0.869, and $P = 0.000$; GC/CC versus GG: OR = 0.865, 95% CI 0.787–0.952, and $P = 0.003$; CC versus GC/GG: OR = 0.835, 95% CI 0.774–0.901, and $P = 0.000$ (Figure 2(a)), and this positive association also was maintained in ethnicity subgroup analysis. 11 out of the 12 included studies were conducted in Asian population. Significant association remained in Asian population in the following genetic models: C versus G: OR = 0.878, 95% CI 0.834–0.925, and $P = 0.000$; CC versus GG: OR = 0.777, 95% CI 0.697–0.867, and $P = 0.000$; GC/CC versus GG: OR = 0.850 95% CI 0.771–0.937, and $P = 0.001$; CC versus GC/GG: OR = 0.834, 95% CI: 0.771–0.901, and $P = 0.000$ (Figure 2(b)).

For miR-196a2 C>T, the results were shown in Table 3. Association between rs11614913 polymorphism and HCC risk was observed in the following models (using the random-effects model): T versus C: OR = 0.891, 95% CI 0.815–0.974, and $P = 0.011$; TT versus CC: OR = 0.783, 95% CI: 0.649–0.943, and $P = 0.010$; CT versus CC: OR = 0.831, 95% CI 0.714–0.967, and $P = 0.017$; CT/TT versus CC: OR = 0.817, 95% CI 0.703–0.949, and $P = 0.008$. The results suggested that miR-196a2 C allele carrier may be susceptible to HCC. In subgroup analysis, there was also significant association in Asian population (using the random-effects model, T versus C: OR = 0.910, 95% CI 0.837–0.990, and $P = 0.029$; TT versus CC: OR = 0.817, 95% CI: 0.684–0.976, and $P = 0.026$; CT versus CC: OR = 0.838, 95% CI 0.712–0.986, and $P = 0.033$; CT/TT versus CC: OR = 0.833, 95% CI 0.712–0.974, and $P = 0.022$).

3.3. Heterogeneity, Sensitivity Analysis, and Publication Bias. Q-test was used in all of the genetic models to test heterogeneity. For miR-146a G>C, it showed no significant heterogeneity between studies during overall comparisons (Table 2). For miR-196a2 C>T, heterogeneity was observed in all models (Table 3): T versus C: $P_h = 0.012$ and $I^2 = 57.7\%$; TT versus CC: $P_h = 0.007$ and $I^2 = 60.6\%$; CT versus CC: $P_h = 0.025$ and $I^2 = 52.7\%$; TC/TT versus CC: $P_h = 0.012$ and $I^2 = 57.6\%$; TT versus CT/CC: $P_h = 0.094$ and $I^2 = 39.6\%$.

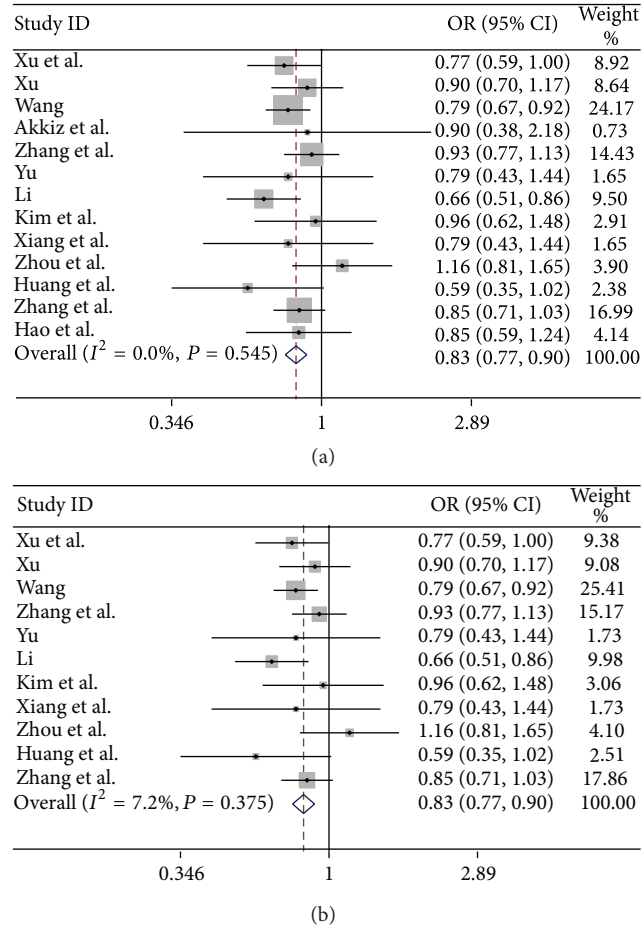


Figure 2: Forest plot of hepatocellular carcinoma risk associated with the *mir-146a G>C* polymorphism (CC versus GC/GG) in overall population (a) and in Asian population (b).

Table 3: Meta-analysis of the miR-196a2 C>T polymorphism associated with hepatocellular carcinoma.

Comparisons	OR	95% CI	P	P_h
Overall				
T versus C	0.891	0.815–0.974	0.011	0.012
TT versus CC	0.783	0.649–0.943	0.010	0.007
CT versus CC	0.831	0.714–0.967	0.017	0.025
CT/TT versus CC	0.817	0.703–0.949	0.008	0.012
TT versus TC/CC	0.907	0.807	1.020	0.094
Asian				
T versus C	0.910	0.837–0.990	0.029	0.036
TT versus CC	0.817	0.684–0.976	0.026	0.021
CT versus CC	0.838	0.712–0.98	0.033	0.017
CT/TT versus CC	0.833	0.712–0.974	0.022	0.012
TT versus TC/CC	0.935	0.846–1.032	0.181	0.270

P_h: P value of Q test for heterogeneity test; OR odds: odds ratio; CI: confidence interval; random-effects model was used when $P_h \leqq 0.10$; otherwise, fix-effects model was used.

Table 4: Metaregression analysis for heterogeneity in studies on the miR-196a2 C>T polymorphism associated with hepatocellular carcinoma.

Sort	P	τ^2	I^2
Ethnicity	0.119	0.0078	51.50%
Source of controls	0.287	0.0097	56.48%
Genotyping method	0.382	0.0134	61.50%
Year	0.976	0.1454	62.34%
Sample size	0.397	0.1194	58.59%

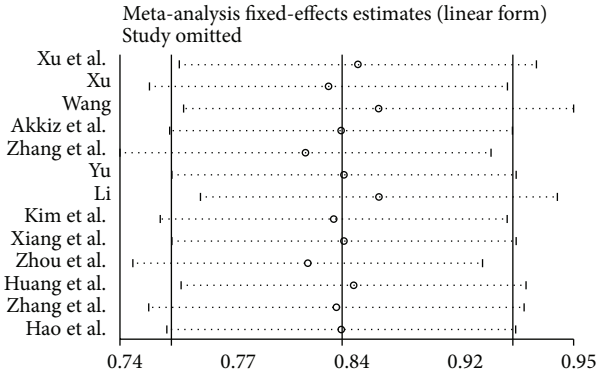


Figure 3: Sensitivity analysis for *mir-146a G>C* polymorphism with hepatocellular carcinoma (CC versus GC/GG).

Then, we assessed the source of heterogeneity by ethnicity, source of controls, genotyping methods, publication year, and sample size (subjects > 500 in both cases and controls). Using metaregression analysis, none of them could explain the significant heterogeneity (Table 4). In addition subgroup analysis was performed; substantial heterogeneity still existed when stratified by ethnicity (P_h = 0.007 and I^2 = 60.6%), source of controls (P_h = 0.009 and I^2 = 61.0%), and sample size (P_h = 0.023 and I^2 = 68.5%).

To assess the influence of each individual study on the pooled ORs, the sensitivity analysis was performed by removing a single study from meta-analysis sequentially. The results indicated that no single study influenced the pooled OR qualitatively (Figure 3). It suggested that the results of this meta-analysis were stable.

The Begg funnel plot and Egger's test were conducted to assess publication bias. The shapes of the funnel plots did not reveal any evidence of obvious asymmetry in all comparison models (Figure 4). Then, Egger's test was used to provide statistical evidence of funnel plot symmetry. The results still did not show any evidence of publication bias (t = −2.00 and P = 0.074 for miR-146a G>C and t = 1.18 and P = 0.273 for miR-196a2 C>T).

4. Discussion

miRNAs are involved in a variety of biological processes and regulate hundreds of gene targets [12]. The study of miRNAs provides a new view of the pathophysiological mechanism of the etiology and development of HCC. SNPs in miRNA

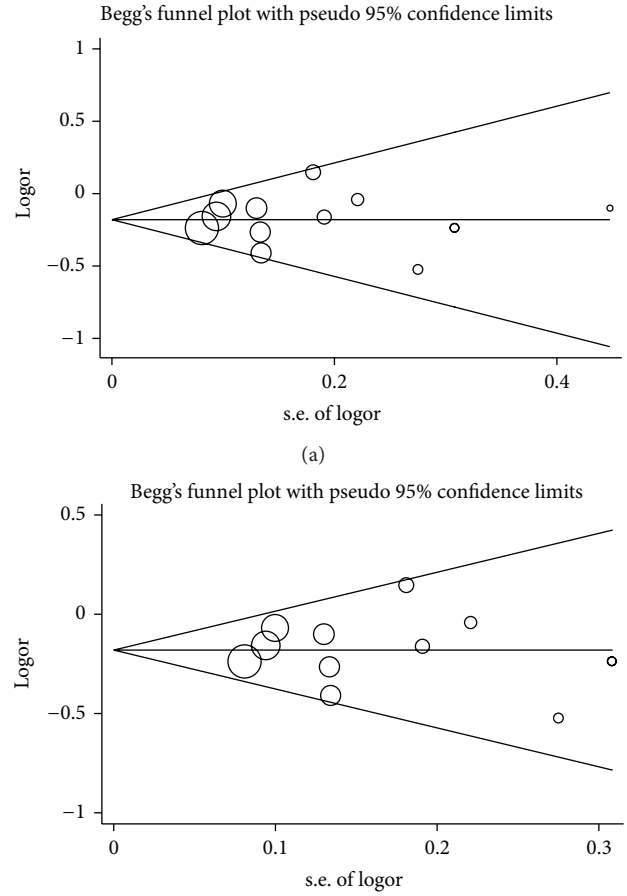


Figure 4: Begg's funnel plot for publication bias test for *mir-146a G>C* polymorphism with hepatocellular carcinoma in overall population (a) and in Asian population (b). s.e.: Standard Error; logor: logOR (logarithms of Odds Ratio).

sequence have the potential to function as new diagnostic and prognostic biomarkers for high risky population in an early stage [42, 43]. Moreover, the identification of SNPs may lead new sights to personalized therapy and small molecular interventions for liver cancer.

MiR-146a G>C, or rs2910164 polymorphism which locates in the passenger strand of miR-146a, can disturb the secondary structure and maturation of miR-146a [26, 44]. Xu et al. [36] found that target genes of miR-146a, such as tumor necrosis factor receptor-associated factor 6 and interleukin-1 receptor-associated kinase 1, are key adapter molecules downstream of the Toll-like and cytokine receptors in the signaling pathways that play crucial roles in cell growth and immune recognition. So individuals with GG genotype of miR-146a gene have an increased level of mature miR-146a and are more susceptible to carcinogens that promote HCC. Li [32] found miR-146a may target DNA repairing genes such as XRCC1, BRCA11, and XPC. G>C polymorphism can affect mature miR-146a expression and associate with HCC susceptibility. However, in contrast, Akkiz et al. [26] and Kim et al. [30] demonstrated that the rs2910164 polymorphism

had no major role in the susceptibility to HCC and they attribute their discrepancy with other studies to ethnic variation in the population. Besides, Zhou et al. [41] indicated polymorphisms of miR-146a were related to the age of onset and Child-Pugh grade in HCC but lacked association with the risk of HCC. Xiang et al. [35] and Zhang et al. [40] also observed no significant difference in ORs of the miRNA-146a variant among HCC patients.

To explain these conflicting results, our meta-analysis, which was based on eleven studies and involved 5689 cases and 6790 controls, was conducted to derive a more precise estimation of the association. Our results suggested that miR-146a G>C polymorphism was associated with decreased risk of hepatocellular carcinoma among the included studies, especially in Asian population. Cochran's Q-test and Egger's test showed no significant heterogeneity or publication bias, which indicated that our results were stable. Since miR-146a regulates hundreds of downstream gene targets, it is biologically plausible that rs2910164 polymorphism may alter the oncogenesis genetic pathway and modulate hepatocellular carcinoma risk.

MiR-196a2 C>T polymorphism is another potential SNP in relevance to HCC. It not only affected the maturation of miR-196a2 but also could enhance the cell response to mutagen challenge [31, 45, 46]. Li et al. [31] and Qi et al. [33] found that C allele carriers have a higher incidence of HCC than T allele carriers, which suggested that the C allele may confer risk to the occurrence of HCC. Studies have indicated that high expression of miR-196a2 could deregulate target genes including homeobox (HOX) gene cluster and annexinA1 (ANXA1) gene and lead to carcinogenesis and malignant transformation of HCC [25, 40, 47]. However, Li [32] and Kim et al. [30] observed no significant difference of the TT, TC, and CC genotypes distribution between HCC patients and controls. Han et al.'s study [27] also showed miR-196a2 polymorphism was not statistically associated with HCC risk, though it may enhance the effects of other SNPs in relevance to HCC.

Our meta-analysis included 9 case-control studies to assess the relationship between MiR-196a2 C>T polymorphism and HCC. The results indicated that T allele carriers had significantly lower HCC susceptibility, especially in Asian population. Identification of heterogeneity is one of the most important goals of meta-analysis and heterogeneity existed in our study. However, through subgroup analysis and meta-regression analysis we could not find the source of heterogeneity, which suggested that these included studies may be different in either clinical, methodological, or statistical components and the quantitative synthesis.

There are three similar meta-analyses about the association between the miR-146a G>C polymorphism or the miR-196a2 C>T polymorphism and the risk of hepatocellular carcinoma, but their studies showed different results from ours. Wang et al. 2012 [48] carried out a meta-analysis to estimate the relevance between these two SNPs and HCC susceptibility and it concluded that neither the rs2910164 nor rs11614913 polymorphism was associated with HCC risk. Their results were in the opposite direction to ours possibly due to the relatively small sample size. Their last search update was on September 10, 2012, and they totally identified 6 studies including 1912 cases and 2149 cases for miR-146a G>C polymorphism and 1790 cases and 1635 controls for miR-196a2 C>T polymorphism. In our study we included a total of 17 studies with 5689 cases and 6790 cases for miR-146a G>C polymorphism and 5249 cases and 5177 controls for miR-196a2 C>T polymorphism. Our sample size was much larger and could lead to the difference. Hu et al. 2013 [49] performed a meta-analysis to assess the contributions of the rs2910164 and rs3746444 polymorphisms to HCC susceptibility. Possibly because of the same reason of Wang's, their study showed no significant association. The meta-analysis of Xu et al. 2013 [50] revealed the miR-146a C variant was associated with a decreased HCC risk and it was consistent with ours. Their study only comprised a total of ten case-control studies involving 3437 cases and 3437 controls. We extracted data from all the published studies and added another 7501 cases and 8530 controls to the analysis, which accounted for 69.9% of the total sample size. Thus our results were more precise and persuasive. With regard to rs11614913, they concluded that the miR196a2 T variant was associated with a decreased risk of HCC. However, heterogeneity existed among studies. Our pooled effects were also statistically significant, but we failed to find the source of heterogeneity by subgroup analysis and metaregression analysis. So we concluded that miR-196a2 C>T polymorphism may contribute to a decreased HCC risk, but the results need to be validated by more qualified studies. In our present meta-analysis, we searched multiple databases and included all eligible studies. It contained the newest data and largest sample size. Compared with previous meta-analyses, we generate more exact and powerful pooled results of the association between SNPs miR-146a G>C and miR-196a2 C>T and risk of hepatocellular carcinoma.

There are some limitations in this meta-analysis that must be addressed. First, in the subgroup analyses, only one study originated from Caucasian, the size of which was small, and there was no African population. So our study mainly suggested the association between the two SNPs and HCC susceptibility in Asian population and may not be generalized to other ethnicities. Further studies on other ethnicities are necessary to validate the results. Second, lack of original data like HBV infection status, alcohol consumption, age, and gender from the included studies limited our further stratified analysis. HBV is one the most important risk factors to HCC [51], and the interactions among gene-gene and gene-environment may relate to cancer risk. Insufficient information prevented us from performing further evaluation. Third, heterogeneity was detected in overall comparisons of miR-196a2 C>T and we could not find its source. Though miR-196a2 C allele carrier was shown to have a higher risk of HCC in our study, more studies using standardized unbiased methods and well-matched controls are needed to draw a more persuasive conclusion. Last, as the two miRNAs have some other more SNPs than miR-146a G>C and miR-196a2 C>T, this analysis cannot tell the contribution of other polymorphisms to the risk of hepatocellular carcinoma.

In conclusion, our meta-analysis provided evidence that the two common polymorphisms miR-146a G>C and miR-196a2 C>T were associated with decreased HCC

susceptibility, especially in Asian population. Additional well-designed, large studies are warranted to validate our findings and further functional studies should be conducted to elucidate its mechanism. More sufficient data such as hepatitis infection status, gene-environment interactions, and multiethnic groups should be considered in future studies to lead to a more comprehensive understanding of the association between miR-146a G>C and miR-196a2 C>T polymorphisms and the risk of HCC.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Zhaoming Wang and Lei Zhang contributed equally to this work. Jianmin Bian designed this study.

Acknowledgment

The authors thank Meilin Wang (Department of Epidemiology, Nanjing Medical University) for critical comments and scientific editing.

References

[1] J. F. Perz, G. L. Armstrong, L. A. Farrington, Y. J. F. Hutin, and B. P. Bell, "The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide," *Journal of Hepatology*, vol. 45, no. 4, pp. 529–538, 2006.

[2] D. M. Parkin, F. Bray, J. Ferlay, and P. Pisani, "Global cancer statistics, 2002," *Ca-A Cancer Journal for Clinicians*, vol. 55, no. 2, pp. 74–108, 2005.

[3] H. B. El-Serag and K. L. Rudolph, "Hepatocellular carcinoma: epidemiology and molecular carcinogenesis," *Gastroenterology*, vol. 132, no. 7, pp. 2557–2576, 2007.

[4] F. Guerrieri, L. Belloni, N. Pediconi, and M. Levrero, "Molecular mechanisms of HBV-associated hepatocarcinogenesis," *Seminars in Liver Disease*, vol. 33, no. 2, pp. 147–156, 2013.

[5] Y. Li, L. Chenc, T. Hon Man Chan et al., "Hepatocellular carcinoma: transcriptome diversity regulated by RNA editing," *The International Journal of Biochemistry & Cell Biology*, vol. 45, no. 8, pp. 1843–1848, 2013.

[6] K. Hagymási and Z. Tulassay, "Epidimiology, risk factors and molecular pathogenesis of primary liver cancer," *Orvosi Hetilap*, vol. 149, no. 12, pp. 541–548, 2008.

[7] C. M. Wong, A. K. Kai, F. H. Tsang, and I. O. Ng, "Regulation of hepatocarcinogenesis by microRNAs," *Frontiers in Bioscience*, vol. 5, pp. 49–60, 2013.

[8] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[9] B. Zhang, X. Pan, G. P. Cobb, and T. A. Anderson, "microRNAs as oncogenes and tumor suppressors," *Developmental Biology*, vol. 302, no. 1, pp. 1–12, 2007.

[10] N. Takebe, P. J. Harris, R. Q. Warren, and S. P. Ivy, "Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways," *Nature Reviews Clinical Oncology*, vol. 8, no. 2, pp. 97–106, 2011.

[11] X. Wang, G. Hu, and J. Zhou, "Repression of versican expression by microRNA-143," *Journal of Biological Chemistry*, vol. 285, no. 30, pp. 23241–23250, 2010.

[12] Y. W. Kong, D. Ferland-McCollough, T. J. Jackson et al., "microRNAs in cancer management," *The Lancet Oncology*, vol. 13, no. 6, pp. e249–e258, 2012.

[13] O. Slaby, J. Bienertova-Vasku, M. Svoboda, and R. Vyzula, "Genetic polymorphisms and microRNAs: new direction in molecular epidemiology of solid cancer," *Journal of Cellular and Molecular Medicine*, vol. 16, no. 1, pp. 8–21, 2012.

[14] C. L. Bartels and G. J. Tsongalis, "Mini-reviews micrornas:novel biomarkers for human cancer," *Clinical Chemistry*, vol. 55, no. 4, pp. 623–631, 2009.

[15] L. Chen, H.-X. Yan, W. Yang et al., "The role of microRNA expression pattern in human intrahepatic cholangiocarcinoma," *Journal of Hepatology*, vol. 50, no. 2, pp. 358–369, 2009.

[16] H. S. Jeon, Y. H. Lee, S. Y. Lee et al., "A common polymorphism in pre-microRNA-146a is associated with lung cancer risk in a Korean population," *Gene*, vol. 534, no. 1, pp. 66–71, 2014.

[17] Y. S. Chae, J. G. Kim, S. J. Lee et al., "A miR-146a polymorphism (rs2910164) predicts risk of and survival from colorectal cancer," *Anticancer Research*, vol. 33, no. 8, pp. 3233–3239, 2013.

[18] S. S. Hasani, M. Hashemi, E. Eskandari-Nasab et al., "A functional polymorphism in the miR-146a gene is associated with the risk of childhood acute lymphoblastic leukemia: a preliminary report," *Tumor Biology*, vol. 35, no. 1, pp. 219–225, 2014.

[19] J. Wei, L. Zheng, S. Liu et al., "MiR-196a2 rs11614913 T > C polymorphism and risk of esophageal cancer in a Chinese population," *Human Immunology*, vol. 74, no. 9, pp. 1199–1205, 2013.

[20] W. J. Wei, Y. L. Wang, D. S. Li et al., "Association between the rs2910164 polymorphism in pre-Mir-146a sequence and thyroid carcinogenesis," *PLoS ONE*, vol. 8, no. 2, Article ID e56638, 2013.

[21] H. H. Handoll, "Systematic reviews on rehabilitation interventions," *Archives of Physical Medicine and Rehabilitation*, vol. 87, no. 6, article 875, 2006.

[22] N. Mantel and W. Haenszel, "Statistical aspects of the analysis of data from retrospective studies of disease," *Journal of the National Cancer Institute*, vol. 22, no. 4, pp. 719–748, 1959.

[23] R. DerSimonian and N. Laird, "Meta-analysis in clinical trials," *Controlled Clinical Trials*, vol. 7, no. 3, pp. 177–188, 1986.

[24] M. Egger, G. D. Smith, M. Schneider, and C. Minder, "Bias in meta-analysis detected by a simple, graphical test," *British Medical Journal*, vol. 315, no. 7109, pp. 629–634, 1997.

[25] H. Akkiz, S. Bayram, A. Bekar, E. Akgöllü, and Y. Ülger, "A functional polymorphism in pre-microRNA-196a-2 contributes to the susceptibility of hepatocellular carcinoma in a Turkish population: a case-control study," *Journal of Viral Hepatitis*, vol. 18, no. 7, pp. e399–e407, 2011.

[26] H. Akkiz, S. Bayram, A. Bekar, E. Akgöllü, O. Üsküdar, and M. Sandikçi, "No association of pre-microRNA-146a rs2910164 polymorphism and risk of hepatocellular carcinoma development in Turkish population: a case-control study," *Gene*, vol. 486, no. 1-2, pp. 104–109, 2011.

[27] Y. Han, R. Pu, X. Han et al., "Associations of pri-miR-34b/c and pre-miR-196a2 polymorphisms and their multiplicative interactions with hepatitis B virus mutations with hepatocellular carcinoma risk," *PLoS ONE*, vol. 8, no. 3, Article ID e58564, 2013.

[28] Y. X. Hao, J. P. Wang, and L. F. Zhao, "Associations between three common MicroRNA polymorphisms and hepatocellular carcinoma risk in Chinese," *Asian Pacific Journal of Cancer Prevention*, vol. 14, no. 11, pp. 6601–6604, 2013.

[29] Q. Huang, T.-R. Huang, J.-L. Li et al., "Correlation between microRNA-146a polymorphism and primary liver carcinoma in the Guangxi Zhuang population," *Chinese Journal of Cancer Prevention and Treatment*, no. 02, pp. 100–104, 2013.

[30] W. H. Kim, K. T. Min, Y. J. Jeon et al., "Association study of microRNA polymorphisms with hepatocellular carcinoma in Korean population," *Gene*, vol. 504, no. 1, pp. 92–97, 2012.

[31] X.-D. Li, Z.-G. Li, X.-X. Song, and C.-F. Liu, "A variant in microRNA-196a2 is associated with susceptibility to hepatocellular carcinoma in Chinese patients with cirrhosis," *Pathology*, vol. 42, no. 7, pp. 669–673, 2010.

[32] Y. Li, *MicroRNA Related SNPs and Genetic Susceptibility to Hepatocellular Carcinoma*, Zhengzhou University, 2012.

[33] P. Qi, T.-H. Dou, L. Geng et al., "Association of a variant in MIR 196A2 with susceptibility to hepatocellular carcinoma in male Chinese patients with chronic hepatitis B virus infection," *Human Immunology*, vol. 71, no. 6, pp. 621–626, 2010.

[34] W. Wang, *Association of MiR-146a Single Nucleotide Polymorphism with Susceptibility to Hepatocellular Carcinoma and the Microarray Analysis of Tumor Related MicroRNAs*, Fudan, 2011.

[35] Y. Xiang, S. Fan, J. Cao et al., "Association of the microRNA-499 variants with susceptibility to hepatocellular carcinoma in a Chinese population," *Molecular Biology Reports*, vol. 39, no. 6, pp. 7019–7023, 2012.

[36] T. Xu, Y. Zhu, Q.-K. Wei et al., "A functional polymorphism in the miR-146a gene is associated with the risk for hepatocellular carcinoma," *Carcinogenesis*, vol. 29, no. 11, pp. 2126–2131, 2008.

[37] Y. Xu, *Association Study of Polymorphisms in MiRNAs Genes with the Susceptibility of Hepatocellular Carcinoma*, Nanjing Medical University, 2010.

[38] L. Yu, *Association of the MicroRNA-499Variants with Susceptibilityto Hepatocellular Carcinomain the Southwest-China Population*, Chongqing Medical University, 2012.

[39] X.-W. Zhang, S.-D. Pan, Y.-L. Feng et al., "Relationship between genetic polymorphism in microRNAs precursor and genetic predisposition of hepatocellular carcinoma," *Zhonghua Yu Fang Yi Xue Za Zhi*, vol. 45, no. 3, pp. 239–243, 2011.

[40] J. Zhang, R. Wang, Y. Y. Ma et al., "Association between single nucleotide polymorphisms in miRNA196a-2 and miRNA146a and susceptibility to hepatocellular carcinoma in a chinese population," *Asian Pacific Journal of Cancer Prevention*, vol. 14, no. 11, pp. 6427–6431, 2013.

[41] J. Zhou, R. Lv, X. Song et al., "Association between two genetic variants in miRNA and primary liver cancer risk in the Chinese population," *DNA and Cell Biology*, vol. 31, no. 4, pp. 524–530, 2012.

[42] S. Giordano and A. Columbano, "MicroRNAs: new tools for diagnosis, prognosis, and therapy in hepatocellular carcinoma?" *Hepatology*, vol. 57, no. 2, pp. 840–847, 2013.

[43] S. Ngamruengphong and T. Patel, "Molecular evolution of genetic susceptibility to hepatocellularcarcinoma," *Digestive Diseases and Sciences*. In press.

[44] Y. Zeng and B. R. Cullen, "Sequence requirements for micro RNA processing and function in human cells," *RNA*, vol. 9, no. 1, pp. 112–123, 2003.

[45] J.-F. Zhan, L.-H. Chen, Z.-X. Chen et al., "A functional variant in MicroRNA-196a2 is associated with susceptibility of colorectal cancer in a chinese population," *Archives of Medical Research*, vol. 42, no. 2, pp. 144–148, 2011.

[46] E. Pavlakis, I. Papaconstantinou, M. Gazouli et al., "MicroRNA gene polymorphisms in pancreatic cancer," *Pancreatology*, vol. 13, no. 3, pp. 273–278, 2013.

[47] R. Luthra, R. R. Singh, M. G. Luthra et al., "MicroRNA-196a targets annexin A1: a microRNA-mediated mechanism of annexin A1 downregulation in cancers," *Oncogene*, vol. 27, no. 52, pp. 6667–6678, 2008.

[48] Z. Wang, Y. Cao, C. Jiang et al., "Lack of association of two common polymorphisms rs2910164 and rs11614913 with susceptibility to hepatocellular carcinoma: a meta-analysis," *PLoS ONE*, vol. 7, no. 6, Article ID e40039, 2012.

[49] M. Hu, L. Zhao, S. Hu et al., "The association between two common polymorphisms in MicroRNAs and hepatocellular carcinoma risk in Asian population," *PLoS ONE*, vol. 8, no. 2, Article ID e57012, 2013.

[50] Y. Xu, L. Li, X. Xiang et al., "Three common functional polymorphisms in microRNA encoding genes in the susceptibility to hepatocellular carcinoma: a systematic review and meta-analysis," *Gene*, vol. 527, no. 2, pp. 584–593, 2013.

[51] A. Ayub, U. A. Ashfaq, and A. Haque, "HBV induced HCC: major risk factors from genetic to molecular level," *BioMed Research International*, vol. 2013, Article ID 810461, 14 pages, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 482926, 13 pages
http://dx.doi.org/10.1155/2014/482926

Research Article

MicroRNA-24 Modulates Aflatoxin B1-Related Hepatocellular Carcinoma Prognosis and Tumorigenesis

Yi-Xiao Liu,[1] Xi-Dai Long,[1,2] Zhi-Feng Xi,[1] Yun Ma,[3] Xiao-Ying Huang,[2] Jin-Guang Yao,[2] Chao Wang,[4] Tian-Yu Xing,[1] and Qiang Xia[1]

[1] *Department of Liver Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Dongfang Road, No. 1630, Shanghai 200127, China*
[2] *Department of Pathology, Youjiang Medical College for Nationalities, Baise 533000, China*
[3] *Department of Pathology, The Affiliated Tumor Hospital, Guangxi Medical University, Nanning 530021, China*
[4] *Department of Medicine, The Affiliated Hospital of Youjiang Medical College for Nationalities, Baise 533000, China*

Correspondence should be addressed to Xi-Dai Long; sjtulongxd@263.net

Received 23 January 2014; Revised 13 March 2014; Accepted 16 March 2014; Published 8 April 2014

Academic Editor: Chunping Jiang

MicroRNA-24 (miR-24) may be involved in neoplastic process; however, the role of this microRNA in the hepatocellular carcinoma (HCC) related to aflatoxin B1 (AFB1) has not been well elaborated. Here, we tested miR-24 expression in 207 pathology-diagnosed HCC cases from high AFB1 exposure areas and HCC cells. We found that miR-24 was upregulated in HCC tumor tissues relative to adjacent noncancerous tissue samples, and that the high expression of miR-24 was significantly correlated with larger tumor size, higher microvessel density, and tumor dedifferentiation. Additionally, this microRNA overexpression modified the recurrence-free survival (relative hazard ratio [HR], 4.75; 95% confidence interval [CI], 2.66–8.47) and overall survival (HR = 3.58, 95% CI = 2.34–5.46) of HCC patients. Furthermore, we observed some evidence of joint effects between miR-24 and AFB1 exposure on HCC prognosis. Functionally, miR-24 overexpression progressed tumor cells proliferation, inhibited cell apoptosis, and developed the formation of AFB1-DNA adducts. These results indicate for the first time that miR-24 may modify AFB1-related HCC prognosis and tumorigenesis.

1. Introduction

Hepatocellular carcinoma (HCC) remains a life-threating malignancy, accounting for more than 90% of primary liver cancer. This tumor is the sixth most commonly occurring cancer worldwide, with an estimated 600,000 new cases per year. More than 80% of all HCC cases occurred in developing countries, and approximately 55% of all cases occur in China (especially in the southeast areas such as Guangxi) [1, 2]. Because of the very poor prognosis resulting from metastasis and reoccurrence, HCC is the third leading cause of cancer-related deaths in the world [3]. Therefore, improvement in the therapy of recurrent or metastatic HCC now depends on improving our understanding of the complex molecular mechanisms governing the progression and aggressiveness of the disease, and looking for valuable prognostic markers and therapeutic targets.

MicroRNAs are small, endogenous, and noncoding RNAs that regulate the translation of protein-coding genes by repressing translation of protein-coding mRNA or enhancing mRNA degradation [4, 5]. Deregulation of microRNAs has been reported to modulate normal cell growth and differentiation, potentially leading to a variety of disorders, including cancer [6, 7]. To date, there are more than 2000 annotated human mature microRNAs in the official registry (the MicroRNA Registry) [8]. Among these microRNAs, miR-24 has been shown to regulate the carcinogenesis of a variety of cancers including HCC [9–13]. However, association between this microRNA and aflatoxin B1 (AFB1)-related HCC prognosis has not yet been elucidated. Here,

we evaluated whether miR-24 expression modified HCC tumorigenesis and prognosis.

2. Materials and Methods

2.1. HCC Patients. During the period from January 2004 to December 2008, all incident cases with the I-II tumor-nodes-metastasis (TNM) stage HCC were recruited at the affiliated hospitals Guangxi Medical University and Youjiang Medical College for Nationalities. All patients were the residents of Guangxi Zhuang Autonomous Region, a high AFB1 exposure area. A total of 207 HCC cases, including 138 patients previously studied [14–16], were included for the present study. All cases were histopathologically confirmed and previously untreated with chemotherapy or radiotherapy. In this study, the response rate for the cases has been about 95%. The study protocol was been carried out in accordance with "Ethical Principles for Medical Research Involving Human Subjects" (World Medical Association Declaration Of Helsinki, 2004) and approved by institutional review boards from Guangxi Cancer Institute and the Medical Research Council from the corresponding hospitals.

2.2. Samples and Data Collection. After informed consent was obtained, surgically removed tumor samples and adjacent noncancerous tissue samples (at least 5 cm from the margin of the tumor) of all cases were collected for analyzing miR-24 expression levels and AFB1-DNA adduct levels. Demographic information (including sex, age, ethnicity, hepatitis B virus [HBV], and hepatitis C virus [HCV] infection) and clinical pathological data (including cirrhosis, tumor size, tumor differentiation, and tumor stage) were collected in the hospitals using a standard interviewer-administered questionnaire and/or medical records by a Youjiang Cancer Institution staff member. In this study, those with hepatitis B surface antigen (HBsAg) positive and anti-HCV positive in their peripheral serum were defined as groups infected with HBV and HCV. Liver cirrhosis was diagnosed by pathological examination, and stages of tumor were confirmed according to the tumor nodes metastasis (TNM) staging system. For tumor grading, Edmondson and Steiner grading system was used to evaluate the differentiation status of HCC in this study [17].

For survival analysis, we followed all HCC cases and more detailed follow-up information was described in our previous studies [14–16, 18]. Briefly, all patients underwent serial monitoring every 2 months for the first 2 years and semiannually thereafter for detection of any recurrence. In the present study, the last follow-up day was set on August 31, 2013, and survival status was confirmed by means of clinic records and patient or family contact. The duration of the duration of overall survival (OS) was defined as from the date of curative treatment to the date of death or last known date alive; whereas the recurrence-free survival (RFS) was defined as from the date of curative treatment to the date of tumor recurrence or last known date alive [14, 15].

2.3. DNA and RNA Detraction. Genomic DNA was extracted from HCC tumor tissues and SMMC-7721 cells in a 1.5 mL microcentrifuge tube for deparaffinization and proteinase K digestion, as described by standard procedures (Protocol #BS474, Bio Basic, Inc., Ontario, Canada). For RNA detraction, total RNA was isolated from tissue or cell cultures, using PureLink RNA minikit (cat number 12183018A, Ambion, USA) according to manufacturer's instructions.

2.4. AFB1 Exposure Analysis. In this study, AFB1 exposure levels were evaluated using the levels of AFB1-DNA adducts in tumor tissues. The amount of AFB1-DNA adducts in cancerous tissues samples was evaluated by means of competitive enzyme-linked immunosorbent assay (ELISA) [19]. To convert any N-7 adduct to AFB1-FAPy adducts, DNA was treated with 15 mM Na_2CO_3 and 30 mM $NaHCO_3$ (pH 9.6) for 2 hours, precipitated with 2.5 volumes of 95% ethanol, and then redissolved in 10 mM Tris-HCl (pH 7.0). The DNA samples were reprecipitated, dissolved in 1 × PBS, and denatured by boiling for 5 min. After that, AFB1-FAPy adducts were quantitated by ELISA using monoclonal antibody 6A10 (Novus Biologicals LLC, catalog number NB600-443). For analysis, AFB1-DNA adduct levels were divided into two groups: low level (≤2.87 μmol/mol DNA) and high level (>2.87 μmol/mol DNA), according to the average value of AFB1-DNA adduct levels among cases.

2.5. The Microvessel Density (MVD) Evaluation. In the present study, the angiogenesis of cancerous tissues was assessed using the IHC staining of CD31 (cat number 2011101101, Gene Tech (Shanghai) Company Limited, Shanghai, China). At ×200 magnification, vessel counts were made of all distinct brown staining endothelial cells in the cancerous regions over five fields in each slide. The microvessel density (MVD) was defined as the average value of the three readings. To analysis the relationship between miR-24 expression and angiogenesis in the cancerous tissues, the angiogenesis status was divided into two groups: low (≤50/×200 magnifications) and high (>50/×200 magnifications), according to the mean MVD of cancerous-tissues vessels.

2.6. MiR-24 Expression Assay. The level of miR-24 expression was analyzed using our previously published TaqMan quantitative reverse transcription-PCR technique [16]. In brief, RNA was reversed transcribed into cDNA using high capacity cDNA reverse transcription kit (cat number 4368814, Invitrogen) and TaqMan microRNA reverse transcription kit (cat number 4366596, Applied Biosystems [ABI], Carlsbad, CA), according to the manufacturer's instructions. In this study, U6 expression was used as an internal control. Real-time quantitative PCR with TaqMan probes (cat number 4427975, ABI) was performed in total volume of 5 μL containing 1 × TaqMAN Universal Master Mix II (cat number 4440041, ABI), 1 × TaqMan microRNA probe and primer Mix, and about 15 ng of cDNA using the running conditions: 95°C for 30 s, and 50 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were conducted in triplicate and controls were performed with no template or no reverse transcription for each gene. The cycle number at which the reaction crossed

an arbitrarily placed threshold (CT) was determined for each gene. For the relative expression of miR-24 in cancer cells, miR-24 expression was normalized to endogenous controls U6 by the comparative CT method ($2^{-\Delta\Delta Ct}$ method). For tissue samples, the relative amount of miR-24 to U6 was calculated as $2^{-\Delta Ct}$ method, where $\Delta Ct = (Ct_{miR\text{-}24} - Ct_{U6})$. To analyse, miR-24 expression levels were divided into two groups: low expression group, $2^{-\Delta Ct} \leq 2.95$; and high expression group, $2^{-\Delta Ct} > 2.95$, according to the average value among HCC cases.

2.7. Cell Lines and Culture. The QSG7701 cells (a kind of peritumoral liver cells) and HCC cells (including SMMC7721, HepG2, and HCCLM3) were obtained from the Cell Bank of Shanghai Institute of Cell Biology of the Chinese Academy of Sciences and Shanghai Xinran Ltd. (Shanghai, China). Cells were cultured in DMEM medium (HyClone, Thermo Fisher Scientific (China) CO., Ltd., Shanghai, China) containing high glucose and L-glutamine supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2/100% humidity. All experimental analyses were done with cells in logarithmic growth. Cells were determined to be free of mycoplasma.

2.8. AFB1 Toxicity Analysis. Cells were transfected with an miR-24 mimic, its inhibitor, its mock, or null control (GenePharma, China) using Lipofectamine 2000 (cat number 11668-027, Invitrogen Grand Island, NY, USA) according to the manufacturer's instructions. According to the types of mimics transfected, cell lines were divided into four groups: control group (control, by null mimics), mock group (mock, by mock mimics); miR-24 group (miR-24, by mature miR-24 mimics), and inhibitor group (inhibitor, by inhibitor of mature miR-24). In this study, transfection efficacy was evaluated as the ratio of transfected cells detected by the LV200 system to total cells obtained from three different regions at random and was about 40%. AFB1 toxicity value was evaluated as our previously published methods [15]. Briefly, 48 hours after transfections, cells were treated with AFB1 (Sigma) at final concentrations of 24 nM for 1 day, and then the DNA was extracted for AFB1-DNA adduct analysis.

2.9. Cell Transfection and Cell Proliferation and Apoptosis Assay. The cell proliferation assay was measured using a cell counting kit (CCK-8) assay (cat number CK04, Dojindo Corp., Japan) according to the manufacturer's instructions. A total of 2500 cells were seeded each well in a 96-well plate. Ten microliters of CCK-8 solution was added into 100 μL of culture media and incubated for 2 hours at 37°C. Finally, the absorbance of optical density (at 450 nm) was measured using UV spectrophotometer at 72 hours after transfection. The assay was performed three times in eight replicates. To analyse, relative proliferation value (RPV) of different groups to control group was calculated as $OD250_{XG}/OD250_{Ref}$, where $OD250_{XG}$ represented OD250 value for cell proliferation analysis for different groups, and $OD250_{Ref}$ represented OD250 value for cell proliferation analysis for control group.

In this study, cell apoptosis was analyzed by the flow cytometry technique using Annexin V, FITC apoptosis detection kit (cat number AD10-10, Dojindo Corp., Japan). Cells were seeded 6-wells and the transfections were performed when cells reached 70% confluent. 48 hours after transfection, cells were harvested, washed, and resuspended for cell apoptosis analysis. To analyse, relative apoptosis value (RAV) of different groups to control group was calculated using the following formula:

$$RAV = \frac{PPC_{XG}}{PPC_{Ref}}, \quad (1)$$

where PPC_{XG} represented the percent of positive cells with apoptosis for different groups, and PPC_{Ref} represented the percent of apoptosis positive cells for control group.

2.10. Statistical Analysis. MiR-24 expression among different tissues and cells was compared by independent two-sample t-test and Mann-Whitney U test for two groups, or one-way ANOVA with Bonferroni corrections for three or more than three groups. Nonconditional logistic regression was used to evaluate odds ratios (ORs) and 95% confidence intervals (CIs) for the effects of miR-24 expression on the pathological features of HCC (including AFB1-DNA adducts, tumor size, tumor differentiation, and MVD). Kaplan-Meier survival analysis (with the log-rank test) was used to evaluate the association between miR-24 expression and HCC prognosis. Hazard ratios (HRs) and 95% CIs for miR-24 expression were calculated from multivariate Cox regression model. In this study, a P value of less than 0.05 was considered statistically significant. All analyses were performed with the statistical package for social science (SPSS) version 18 (SPSS Institute, Chicago, IL, USA).

3. Results

3.1. The Characteristics of HCC Patients. Table 1 showed the demographic and clinic-pathological data of the cases. The present study comprised 207 HCC patients with 189 (91.3%) males and 18 (8.7%) females. The mean age was 46.9 ± 11.5 years. Among these patients, more than 80% of cases were infected by HBV, and most of them had liver cirrhosis. One hundred percent of patients featured HCC with I-II TNM stage and received the same curative resection treatment, according to Chinese Manage Criteria of HCC. During the follow-up period of these patients, 72 faced tumor recurrence with 61.3% of the 5-year RFS rate, and 115 died with 47.5% of the five-year OS rate.

3.2. AFB1 Exposure Levels Related to Poor Prognosis of HCC Patients. In this study, we elucidated AFB1 exposure levels through testing AFB1-DNA adducts of DNA samples from cancerous tissue of the patients and found the mean of 2.87 ± 1.60 μmol/mol DNA. To analyze the effects of AFB1 exposure on HCC prognosis, this variable was divided into two groups: low and high level. Results showed high AFB1 exposure levels were associated with decreasing 1-year, 3-year, and 5-year survival rate (Figure 1 and Table 2). Multivariate Cox

Table 1: Characteristics of the patients with HCC.

Characteristics	
Age, year	
Mean ± SE	46.9 ± 11.5
Range	15–75
Sex	
Man, *n* (%)	189 (91.3)
Female, *n* (%)	18 (8.7)
Ethnicity	
Han, *n* (%)	163 (78.7)
Minority, *n* (%)	44 (21.3)
HBV infection	
HBsAg (−), *n* (%)	33 (15.9)
HBsAg (+), *n* (%)	174 (84.1)
HCV infection	
anti-HCV (−), *n* (%)	204 (98.6)
anti-HCV (+), *n* (%)	3 (1.4)
Smoking status	
No, *n* (%)	165 (79.7)
Yes, *n* (%)	42 (20.3)
Drinking status	
No, *n* (%)	167 (80.7)
Yes, *n* (%)	40 (19.3)
Liver cirrhosis	
No, *n* (%)	41 (19.8)
Yes, *n* (%)	166 (80.2)
TNM stage	
I, *n* (%)	13 (6.3)
II, *n* (%)	194 (93.7)
Tumor size	
≤5 cm, *n* (%)	101 (48.8)
>5 cm, *n* (%)	106 (51.2)
Tumor grade	
I	44 (21.3)
II	106 (51.2)
III	54 (26.1)
IV	3 (1.4)
Total, *n* (%)	207 (100)

regression analysis (with stepwise forward selection based on likelihood ratio test) exhibited that this variable increased dying risk and tumor reoccurring risk of HCC, with an HR of 2.12 and 2.40 ($P < 0.01$; Figure 1), respectively.

3.3. MiR-24 Was Upregulated in HCC Samples and in HCC Cells. We performed the real-time PCR experiment to detect the expression of mature miR-24 RNA in HCC tumor tissues and adjacent noncancerous tissues. Through comparing Ct values in these two types of tissues, we evaluated the significance of different miR-24 expression and observed that miR-24 expression was significantly higher in tumour tissues (TT) than in nonmalignant adjacent liver tissues (NT), with a relative expression value of 2.95 ± 1.88 versus 1.73 ± 0.92 ($P < 0.01$, Figure 2(a)). We also found similar results in the expression analysis of this microRNA in HCC cell lines and nontumor liver cell lines QSG7701 ($P = 3.82 \times 10^{-4}$, Figure 2(b)). Furthermore, higher expression of miR-24 was observed in the HCCLM3 (a kind of poor differentiated HCC cells with high infiltrating capacity) cells compared to in the HepG2 (a kind of good differentiated HCC cells with low infiltrating capacity).

3.4. MiR-24 Expression Was an Independent Factor of HCC Prognosis. To investigate the effects of miR-24 expression on outcome of HCC patients, we divided miR-24 expression in cancerous tissues into two groups: low expression group (relative level ≤ 2.95) and high expression group (relative level > 2.95), according to the average relative expression levels. Kaplan-Meier survival analysis showed that patients with high miR-24 expression featured a significantly poorer prognosis than those with low miR-429 expression ($P = 2.00 \times 10^{-10}$ for RFS and $P = 1.92 \times 10^{-12}$ for OS, resp., Figure 3). Multivariate Cox regression analysis (with stepwise forward selection based on likelihood ratio test) was next performed to determine whether miR-24 expression was an independent predictor of HCC cases. The results exhibited that high miR-24 expression increased the risk of tumor reoccurrence compared with low expression (HR = 4.75, 95% CI = 2.66–8.47, $P = 1.38 \times 10^{-7}$, Figure 3(a)). Risk role was also found in the OS analysis; the corresponding HR (95% CI) was 3.58 (2.34–5.46), with a P value of 3.51×10^{-9} (Figure 3(b)). Taken together, these results suggested that this microRNA could be used as an independent prognostic marker for HCC.

3.5. Joint Effects of miR-24 Expression and AFB1 Exposure on HCC Prognosis. To investigate the joint effects between miR-24 expression and AFB1 exposure on HCC prognosis, we performed a stratified analysis based on AFB1 exposure levels (Figure 4). We found lower 5-year RFS and OS for high miR-24 expression among these cases with high level of AFB1-DNA adducts (Figures 4(b) and 4(d)) than among those without high level of AFB1-DNA adducts (Figures 4(a) and 4(c)), suggesting high miR-24 expression might interact with AFB1 exposure in the process of HCC carcinogenesis. Next, a joint analysis between miR-24 expression and AFB1 exposure was accomplished (Figure 5). In this analysis, we used as a reference the lowest risk group: those who had both low AFB1-DNA adducts level and low miR-24 expression (LALM). Results showed that increasing adducts amount decreased 5-year survival rate of HCC; moreover, this effect was more pronounced among the high miR-24 expression subjects (Figures 5(a) and 5(c)). Moreover, compared to those with LALM, these with high AFB1-DNA adducts level and high miR-24 expression (HAHM) featured increasing tumor reoccurring risk (HR = 11.75, 95% CI = 5.15–26.79, Figure 5(b)) and death risk (HR = 8.13, 95% CI = 4.46–14.84, Figure 5(d)).

3.6. MiR-24 Expression Associated with the Clinic-Pathological Features of HCC Patients. To explore possible pathogenesis

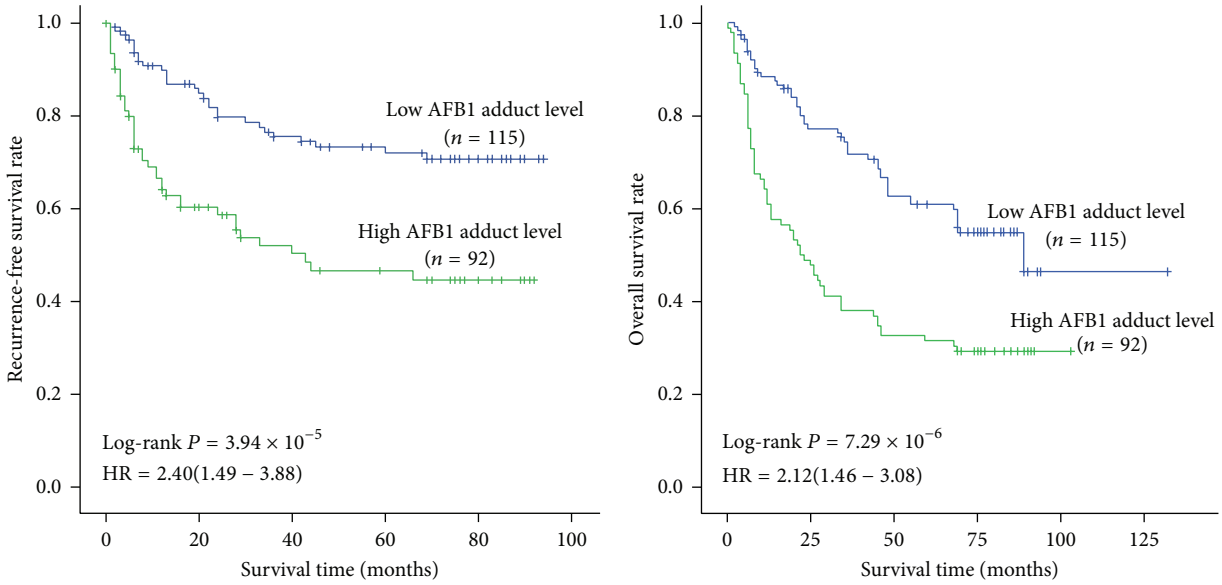


Figure 1: Association between survival and AFB1 exposure in 207 HCC cases receiving curative treatment. According to the average level of AFB1-DNA adducts in cancerous tissues, AFB1 exposure was divided into two groups: low exposure group (relative level ≤ 2.87 μmol/mol DNA) and high exposure group (relative level > 2.87 μmol/mol DNA). AFB1 exposure levels were associated with tumor reoccurrence-free survival (a) and overall survival (b) of HCC. Cumulative hazard function was plotted by Kaplan-Meier's methodology, and P value was calculated with two-sided log-rank tests. Relative hazard ratio (HR) and corresponding 95% CI of high AFB1 exposure (compared with low exposure) was calculated using multivariable cox regression model (including all significant variables).

Table 2: RFS rate and OS rate of HCC cases.

		RFS rate (%)			OS rate (%)		
		1 year	3 years	5 years	1 year	3 years	5 years
Figure 1	AFB1-DNA adduct level						
	Low	89.9	75.5	70.8	88.4	74.4	60.9
	High	66.7	53.9	46.7	64.1	41.3	31.5
Figure 3	miR-24 expression						
	Low	97.8	88.1	82.9	94.5	82.3	72.0
	High	65.2	44.4	41.2	61.1	37.7	27.3
Figures 4(a) and 4(c)	miR-24 expression						
	Low	100	91.1	87.3	96.6	89.7	84.3
	High	86.8	55.7	52.6	79.2	56.5	33.5
Figures 4(b) and 4(d)	miR-24 expression						
	Low	93.8	81.9	73.2	90.6	68.8	50.0
	High	50.7	33.9	30.5	48.3	25.0	20.0

of miR-24 expression modifying the outcome of AFB1-related HCC, we analyzed the distribution difference of this microRNA expression among different clinic-pathological characteristics of cases. Results showed these HCC cases with high miR-24 expression, compared to those with low miR-24 expression, faced larger tumor size (OR = 2.01), lower tumor differentiation (OR = 2.10), and higher MVD (OR = 2.63, Table 3). However, the expression of this microRNA did not affect other features (data not shown).

3.7. MiR-24 Expression Modified HCC Cell Proliferation. We evaluated the functional role of miR-24 in HCC cells by means of measuring cell proliferation in HCC cells which were transfected with miR-24 mimics and its inhibitor. Overexpression of miR-24 in HCC cells promoted proliferation while downregulation of miR-24 in HCC cells inhibited cell proliferation. On the other hand, compared with the mock group, the proliferation of tumor cells in the inhibitor groups was inhibited significantly ($P < 0.05$, Figure 6(a)).

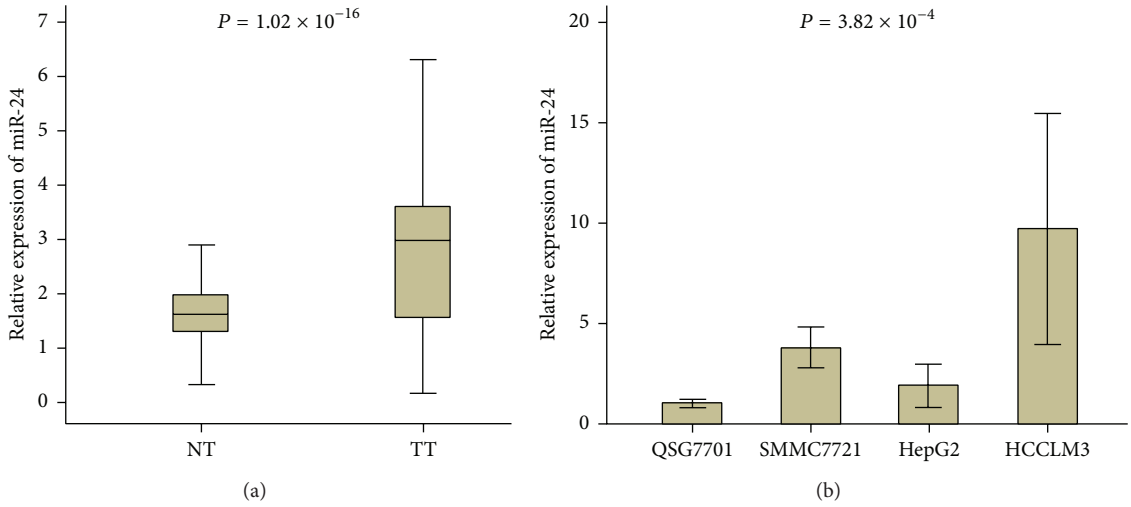


FIGURE 2: MiR-24 expression related with HCC tumorigenesis. (a) miR-24 expression was evaluated in the tumor tissues versus in the adjacent noncancerous tissues. The relative expression of miR-24 is shown as box plots, with horizontal lines representing the median, the bottom, and the top of the boxes representing the 25th and 75th percentiles, respectively, and vertical bars representing the range of data. We compared the difference among group using the Mann-Whitney U test. (b) miR-24 expression was higher in cancer cell line SMMC-7721 than in noncancer cell line QSG-7701.

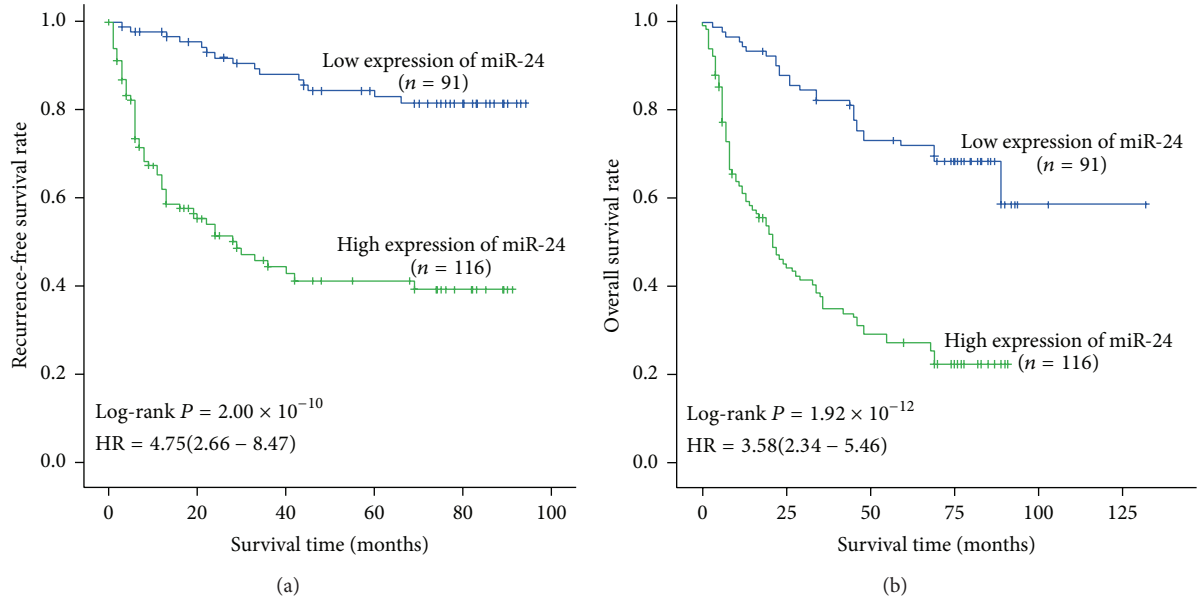


FIGURE 3: Association between survival and miR-24 expression in 207 HCC cases receiving curative treatment. According to the average expression in cancerous tissues, miR-24 expression was divided into two groups: low expression group (relative level ≤ 2) and high expression group (relative level > 2). MiR-24 expression was associated with tumor reoccurrence-free survival (a) and overall survival (b) of HCC. Cumulative hazard function was plotted by Kaplan-Meier's methodology, and P value was calculated with two-sided log-rank tests. Relative hazard ratio (HR) and corresponding 95% CI of high miR-24 expression (compared with low expression) was calculated using multivariable cox regression model (including all significant variables).

3.8. MiR-24 Expression Modulated the Apoptosis of HCC Cells. We also explored the function of miR-24 in HCC cells through analyzing changes in apoptosis after the HCC cells were transfected with miR-24 mimics and its inhibitor. DNA content of transiently microRNA-transfected cells was analyzed by flow cytometry. the RAV of SMMC7721 cell lines in the miR-24 group was significantly decreased ($P < 0.05$) compared with the control group. Tumor cell apoptosis in the inhibitor group, compared with the mock group, was promoted significantly ($P < 0.05$) (Figure 6(b)). Similar

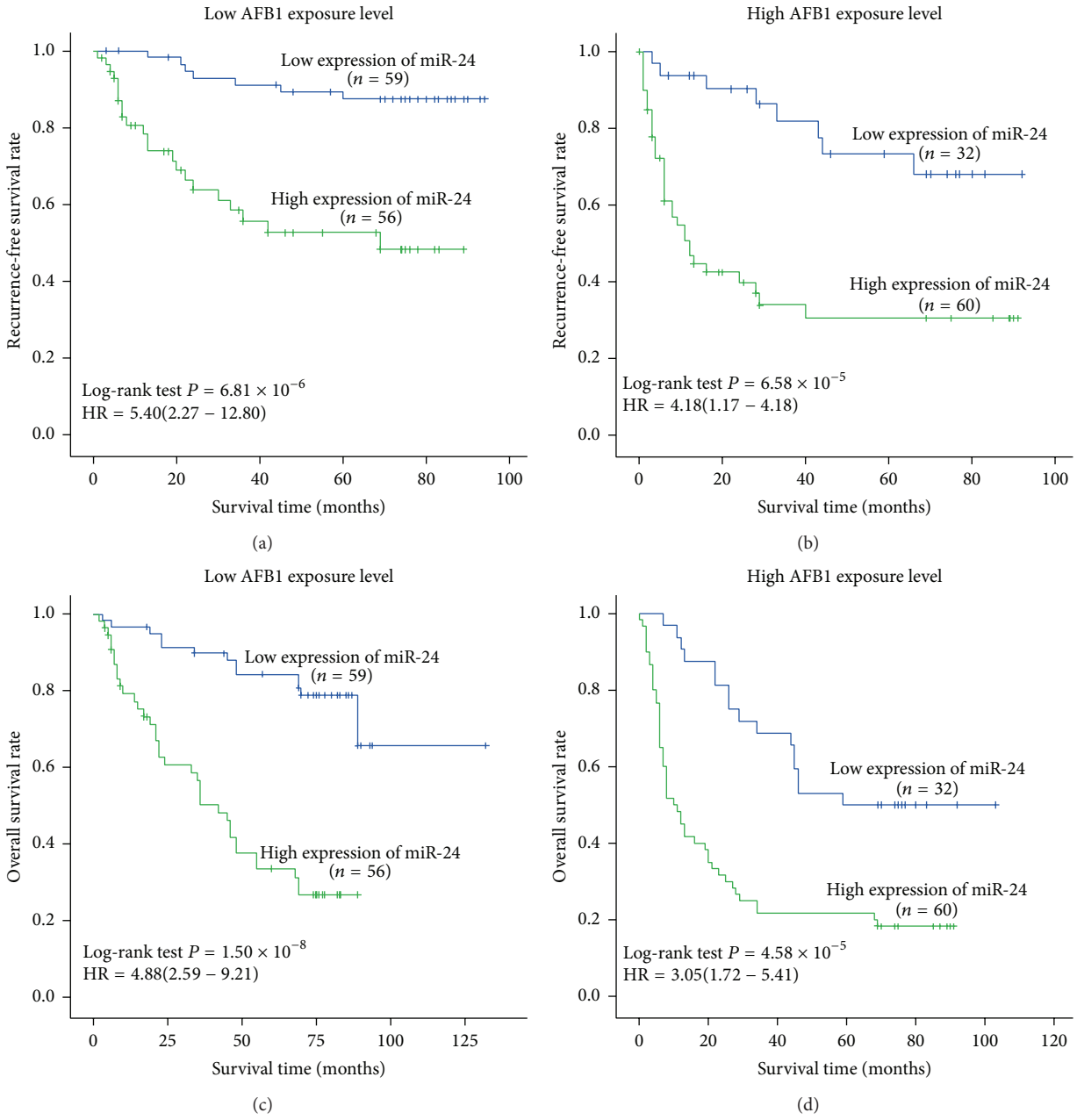


FIGURE 4: Survival analysis of miR-24 expression in strata of AFB1-DNA adduct. (a)-(b) Tumor recurrence-free survival (RFS) and miR-24 expression in strata of AFB1-DNA adduct levels. (c-d) Overall survival (OS) and miR-24 expression in strata of AFB1-DNA adduct levels. Cumulative hazard function was plotted by Kaplan-Meier's methodology, and P value was calculated with two-sided log-rank tests. Relative hazard ratio (HR) and corresponding 95% CI of high miR-24 expression (compared with low expression) was calculated using multivariable cox regression model (including all significant variables).

results were observed in the two other cells HepG2 and HCCLM3.

3.9. MiR-24 Expression Increased AFB1-DNA Adducts in the HCC Samples and SMMC-7721 Cells. To investigate the effects of miR-24 expression on AFB1-DNA formation, we analyzed the effects of miR-24 expression on AFB1-DNA formation in liver cancer tissues. Results showed that these persons having high miR-24 expression in their tumor tissues faced increasing DNA adducts levels (3.27 ± 1.81 μmol/mol DNA) compared with those with low miR-24 expression (2.37 ± 1.12 μmol/mol DNA, $P < 0.01$, Figure 7(a)). A toxin experiment of AFB1 was next performed through transfecting different mimics into the SMMC7721 cells. We found that group with overexpression of miR-24 had elevated levels of AFB1-DNA adducts (0.795 ± 0.005 nmol/μg DNA) compared with control group (0.394 ± 0.005 nmol/μg DNA, $P < 0.05$, Figure 6(b)). On the other hand, compared with mock group

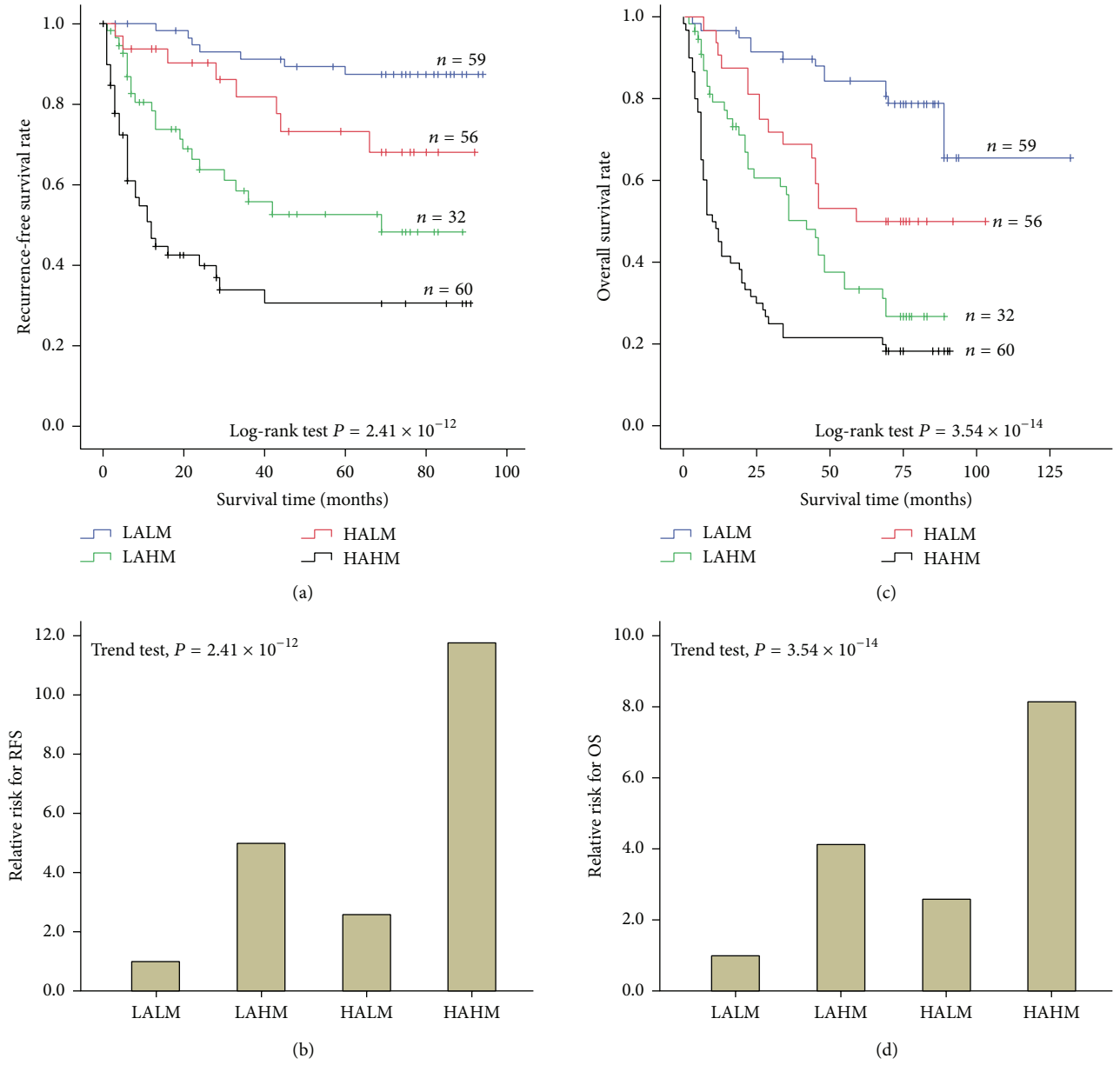


FIGURE 5: The joint effects of AFB1 exposure and miR-24 expression on HCC prognosis. (a and c) The joint effects of AFB1 exposure and miR-24 expression on tumor recurrence-free survival (RFS) and overall survival (OS) of HCC patients. Cumulative hazard function was plotted by Kaplan-Meier's methodology, and P value was calculated with two-sided log-rank tests. (b and d) Relative hazard ratio (HR) of both AFB1 exposure and miR-24 expression on HCC. HR was calculated using multivariable cox regression model (including all significant variables). Abbreviations: LALM, the combination of low AFB1 exposure level and low miR-24 expression; LAHM, the combination of low AFB1 exposure level and high miR-24 expression; HALM, the combination of high AFB1 exposure level and low miR-24 expression; HAHM, the combination of high AFB1 exposure level and high miR-24 expression.

(0.412 ± 0.002 nmol/μg DNA), cells transfected by miR-24 inhibitor featured decreased levels of DNA adducts (0.181 ± 0.002 nmol/μg DNA, $P < 0.05$, Figure 7(b)).

4. Discussion

In Guangxi Zhuang Autonomous Region, China, HCC is the most common cancer type, with an incidence rate of 53/100,000 per year and a death rate of 37–55/100,000 annually [1, 2]. Clinical-epidemiologic evidence suggests AFB1 exposure is a major risk factor for liver cancer in Guangxi Region [1]. AFB1 is an important I-type chemical carcinogen produced by some strains of the moulds aspergillus parasiticus and aspergillus flavus that grow readily on such foodstuffs as corn and groundnuts stored in damp conditions. Once ingested, this toxin is metabolized mainly by cytochrome

Table 3: Expression levels of miR-24 and clinicopathological features of cases.

	Low expression		High expression		OR (95% CI)[a]	P
	n	%	n	%		
AFB1 exposure						3.10×10^{-2}
Low level	59	64.8	56	48.3	Reference	
High level	32	35.2	60	51.7	1.92 (1.06–3.46)	
Tumor size						1.91×10^{-2}
≤5 cm	53	58.2	48	41.4	Reference	
>5 cm	38	41.8	68	58.6	2.01 (1.12–3.60)	
Tumor grade						3.92×10^{-2}
I-II	72	79.1	78	67.3	Reference	
III-IV	19	20.9	38	32.7	2.10 (1.04–4.27)	
MVD						1.93×10^{-2}
Low	50	54.9	38	32.8	Reference	
High	41	45.1	78	67.2	2.62 (1.43–4.81)	

[a]Adjusted by age, sex, race, HBV and HCV infection status, and smoking and drinking status.

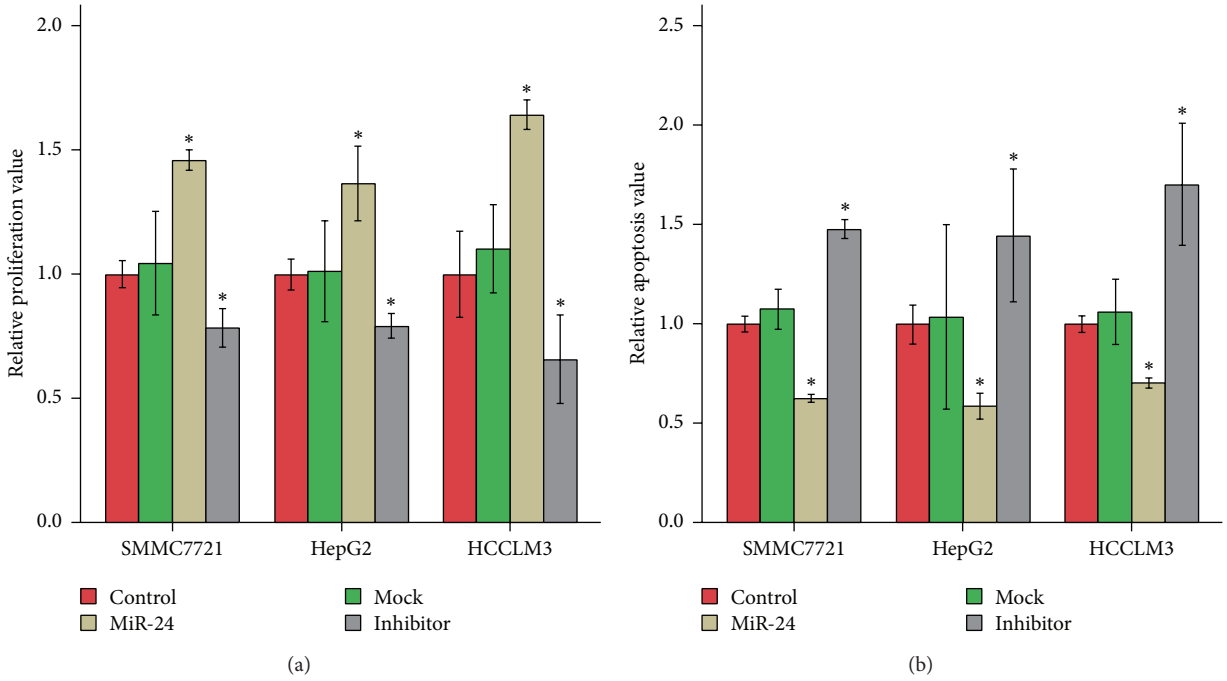


Figure 6: MiR-24 expression modified the proliferation and the apoptosis of HCC cancer cells. According to the types of mimics transfected, cell lines were divided into four groups: control group (control, by null mimics), mock group (mock, by mock mimics); miR-24 group (miR-24, by mature miR-24 mimics), and inhibitor group (inhibitor, by inhibitor of mature miR-24). (a) Association between miR-24 expression and cancer cell proliferation was elucidated using the CCK-8 assays. Relative proliferation value was calculated using control group as a reference. (b) Relationship between miR-24 expression and cancer cell apoptosis was evaluated by flow cytometry technique. Relative apoptosis value was calculated using control group as a reference. Data were analyzed using one-way ANOVA with Bonferroni corrections. Asterisk, $P < 0.05$.

P450 into the genotoxic metabolic aflatoxin B1-*exo*-8,9-epoxide (AFBO). AFBO is able to bind to DNA and causes genomic DNA damage and induces HCC [1, 2, 20]. In our study, about 2.9 μmol/mol DNA of AFB1 adducts was tested in the liver cancer tissue samples, and AFB1-exposure status was also found to be associated with the poorer outcome of HCC. These results suggest that AFB1 is an important marker for HCC prognosis. However, because of metastasis or other causes, most AFB1-related HCC cases are already in an incurable stage with an extremely poor prognosis at the time of diagnosis [21]. Therefore, new prognosis biomarkers and therapies have been expected, but no remarkable advances have been made in the treatment and prognostic prediction of this malignant tumor.

Increasing evidence has shown that microRNAs may be a type of significant prognosis factor and potential therapeutic

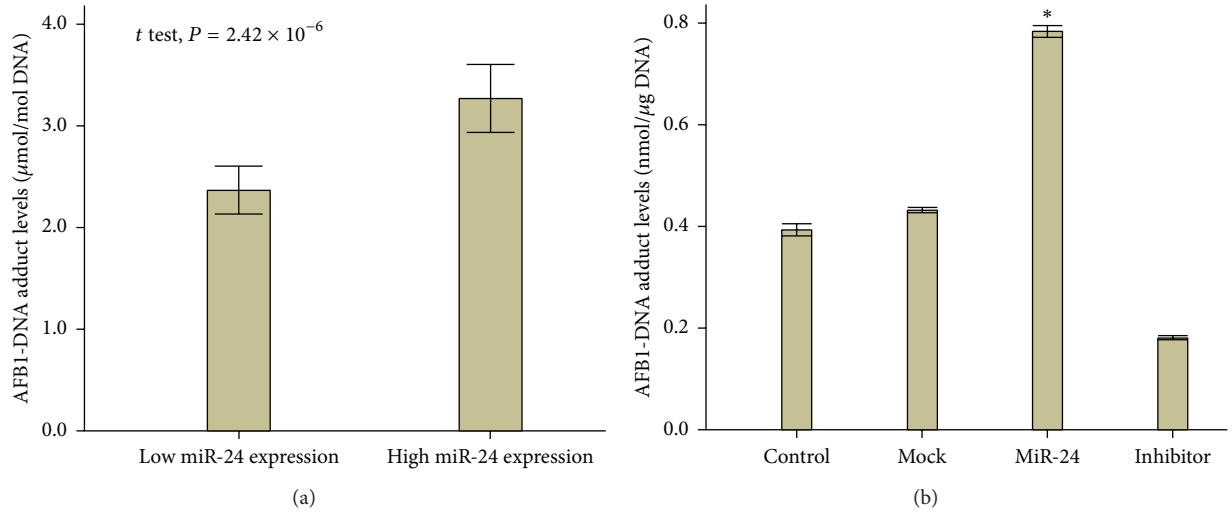


Figure 7: AFB1-DNA adducts formation in AFB1-treated SMMC7721 cells with overexpression of miR-24 (see Section 2). Levels of AFB1-DNA adducts were tested using comparative ELISA. Data were analyzed from three independent tests using one-way ANOVA with Bonferroni corrections. Asterisk, $P < 0.05$.

target for some malignant tumors including HCC [16, 22, 23]. MicroRNAs, a class of small noncoding single-stranded RNA with about 20 nucleotide sequence, are formed from the sequential processing of primary transcripts by two RNase enzymes, Drosha and Dicer [6, 24]. Through regulating gene expression, they functionally involve in cell differentiation, cell proliferation, cell apoptosis, physiological timing, metabolism, and hormone secretion [25, 26]. Moreover, increasing reports have exhibited that microRNAs may play a role in the aetiology and pathogenesis of various cancers by targeting a number of oncogenes or tumour suppressor genes [7]. The dysregulation of microRNA expression may correlate with the prognosis of some tumors such as HCC [16, 23].

Of particular recent interest is the possible contribution of miR-24 to tumor prognosis and tumorigenesis [23, 27, 28]. MiR-24, an important abundant microRNA encoded by the corresponding gene that maps to human chromosome 9q22 and 19p13 regions, is well conserved between various species and is expressed in normal tissues such as adipose tissue, mammary gland, kidney, and differentiated skeletal muscles [29]. Increasing evidence has shown that the miR-24 is frequently altered in liver cancer [11–13, 22, 23]. The dysregulation of miR-24 expression may modify tumor prognosis [22, 23]. In the present study, we collected 207 HCC tissue samples from Guangxi Zhuang Autonomous Region, both a high AFB1 exposure area and a high epidemic area of HCC in China, and investigated the possible effects of miR-24 expression on HCC prognosis. We found that HCC patients having high miR-24 expression in the tumor tissues had a significant poor RFS and OS compared with those with low expression of miR-24. Multivariate Cox regression analysis showed high miR-24 expression increased 3.75-times tumor reoccurrence risk and 2.58-times death risk; moreover, this risk did not depend on the clinic-pathological change. Supporting our results, a recent study has shown the dysregulation of miR-24 expression can modify the prognosis of cirrhotic HCC [22]. These data implied that miR-24 expression might be an independent prognostic factor for HCC and that its abnormal expression could be used as a prognostic marker for HCC.

In this study, we stratified HCC patients with respect to AFB1-DNA adducts levels and investigated the effects of miR-24 expression on HCC prognosis in different AFB1 exposure status. This was done primarily because the cause of HCC might modify the levels of miR-24 expression. Poorer outcome was found in these patients having high AFB1 exposure, suggesting possible interactive effects between environment exposure and gene expression on HCC prognosis. Next joint analysis proved this positive interaction. Therefore, it is well known that postoperative adjuvant therapy might significantly improve the prognosis of HCC cases, especially with high AFB1 exposure status.

Recent reports have exhibited that miR-24 may be involved in different cancers and play important role in carcinogenesis, such as gastric cancer [30], colorectal cancer [31], cervical cancer [32], oral squamous cell carcinoma [33], breast cancer [9], leukemia [34, 35], glioma [36], and lung cancer [10]. Several target genes of miR-24 have been discovered, including PKC-alpha, MXI 1, DHFR, ALK4, FAF1, DND1, AE1, p14ARF, and XIAP [9, 10, 27, 28, 30–42]. In this study, we also explored the association between miR-24 expression and HCC tumorigenesis through testing the expression difference of miR-24 in the different tissues (including tumor tissues and paired noncancerous matched tissues). Higher expression of miR-24 was observed in the tumor tissues, and this increasing expression of miR-24 was correlated with larger tumor size, tumor dedifferentiation, and increasing MVD. Our results also exhibited that the overexpression of miR-24 progressed cell proliferation and inhibited cell apoptosis. On the contrary, the suppression of miR-24 expression hindered cell proliferation and promoted cell apoptosis. Furthermore, different expression levels of

miR-24 were found in the different degrees of differentiation; and HCC cells with poor differentiation and high infiltrating capacity had an increasing expression of miR-24. In accordance with our results, several recent studies have demonstrated that upregulation of miR-24 is involved in the tumorigenesis of HCC [11–13, 23, 43, 44]. Taken together, these results suggested that the dysregulation of miR-24 expression might play an important role in the tumorigenesis of HCC through promoting tumor angiogenesis, proliferation, tumor invasion, and metastasis [11–13].

Interestingly, we found that high expression of miR-24 could promote AFB1-DNA formation and increase adducts mount. This is possibly because miR-24 can target some detoxification enzyme genes [45] and reduce their detoxification capacity and subsequently result in the accumulation of AFB1-DNA adducts. Additionally, our previous reports showed that low expression of DNA repair by microRNA would decrease the DNA repair capacity and subsequently increase DNA damage amount and HCC risk [14–16]. These results provided new insights into the mechanism of HCC induced by AFB1.

The present study had several limitations. Only 207 HCC patients were enrolled in the analysis of the clinicpathological characteristics and prognosis. We would like to confirm the findings in a larger HCC patient population. Another important limitation was that we did not do migration and invasiveness assays to validate the involvement of miR-24 in tumour migration and invasion. Although the status of miR-24 expression was investigated in cases of HCC, other microRNAs, such as microRNA-629 and microRNA-124, which may be involved in HCC tumorigenesis and modify HCC prognosis [11], were not evaluated. Additionally, because the liver disease itself may affect the metabolism of AFB1 and modify the levels of AFB1 DNA adducts, the increased death risk and tumor reoccurring risk with AFB1 exposure status noted in this study was probably underestimated. Therefore, more microRNAs deserve further elucidation based on a large sample and the combination of genes and AFB1 exposure.

5. Conclusions

In summary, this study is, to the best of our knowledge, the first report that describes miR-24 expression in AFB1-related liver cancer and its associations with HCC prognosis. Our results showed that miR-24, as an oncogene, was overexpressed in liver cancer tissues and could be considered as a potential prognostic factor for HCC. Furthermore, overexpression of this microRNA was associated with AFB1-related HCC tumorigenesis. Therefore, more detailed molecular pathogenesis analysis deserves elucidation based on the results from large samples. Expanding insights into the key role of dysregulated microRNAs involved in liver tumorigenesis will yield important clues for the complicated molecular pathogenesis of HCC and may assist in the development of new therapeutic regimens for HCC patients, especially from high AFB1 exposure areas.

Conflict of Interests

The authors declare no competing financial interests.

Authors' Contribution

Yi-Xiao Liu, Xi-Dai Long, and Zhi-Feng Xi contributed equally to this work.

Acknowledgments

The authors thank Dr. Qiu-Xiang Liang, Dr. Yun Yi, and Dr. Yuan-Feng Zhou for sample collection and management and Dr. Hua Huang for molecular biochemical technique. They also thank all members of Department of Medical Test and Infective Control, Affiliated Hospital of Youjiang Medical College for Nationalities for their help. This study was supported in part by the National Natural Science Foundation of China (nos. 81160255 and 81372639), the Innovation Program of Guangxi Municipal Education Department (no. 201204LX674), Innovation Program of Shanghai Municipal Education Commission (no. 13YZ035), the Natural Science Foundation of Guangxi (no. 2013GXNSFAA019251), and "Shu Guang" Project (supported by Shanghai Municipal Education Commission and Shanghai Education Development Foundation, no. 13SG19).

References

[1] Q. Xia, X. Y. Huang, F. Xue et al., "Genetic polymorphisms of DNA repair genes and DNA repair capacity related to aflatoxin b1 (AFB1)-induced DNA damages," in *New Research Directions in DNA Repair*, C. Chen, Ed., pp. 377–412, InTech, Rijeka, Croatia, 1 edition, 2013.

[2] X. D. Long, J. G. Yao, Z. Zeng et al., "DNA repair capacity-related to genetic polymorphisms of DNA repair genes and aflatoxin B1-related hepatocellular carcinoma among Chinese population," in *DNA Repair, (Amst)*, I. Kruman, Ed., pp. 505–524, InTech, Rijeka, Croatia, 2011.

[3] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[4] J. Vera, X. Lai, U. Schmitz, and O. Wolkenhauer, "MicroRNA-regulated networks: the perfect storm for classical molecular biology, the ideal scenario for systems biology," *Advances in Experimental Medicine and Biology*, vol. 774, pp. 55–76, 2013.

[5] B. D. Aguda, "Modeling microRNA-transcription factor networks in cancer," *Advances in Experimental Medicine and Biology*, vol. 774, pp. 149–167, 2013.

[6] N. Lynam-Lennon, S. G. Maher, and J. V. Reynolds, "The roles of microRNA in cancer and apoptosis," *Biological Reviews*, vol. 84, no. 1, pp. 55–71, 2009.

[7] B. Zhang, X. Pan, G. P. Cobb, and T. A. Anderson, "microRNAs as oncogenes and tumor suppressors," *Developmental Biology*, vol. 302, no. 1, pp. 1–12, 2007.

[8] S. Griffiths-Jones, "The microRNA registry," *Nucleic Acids Research*, vol. 32, pp. D109–D111, 2004.

[9] E. C. Martin, S. Elliott, L. V. Rhodes et al., "Preferential star strand biogenesis of pre-miR-24-2 targets PKC-alpha and

suppresses cell survival in MCF-7 breast cancer cells," *Molecular Carcinogenesis*, vol. 53, no. 1, pp. 38–48, 2014.

[10] T. Franchina, V. Amodeo, G. Bronte et al., "Circulating miR-22, miR-24 and miR-34a as novel predictive biomarkers to pemetrexed-based chemotherapy in advanced non-small cell lung cancer," *Journal of Cellular Physiology*, vol. 229, no. 1, pp. 97–99, 2014.

[11] M. Hatziapostolou, C. Polytarchou, E. Aggelidou et al., "An HNF4α-miRNA inflammatory feedback circuit regulates hepatocellular oncogenesis," *Cell*, vol. 147, no. 6, pp. 1233–1247, 2011.

[12] C. E. Rogler, L. LeVoci, T. Ader et al., "MicroRNA-23b cluster microRNAs regulate transforming growth factor-beta/bone morphogenetic protein signaling and liver stem cell differentiation by targeting Smads," *Hepatology*, vol. 50, no. 2, pp. 575–584, 2009.

[13] X. Z. Meng, T. S. Zheng, X. Chen et al., "microRNA expression alteration after arsenic trioxide treatment in HepG-2 cells," *Journal of Gastroenterology and Hepatology*, vol. 26, no. 1, pp. 186–193, 2011.

[14] X. D. Long, D. Zhao, C. Wang et al., "Genetic polymorphisms in DNA repair genes XRCC4 and XRCC5 and aflatoxin B1-related hepatocellular carcinoma," *Epidemiology*, vol. 24, no. 5, pp. 671–681, 2013.

[15] X. D. Long, J. G. Yao, Z. Zeng et al., "Polymorphisms in the coding region of X-ray repair complementing group 4 and aflatoxin B1-related hepatocellular carcinoma," *Hepatology*, vol. 58, no. 1, pp. 171–181, 2013.

[16] X. Y. Huang, J. G. Yao, H. D. Huang et al., "MicroRNA-429 modulates hepatocellular carcinoma prognosis and tumorigenesis," *Gastroenterology Research and Practice*, vol. 2013, Article ID 804128, 10 pages, 2013.

[17] D. J. Dabbs, K. R. Geisinger, F. Ruggiero, S. S. Raab, M. Nalesnik, and J. F. Silverman, "Recommendations for the reporting of tissues removed as part of the surgical treatment of malignant liver tumors," *Human Pathology*, vol. 35, no. 11, pp. 1315–1323, 2004.

[18] X. D. Long, Y. Ma, Y. F. Zhou, A. Ma, and G. Fu, "Polymorphism in xeroderma pigmentosum complementation group C codon 939 and aflatoxin B1-related hepatocellular carcinoma in the Guangxi population," *Hepatology*, vol. 52, no. 4, pp. 1301–1309, 2010.

[19] X. D. Long, Y. Ma, Y. F. Zhou et al., "XPD codon 312 and 751 polymorphisms, and AFB1 exposure, and hepatocellular carcinoma risk," *BMC Cancer*, vol. 9, no. 1, p. 400, 2009.

[20] T. W. Kensler, B. D. Roebuck, G. N. Wogan, and J. D. Groopman, "Aflatoxin: a 50-year Odyssey of mechanistic and translational toxicology," *Toxicological Sciences*, vol. 120, supplement 1, pp. S28–S48, 2011.

[21] Y. F. Sun, Y. Xu, X. R. Yang et al., "Circulating stem cell-like epithelial cell adhesion molecule-positive tumor cells indicate poor prognosis of hepatocellular carcinoma after curative resection," *Hepatology*, vol. 57, no. 4, pp. 1458–1468, 2013.

[22] A. Salvi, E. Abeni, N. Portolani, S. Barlati, and G. De Petro, "Human hepatocellular carcinoma cell-specific miRNAs reveal the differential expression of miR-24 and miR-27a in cirrhotic/non-cirrhotic HCC," *International Journal of Oncology*, vol. 42, no. 2, pp. 391–402, 2013.

[23] Z. B. Han, L. Zhong, M. J. Teng et al., "Identification of recurrence-related microRNAs in hepatocellular carcinoma following liver transplantation," *Molecular Oncology*, vol. 6, no. 4, pp. 445–457, 2012.

[24] S. K. Shenouda and S. K. Alahari, "MicroRNA function in cancer: oncogene or a tumor suppressor?" *Cancer and Metastasis Reviews*, vol. 28, no. 3-4, pp. 369–378, 2009.

[25] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[26] V. Ambros, "The functions of animal microRNAs," *Nature*, vol. 431, no. 7006, pp. 350–355, 2004.

[27] Y. Xie, L. A. Tobin, J. Camps et al., "MicroRNA-24 regulates XIAP to reduce the apoptosis threshold in cancer cells," *Oncogene*, vol. 32, no. 19, pp. 2442–2451, 2013.

[28] N. Srivastava, S. Manvati, A. Srivastava et al., "MiR-24-2 controls H2AFX expression regardless of gene copy number alteration and induces apoptosis by targeting antiapoptotic gene BCL-2: a potential for therapeutic intervention," *Breast Cancer Research*, vol. 13, no. 2, p. R39, 2011.

[29] J. H. Gibcus, L. P. Tan, G. Harms et al., "Hodgkin lymphoma cell lines are characterized by a specific miRNA expression profile," *Neoplasia*, vol. 11, no. 2, pp. 167–176, 2009.

[30] Y. Tsukamoto, C. Nakada, T. Noguchi et al., "MicroRNA-375 is downregulated in gastric carcinomas and regulates cell survival by targeting PDK1 and 14-3-3ζ," *Cancer Research*, vol. 70, no. 6, pp. 2339–2349, 2010.

[31] P. J. Mishra, B. Song, P. J. Mishra et al., "Mir-24 tumor suppressor activity is regulated independent of p53 and through a target site polymorphism," *PLoS ONE*, vol. 4, no. 12, Article ID e8445, 2009.

[32] X. Wang, S. Tang, S. Le et al., "Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth," *PLoS ONE*, vol. 3, no. 7, Article ID e2557, 2008.

[33] S. C. Lin, C. J. Liu, J. A. Lin, W. F. Chiang, P. Hung, and K. Chang, "miR-24 up-regulation in oral carcinoma: positive association from clinical and in vitro analysis," *Oral Oncology*, vol. 46, no. 3, pp. 204–208, 2010.

[34] T. Nguyen, A. Rich, and R. Dahl, "MiR-24 promotes the survival of hematopoietic cells," *PLoS ONE*, vol. 8, no. 1, Article ID e55406, 2013.

[35] Q. Wang, Z. Huang, H. Xue et al., "MicroRNA miR-24 inhibits erythropoiesis by targeting activin type I receptor ALK4," *Blood*, vol. 111, no. 2, pp. 588–595, 2008.

[36] W. Xu, M. Liu, X. Peng et al., "miR-24-3p and miR-27a-3p promote cell proliferation in glioma cells via cooperative regulation of MXI1," *International Journal of Oncology*, vol. 42, no. 2, pp. 757–766, 2013.

[37] A. Talasila, H. Yu, M. Ackers-Johnson et al., "Myocardin regulates vascular response to injury through miR-24/-29a and platelet-derived growth factor receptor-beta," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 10, pp. 2355–2365, 2013.

[38] L. Chen, A. Zhang, Y. Li et al., "MiR-24 regulates the proliferation and invasion of glioma by ST7L via beta-catenin/Tcf-4 signaling," *Cancer Letters*, vol. 329, no. 2, pp. 174–180, 2013.

[39] S. Giglio, R. Cirombella, R. Amodeo, L. Portaro, L. Lavra, and A. Vecchione, "MicroRNA miR-24 promotes cell proliferation by targeting the CDKs inhibitors p27Kip1 and p16INK4a," *Journal of Cellular Physiology*, vol. 228, no. 10, pp. 2015–2023, 2013.

[40] W. W. Du, L. Fang, M. Li et al., "MicroRNA miR-24 enhances tumor invasion and metastasis by targeting PTPN9 and PTPRF to promote EGF signaling," *Journal of Cell Science*, vol. 126, part 6, pp. 1440–1453, 2013.

[41] W. Qin, Y. Shi, B. Zhao et al., "miR-24 regulates apoptosis by targeting the Open Reading Frame (ORF) region of FAF1 in cancer cells," *PLoS ONE*, vol. 5, no. 2, Article ID e9429, 2010.

[42] K. H. To, S. Pajovic, B. L. Gallie, and B. L. Thériault, "Regulation of p14ARF expression by miR-24: a potential mechanism compromising the p53 response during retinoblastoma development," *BMC Cancer*, vol. 12, p. 69, 2012.

[43] A. Gougelet and S. Colnot, "MicroRNA-feedback loop as a key modulator of liver tumorigenesis and inflammation," *World Journal of Gastroenterology*, vol. 19, no. 4, pp. 440–444, 2013.

[44] X. Liu, T. Wang, T. Wakita, and W. Yang, "Systematic identification of microRNA and messenger RNA profiles in hepatitis C virus-infected human hepatoma cells," *Virology*, vol. 398, no. 1, pp. 57–67, 2010.

[45] Y. Oda, M. Nakajima, T. Mohri et al., "Aryl hydrocarbon receptor nuclear translocator in human liver is regulated by miR-24," *Toxicology and Applied Pharmacology*, vol. 260, no. 3, pp. 222–231, 2012.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 742417, 12 pages
http://dx.doi.org/10.1155/2014/742417

Research Article

Reexpression of Let-7g MicroRNA Inhibits the Proliferation and Migration via K-Ras/HMGA2/Snail Axis in Hepatocellular Carcinoma

Ke-ji Chen, Ying Hou, Kui Wang, Jun Li, Yong Xia, Xiao-yu Yang, Gang Lv, Xiang-Lei Xing, and Feng Shen

Department of Hepatic Surgery, Eastern Hepatobiliary Hospital, Second Military Medical University, 225 Changhai Road, Shanghai 200438, China

Correspondence should be addressed to Feng Shen; doctor_fengshen@163.com

Received 9 December 2013; Revised 16 January 2014; Accepted 26 January 2014; Published 4 March 2014

Academic Editor: Chunping Jiang

Let-7 family microRNAs have been reported to be downregulated in human hepatocellular carcinoma in comparison with normal hepatic tissues. Among them, let-7g was identified as the lowest expression using real-time RT-PCR. However, the mechanism by which let-7g works in hepatocellular carcinoma remains unknown. Here, in our present study, we have had let-7g reexpressed *in vitro* in hepatocellular carcinoma cell lines MHCC97-H and HCCLM3 via transfection. The proliferation after reexpression of let-7g was assayed using MTT method; the migration and invasion after restoration were detected by wound-healing and Transwell assay, respectively. We found using Western-blotting that let-7g can regulate epithelial-mesenchymal transition (EMT) by downregulating K-Ras and HMGA2A after reexpresssion. Xenografted nude mice were used to observe whether or not reexpression of let-7g could have potential therapeutic ability. *In vivo*, to observe the association with let-7g expression and overall prognosis, 40 paired cases of hepatocellular carcinoma were analyzed using in situ hybridization (ISH). It was found that reexpression of let-7g can inhibit the proliferation, migration, and invasion significantly, and that low expression of let-7g was significantly associated with poorer overall survival. Taken together, let-7g could be used as a promising therapeutic agent *in vivo* in the treatment of hepatocellular carcinoma at the earlier stage.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common and most aggressive malignancy and is the third leading cause of cancer-related deaths worldwide [1], with 5-year survival being poor. The tumorigenesis of HCC is a multistage process where noncoding genes, particular microRNAs (miRNAs), and protein-coding genes were found to be deregulated in the development [2]. It has been reported that aberrant expression of miRNAs may also contribute to the development and progression of HCC [3]. Growing reports suggest that miRNAs may function as oncogenes whose expressions usually were found to be upregulated in HCC tissues [4, 5] or as tumor suppressor genes whose expressions were discovered to be downregulated in cancer tissues as compared with normal tissues in the development and progression of HCC [6, 7].

Let-7 family has been reported to be downregulated significantly in HCC [8] whose 9 members have been found in humans [9]. Among the 9 members of let-7 family, let-7g was reported to be significantly associated with metastasis of HCC and breast cancer [10, 11]. Despite the let-7g being reported to negatively regulate Bcl-xL expression and induce apoptosis in cooperation with anticancer drug targeting Mcl-1 in HCC [12] and to inhibit the cell migration in HCC through targeting collagen type I $\alpha 2$ [10], the underlying mechanism by which let-7g works to inhibit proliferation and migration in HCC remains largely unknown.

In the present study, we have identified that expression of let-7g was lowest among the seven let-7 family members that we have chosen in terms of basal expression in clinical HCC tissues using the real-time RT-PCR, and that it is only let-7g that is significantly associated with metastasis of HCC. We

found that reexpression of let-7g could alleviate the epithelial-mesenchymal transition (EMT) via downregulating the K-Ras/HMGA2A pathway. In order to evaluate the potential therapeutic ability of let-7g, xenografted nude mice were employed. It was found that let-7g can significantly suppress the tumorigenesis of HCC *in vitro* at the earlier stage. Furthermore, we found that low expression of let-7g was significantly associated with poorer survival outcomes.

2. Materials and Methods

2.1. HCC Cell Lines and Clinical Tissues Samples. The human HCC cell lines MHCC97-H and HCCLM3 (Shanghai fmg-bio, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitorgen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) in a humidified incubator at 37 with an atmosphere of 5% CO_2. 40 pairs of fresh samples from HCC were recruited from surgical specimens collected from 2007 to 2013, fixed in formalin, and embedded in paraffin. The current study was approved by the local Medical Ethics Committee and signed informed consent was obtained. None of the recruited patients received treatment before surgery, and for all patients clinical-pathological information was available.

2.2. RNA Isolation and qRT-PCR. Total RNA of cells was isolated using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA was reversely transcripted into cDNAs by PrimeScript one-step RT-PCR kit (TAKARA, Dalian, China). The expression of let-7g was measured by qRT-PCR assays. All samples with SYBR Green PCR Master Mix (TAKARA, Dalian, China), U6 expression was assayed for normalization, and relative gene expression determinations were made with the comparative delta-delta CT method ($2^{-\Delta\Delta Ct}$). The reaction mixtures of let-7g and U6 were incubated at thermal cycling conditions comprised 95°C for 30 sec, and 40 cycles at 95°C for 5 sec followed by 56.5°C for 30 sec. The primers used for quantitative RT-PCR were provided in Supplementary Table 1 (available online at http://dx.doi.org/10.1155/2014/742417).

2.3. In Situ Hybridization (ISH). Expression of let-7g in 40 paired of HCC tissues and normal controls were detected by ISH with probes for let-7g (Exiqon, Woburn, Massachusetts). Melted paraffin was in an oven at 60°C for 45 min then stored as slides overnight (O/N) at 4°C. Deparaffinized slides were in xylene and ethanol solutions at room temperature and then incubated with Proteinase-K for 10 min at 37°C. Slides were hybridized with 20 nmol/L let-7g probe in a hybridization buffer for 2 h at 55°C then washed with SSC buffers. The remaining procedures were performed with a modified version of the manufacturer's protocol. The slides were counterstained with hematoxylin and eosin (H&E) and visualized under a microscope. Each slide was examined by an observer blinded to the diagnosis and clinicopathologic data and reviewed and confirmed by a second blinded observer. Staining intensity and percentage of positive cells were noted as follows: four grades were used for the staining intensity (0: no intensity; 1: weak intensity; 2: moderate intensity; 3: strong intensity), and four grades were used for the percentage of positive cells (0: less than 10%; 1: between 10%–25%; 2: between 25%–40%; 3: more than 40%). HCC patients were classified into two groups according to the total score of staining intensity plus percentage of positive cells: low expression group (total score: 0–2) and high expression group (total score: 3–6), in order to better analyze the prognosis between groups.

2.4. Transfection. Plasmid vector pCMV-let-7g harboring let-7g precursor and scramble sequence were purchased from OriGene (SC400010, OriGene, USA). MHCC97-H and HCCLM3 cells were grown to 80–90% confluence in 6-well plates and were transfected with 4.0 μg of pCMV-let-7g or scramble control vector together with 10 μL Lipofectamine 2000 (Invitrogen, CA, USA) in Opti-MEM (Invitrogen, CA, USA) following the manufacturer's instruction (Invitrogen Life Technologies, CA, USA). All groups were performed in triplicate. Cells were lysed for RNA at 48 h and protein at 72 h after transfection.

2.5. Western Blotting. Seventy-two hours after transfection, MHCC97-H and HCCLM3 cells were harvested in RIPA Lysis buffer (Bioteke, Beijing, China) and 80 μg of cellular protein was subjected to 10% SDS-PAGE separation. Proteins were transferred to PVDF microporous membrane (Millipore, Boston, MA, USA) and blots were probed with rabbit polyclonal antibody against K-Ras (#3339), ERK1/2 (#4695), p-ERK1/2 (#4370), Snail (#4719), E-cadherin (#3195), N-cadherin (#4061), and HMGA2 (#8179); they were all from cell signaling technology (Cell Signaling Technology, USA). β-Tubulin (sc-9104), GAPDH (sc-25778), Bax (sc-20067), Bak (sc-1035), and Bcl-xL (sc-8392) were all from Santa Cruz Biotechnology (CA, USA). β-Tubulin and GAPDH were chosen as an internal control. the blots were visualized using WesternBreeze Kit (WB7105, Invitrogen Life Technologies, CA, USA), and further quantified using Quantity One Software (Bio-Rad Laboratories, USA) by measuring the band intensity for each group and normalizing to β-Tubulin and GAPDH as internal control. The final results were expressed as fold changes by normalizing the data to the control values.

2.6. Cell Proliferation Assay. The methyl thiazolyl blue tetrazolium (MTT; Sigma-Aldrich, St Louis, MO) spectrophotometric dye assay was used to detect cell proliferation ability. MHCC97-H and HCCLM3 cells were plated in 96-well plates at a density of 5×10^3 cells per well. After transfection experiments, cell proliferation was assessed. Cells were incubated for 4 h in 20 μL MTT at 37°C. The color was developed by incubating the cells in 150 μL dimethyl sulfoxide, the absorbance was detected at 490 nm wave length. The data were obtained from three independent experiments.

2.7. Cell Migration and Invasion Assays In Vitro. Cell migration was assayed using the wound healing assay. MHCC97-H and HCCLM3 cells were plated in 6-well plate at a concentration of 5×10^5 cells/well and allowed to form a confluent

monolayer for 24 h. After transfection, the monolayer was scratched with a sterile pipette tip (10 μL) then washed with serum free medium to remove the floating and detached cells and photographed (time 0 h, 24 h and 48 h) using inversion fluorescence microscope (Olympus, Japan). Cell culture inserts (24-well, pore size 8 μm; BD Biosciences) were seeded with 5×10^3 cells in 100 μL of medium with 0.1% FBS. Inserts precoated with Matrigel (40 μL, 1 mg/mL; BD Biosciences) were used for invasion assays. Medium with 10% FBS (400 μL) was added to the lower chamber and served as a chemotactic agent. Noninvasive cells were wiped from the upper side of the membrane and cells on the lower side were fixed in cold methanol (−20°C) and air dried. Cell were stained with 0.1% crystal violet (dissolved in methanol) and counted using the inverted microscope. Each individual experiment had triplicate inserts, and 4 microscopic fields were counted per insert.

2.8. Cell Cycle and Apoptosis Analysis. The cell cycle and apoptosis were analyzed using flow cytometry (FCM). For cell cycle analysis, cells were plated at a density of 3×10^5 per well in 6-well plate and transfected with either PCMV-let-7g overexpression plasmid or a blank vector control. Cells were washed twice with cold Phosphate buffer saline (PBS) and fixedin 70% cold ethanol O/N at 4°C. After incubation with RNase for 1 h at 4°C, DNA was stained with 2 μL propidium iodide (PI) (400 mg/mL) for 15 min then was analyzed by FCM. For cell cycle analysis, Annexin V-FITC apoptosis detection kit was used (Invitrogen Life Technologies, CA, USA), and AnnexinV-FITC staining was performed following the instructions provided by the manufacturer. Briefly, cells were washed twice with cold PBS and resuspended in 400 μL with 1× binding buffer at a concentration of 1×10^6 cells/mL. Cells were then mixed with 5 μL of the Annexin V-FITC solution and 2 μL of propidium iodide (PI). Cells were incubated for 15 min at 4°C in the dark then analyzed by FCM.

2.9. Tumor Xenografts. To evaluate *in vivo* tumorigenesis, HCC xenografting mouse model was developed. Male BALB/c-nude mice of 4 weeks were prepared for tumor implantation. All animals were maintained in a sterile environment on a daily 12-h light/12-h dark cycle and categorized into 2 groups with each group being 8 nude mice. After resuspension in PBS, HCCLM3 cells (3×10^6/mouse) were injected subcutaneously into the flanks of the nude mice. One week after implantation when the tumor became visually palpable at the size of 2 mm in diameter, intratumoral injection with 40 μg of let-7g precursor plasmid dissolved in 100 μL of DMEM mixed with 5 μL of Lipofectamine 2000 (Invitrogen) was done every two days. Tumor volume (TV) was measured every two days according to the formula: TV (mm^3) = length × $width^2$ × 0.5 [13].

2.10. Statistics. Data were expressed as mean ± SD and were analyzed by Student's t-test, one-way ANOVA, and χ^2 test using SPSS for Windows version 16.0 (SPSS, Chicago, USA). Kaplan-Meier survival curves were plotted and log rank test was done. The significance of various variables for survival was analyzed by Cox proportional hazards model in a multivariate analysis. $P < 0.05$ in all cases was considered statistically significant.

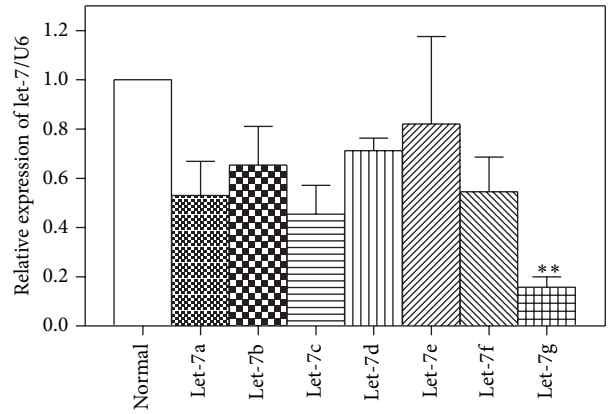


FIGURE 1: The endogenous expression level of let-7g in HCC tissues. The basal expression of let-7 family members, including let-7a, let-7b, let-7c, let-7d, let-7e, and let-7f was detected using quantitative real-time Reverse Transcription PCR (qRT-PCR) in 40 paired HCC tissues and their normal controls. Total RNA was extracted using Trizol reagent after 48 h. The relative expression of let-7 family members, normalized to U6, was calculated using the formula $2^{-\Delta\Delta Ct}$ (relative expression). $^{**}P < 0.01$ versus normal control.

3. Results

3.1. Let-7 Family Were Reduced in HCC. To observe the basal expression of let-7 family in HCC, we performed real-time RT-PCR to detect the 7 members of let-7 miRNA family in 40 paired HCC clinical tissues and paired normal control. It was found that the levels of miRNA let-7 family including let-7a, let-7b, let-7c, let-7e, let-7f, and let-7g were all consistently reduced in HCC tissues as compared with the normal hepatocellular tissues. Among them, relative expression of let-7g was significantly the lowest (Figure 1). Hence, let-7g was chosen as miRNA of interest in our following experiment.

3.2. Low Expression of Let-7g Was Significantly Associated with Prognosis. To investigate the expression and localization of let-7g in patients with HCC, in situ hybridization (ISH) was performed. It can be seen that let-7g was mainly localized in cytoplasm of HCC cells and let-7g was heterogeneously expressed, with some cases being high and others being low expression in HCC tissues (Figure 2(a)). Kaplan-Meier analysis was conducted illustrating that there was significant correlation between poor overall survival and low expression of let-7g in the 40 cases of HCC cohort using ISH method (Figure 2(b)).

3.3. Reexpression of Let-7g Inhibited Proliferation in HCC Cell Lines. To elucidate the functional role of let-7g in HCC, eukaryotic expression vector harboring let-7g precursor sequence and control vector harboring scramble sequence of

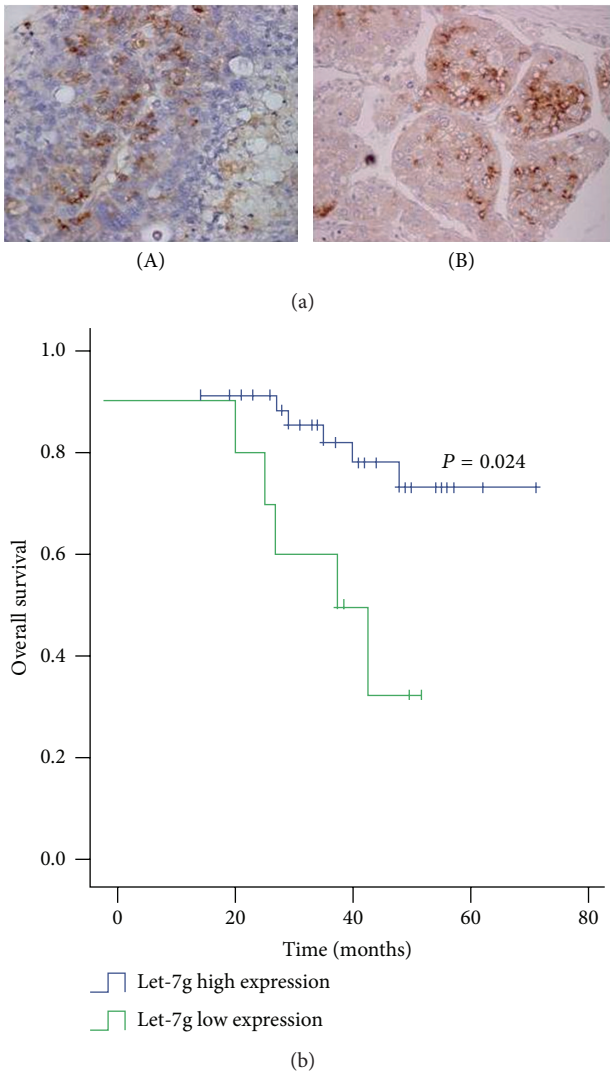


Figure 2: Low expression of let-7g was significantly associated with poorer overall survival. (a) Heterogeneous expression of let-7g was detected using in situ hybridization method in HCC tissues. Figure (A) showed that let-7g was low in HCC whereas figure (B) showed let-7g high in HCC. The magnification fold was (×100). (b) Kaplan-Meier survival curves were plotted. There is strikingly significant difference between let-7g positive group and let-7g negative group ($P < 0.01$, using log rank test), after analysis using ISH in 40 cases of HCC tissues.

let-7g were transfected into two different kinds of HCC cell lines, MHCC97-H and HCCLM3, respectively. Both the two vectors had green fluorescent protein (GFP), which can be indirectly monitoring the expression rate after transfection (Supplementary Figure 1). Efficiency of transfection was measured using quantitative real-time RT-PCR (Supplementary Figure 2). The cell proliferation was monitored using MTT method continuously for 4 days after transfection. No significant changes in size and morphology of MHCC97-H and HCCLM3 were found using light microscope (data not shown). However, the proliferation in the group transfected with let-7g precursor plasmid was significantly suppressed as compared with the group transfected with scramble sequence of let-7g for MHCC97-H and HCCLM3 cell lines (Figure 3).

3.4. Reexpression of Let-7g Inhibited Migration and Invasion of HCC Cell Lines. To test whether or not let-7g reexpression could have an effect on the metastasis of HCC cell lines, we examined the rate of migration and invasion through wound-healing and Transwell approach. It was found that the migration ($P < 0.01$, Figure 4) and invasion ($P < 0.05$, Figure 5) were both significantly inhibited in MHCC97-H and HCCLM3 cell lines after reexpression of let-7g.

3.5. Reexpression of Let-7g Induced Apoptosis and Cell Cycle Change of HCC Cell Lines. To examine whether or not reexpression of let-7g could induce cell apoptosis and cell cycle variation, both MHCC97-H and HCCLM3 cell lines were subjected to Flow Cytometry analysis after reexpression of let-7g for 48 hours. We found that significant apoptosis occurred to both of the two different HCC cell lines ($P < 0.01$, Figures 6(a) and 6(b)). With regard to variation of the cell cycle, it was found that cell cycles were arrested at G1 stage ($P < 0.01$, Figures 6(c) and 6(d)). Based on the earlier reports [12], several molecular markers involved in the regulation of apoptosis and cell cycle were detected using Western-blotting. What was previously reported was confirmed; that is, apoptosis related proteins BaX and BaK were upregulated, whereas apoptosis inhibiting protein Bcl-xL was downregulated after reexpression of let-7g precursor for 72 hours (Figure 6(e)), suggesting that it is through upregulating the Bax and BaK and downregulating Bcl-xL that let-7g induced apoptosis of HCC cell lines, MHCC97-H and HCCLM3.

3.6. Reexpression of Let-7g Suppressed the Expression of HMGA2 and EMT Markers. To explore the possible mechanism of let-7g, we hypothesized that let-7g reexpression inhibited malignant cellular behaviors via downregulating K-Ras-mediated mitogen-activated protein kinase (MAPK) signaling pathway, based on the analysis and reanalysis of published reports available [14–16]. Additionally, a recent study has shown that HMGA2, one of the targets of let-7g, was involved in the process of epithelial-mesenchymal transition (EMT) [17]. In the light of these peer findings mentioned; we detected some of the key protein markers which were reported to be associated highly with MAPK and EMT using immunoblotting approach. it was shown that K-Ras and phosphorylated ERK1/2 (p-ERK1/2) were decreased in both MHCC97-H and HCCLM3 cell lines after reexpression of let-7g as compared with control group. On reexpressing the let-7g, E-cadherin that has been a typical pathological marker for epithelial trait was upregulated; meanwhile, N-cadherin and Snail were downregulated. HMGA2A expression was expectedly and significantly decreased after upregulation of let-7g (Figure 7), which was wholly consistent with Zucchini-Pascal et al.'s observation [18].

3.7. Reexpression of Let-7g Suppressed Tumorigenesis of HCC Xenografts. Next, to test whether or not let-7g re-expression

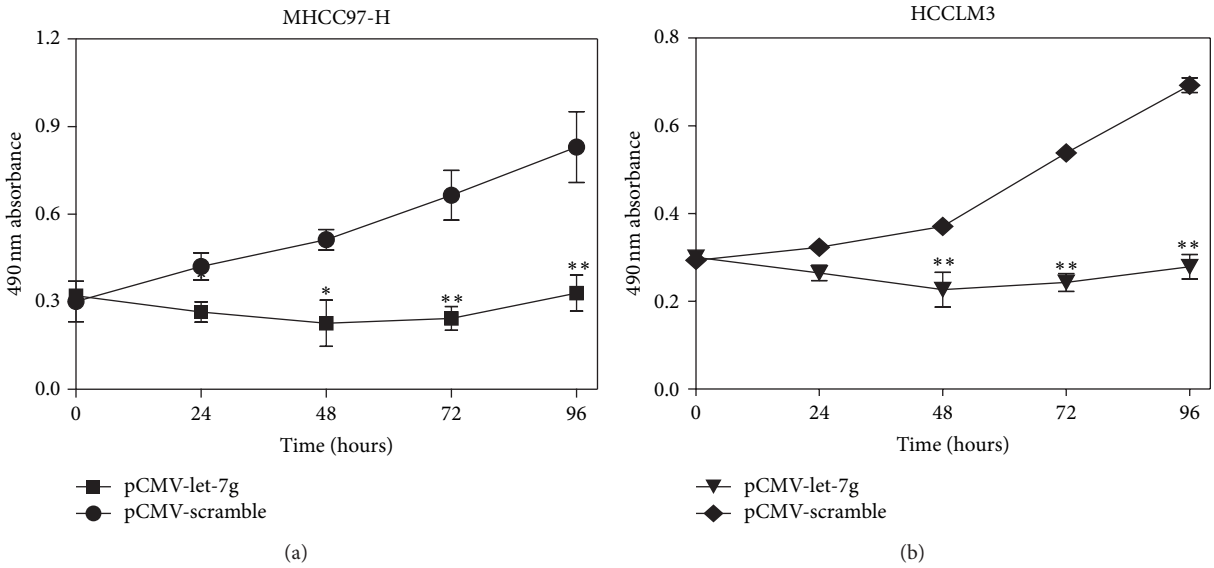


Figure 3: Reexpression of let-7g inhibited the proliferation of MHCC97-H and HCCLM3 cells. Cell proliferation of MHCC97-H and HCCLM3 cells after reexpression of let-7g for 0 h, 24 h, 48 h, 72 h, and 96 h were examined by MTT assay. Let-7g can obviously retard the proliferation as compared with control group ($^{**}P < 0.01$, one-way ANOVA analysis). The mean and standard error from triplicate experiments are indicated.

could suppress the tumor formation *in vivo*, nude mice xenografted with HCCLM3 cells were employed. Intratumoral injection with let-7g precursor plasmid at two-day interval was as experimental group. Injection with plasmid having scramble sequence as in the same way control. Tumor size was calculated according to the reference [13]. It can be seen that injection with let-7g precursor plasmid can significantly reduce the tumor volume of HCCLM3 before or at the 10th day (Figure 8), suggesting that reexpression of let-7g could be used as ideal therapeutic agent in the treatment of HCC at the earlier stage.

4. Discussion

In our present study, we found that among the let-7 family miRNAs, endogenous expression of let-7g was lowest in the clinical HCC tissues. Reexpression of let-7g can significantly inhibit the malignant behaviors of HCC cells *in vitro* and suppress HCC tumorigenesis at the earlier stage *in vivo*. Further, in HCC cell lines, we found it is the way through K-Ras/HMGA2A/Snail axis that reexpression of let-7g inhibitied the proliferation, migration, and invasion of HCC cells. Additionally, low expression of let-7g was significantly associated with inferior overall survival of patients with HCC. Our results suggest that reexpression of let-7g could be used as an ideal therapeutic agent in the management of HCC at earlier stage.

Let-7 is a family consisting of 13 members located on nine different chromosomes whose expression usually has been lost, reduced, or deregulated in the majority of human cancers [2]. A growing evidence suggests that restoration of let-7 expression has an antiproliferative effect on cancer cells of different kinds [8], thus indicating that let-7 restoration may be a useful therapeutic option in HCC. In light of the conflicting expression patterns of let-7 family across human cancers [8], we first tested the expression levels of seven kinds of let-7 family members we have chosen in our study in 40 paired HCC tissues and their corresponding normal controls. We observed that the 7 different members of let-7 family were all reduced in HCC tissues as compared with normal control tissues. Among these, we have identified that only let-7g was significantly correlated with HCC metastasis, which was highly consistent with result obtained by Zhao and colleagues that let-7g was found to be reduced and associated with metastasis of HCC [19]. We therefore hypothesized that the low let-7g may contribute to the high metastasis rate of HCC cells.

To test the hypothesis we proposed, we transfected the two different HCC cell lines, MHCC97-H and HCCLM3 with the similar metastatic ability, with let-7g precursor plasmid and control plasmid, respectively. It is shown that reexpression of let-7g in MHCC97-H and HCCLM3 can significantly suppress the migration and invasion ability of MHCC97-H and HCCLM3 cells in culture system, which was in line with and in agreement with Qian and coworkers' report in spite of whose consistent results were found in breast carcinoma [11].

In the following, we have analyzed whether or not there were variations of cellular apoptosis and cell cycle after restoration of let-7g. We found that reexpression of let-7g can substantially induce apoptosis rate as compared with control group where scramble sequence of let-7g was transfected, and cell cycles were significantly arrested at G1 stage. Based on the earlier report [12] that let-7g could induce cellular apoptosis via downregulation of Bcl-xL and meanwhile upregulation of Bax. To verify the proposal, we have tested the protein

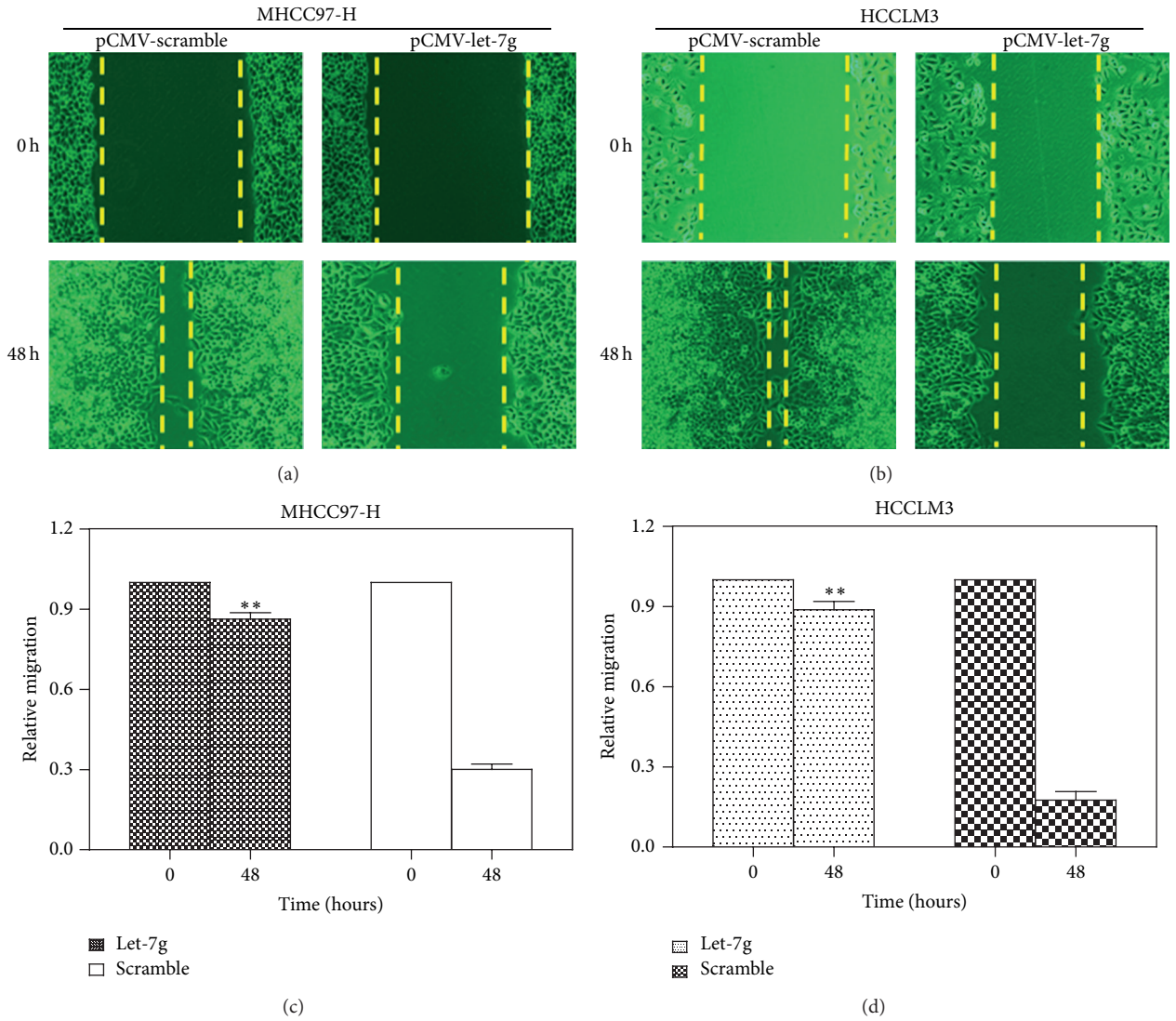


Figure 4: Reexpression of let-7g inhibited the migration of MHCC97-H and HCCLM3 cells. (a) Qualification of wound-healing assay of MHCC97-H after re-expression of let-7g for 0 and 48 hours. (b) Qualification of wound-healing assay of HCCLM-3 after re-expression of let-7g for 0 and 48 hours. (c) Quantitation of wound-healing assay of MHCC97-H. Migration of MHCC97-H cells was significantly retarded in comparison with scramble sequence control group. There is strikingly significant difference between group transfected with scramble sequence and group transfected with let-7g group ($^{**}P < 0.01$, one-way ANOVA analysis). (d) Quantitation of wound-healing assay of HCCLM-3. Highly similarly, migration of HCCLM-3 cells was also significantly suppressed compared with scramble sequence group. ($^{**}P < 0.01$, one-way ANOVA analysis). The mean and standard error from triplicate experiments are indicated.

markers mentioned previously in HCCLM3 after reexpression of let-7g. It turns out to be that our results confirmed the previous findings.

What is the potential mechanism by which reexpression of let-7g inhibited migration and invasion in HCC cell lines? So far, the potential mechanism by which let-7g work in the antiproliferation and antimetastasis of HCC still remains unknown, despite the recent peer findings available that let-7g may suppress HCC metastasis partially through targeting COL1A2 [10] and that let-7g inhibit cell proliferation of HCC by downregulating the c-Myc and upregulating p16 (INK4A) [20]. Thus, to better understand the potential mechanism of let-7g, we explored the variations of the several possible important protein markers that were reported to be involved in EMT process based on the evidence available [21, 22]. We found that EMT happened to both MHCC97-H and HCCLM3 cells. The epithelial trait marker, E-cadherin, was upregulated whereas at the same time N-cadherin and Snail which belonged to specific mesenchymal biomarker were correspondingly decreased in a different level, suggesting that restoration of let-7g could alleviate the EMT extent that is crucial event in HCC progression and recurrence [22]. Liu et al., in the investigation of EMT of breast cancer, discovered the Ras was heavily involved in EMT process [23]. Based on reports above mentioned, we found that E-cadherin, N-cadherin, and Snail were changed as reported

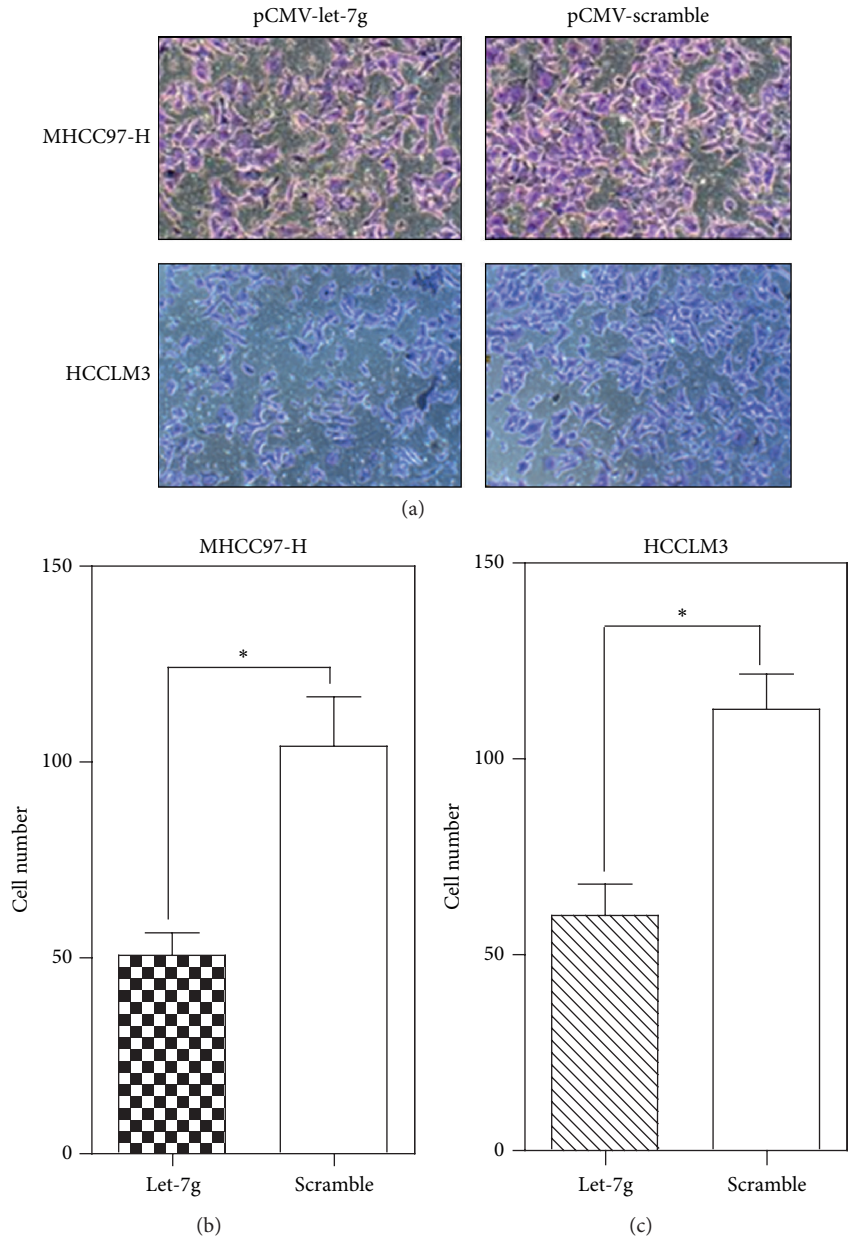
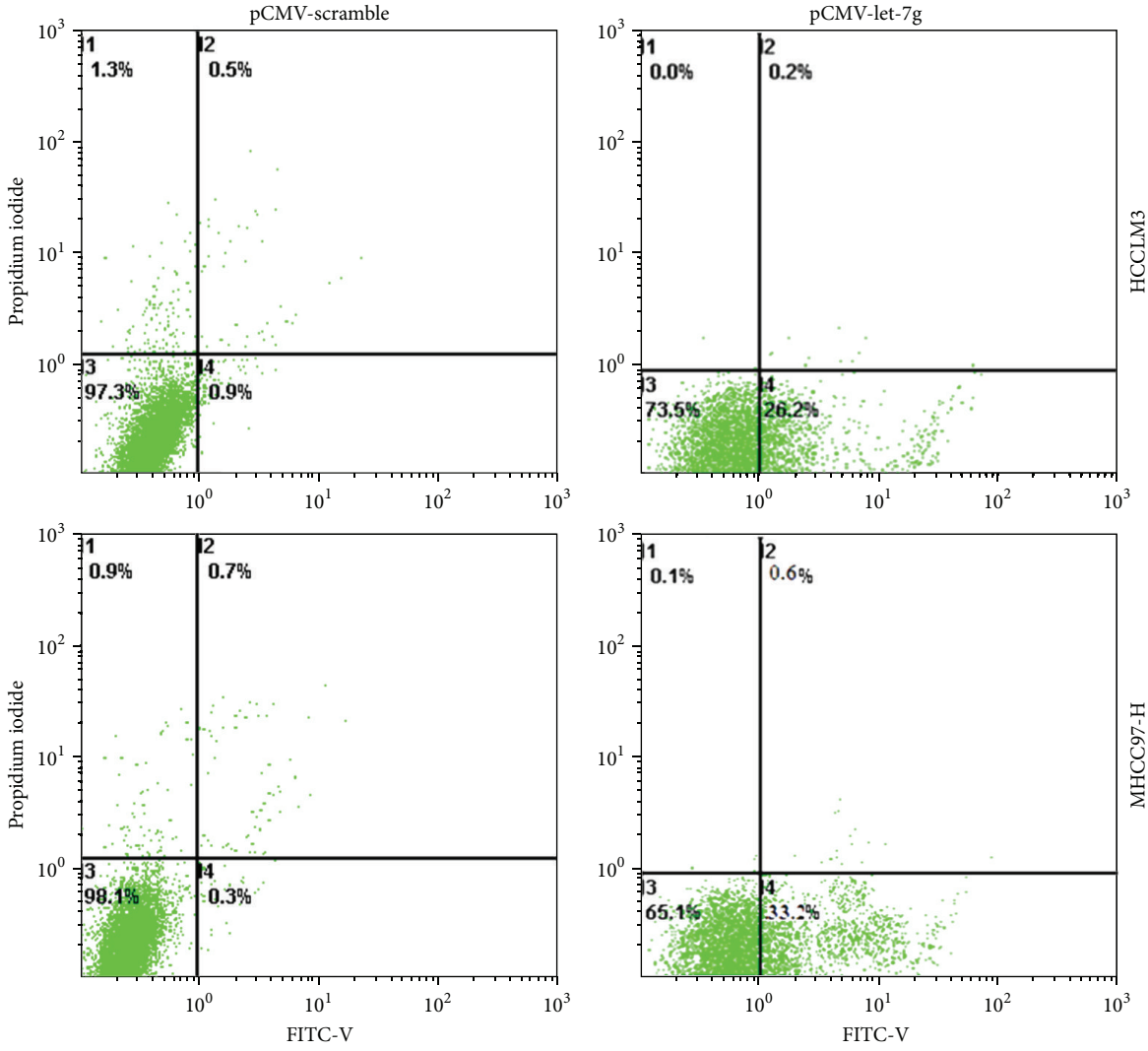


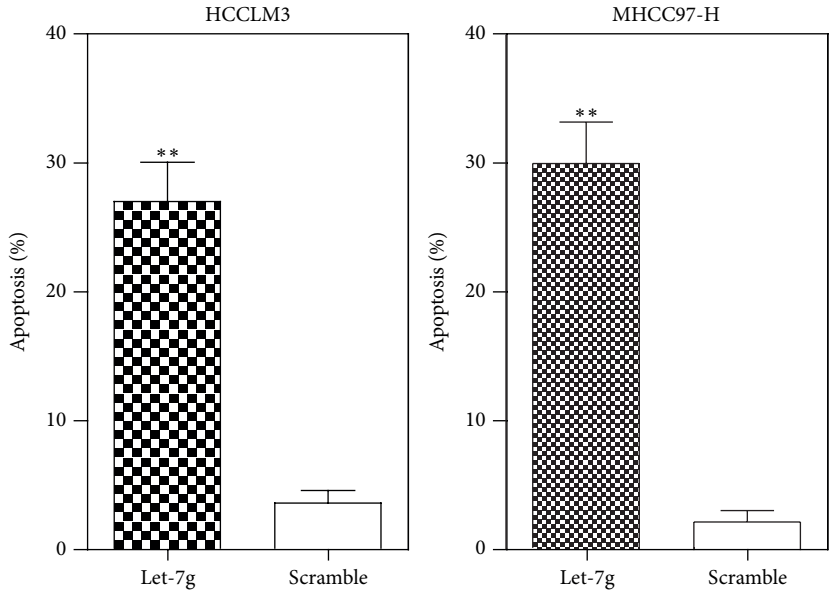
Figure 5: Reexpression of let-7g inhibited the invasion of MHCC97-H and HCCLM3 cells. (a) Qualification of Transwell assays for MHCC97-H and HCCLM3 after reexpression of let-7g for 48 hour. (b) Quantitation of Transwell assay for MHCC97-H ($^*P < 0.05$, Student's t-test). (c) Quantitation of Transwell assay for HCCLM3 ($^*P < 0.05$, Student's t-test). The mean and standard error from triplicate experiments are indicated.

after K-Ras was transiently silenced using specific siRNA. Tan and colleagues made serial discoveries that HMGA2 can regulate important transcription factor Twist [24] and Snail [25] in a single or combinational with Smads in the induction of EMT. Therefore, based on findings of our own as well as other peer findings, results suggest that reexpression of let-7g could suppress the EMT process via downregulation of K-Ras/ERK1/2 signaling pathway.

Given the significant anti-proliferation effect of let-7g, we have developed the xenografted nude mice to evaluate the effect after reexpression of let-7g *in vivo* in the tumorigenesis of HCC. It was shown that let-7g reexpression can significantly inhibit the tumorigenesis of HCC in nude mice, which is highly consistent and similar with Kumar et al., in their study in the evaluation of suppression of tumorigenesis of let-7g in nonsmall cell lung cancer [14]. Nonetheless, one dilemma that is inevitable and we fell into in the experimentation of xenografted nude mice is that let-7g reexpression was only workable at the earlier stage (at or before the 10th days). After the 10th day, it turns out to be insufficient or incapable of inhibiting the tumorigenesis of HCC. Thus, it can be seen that the continuous let-7g delivery

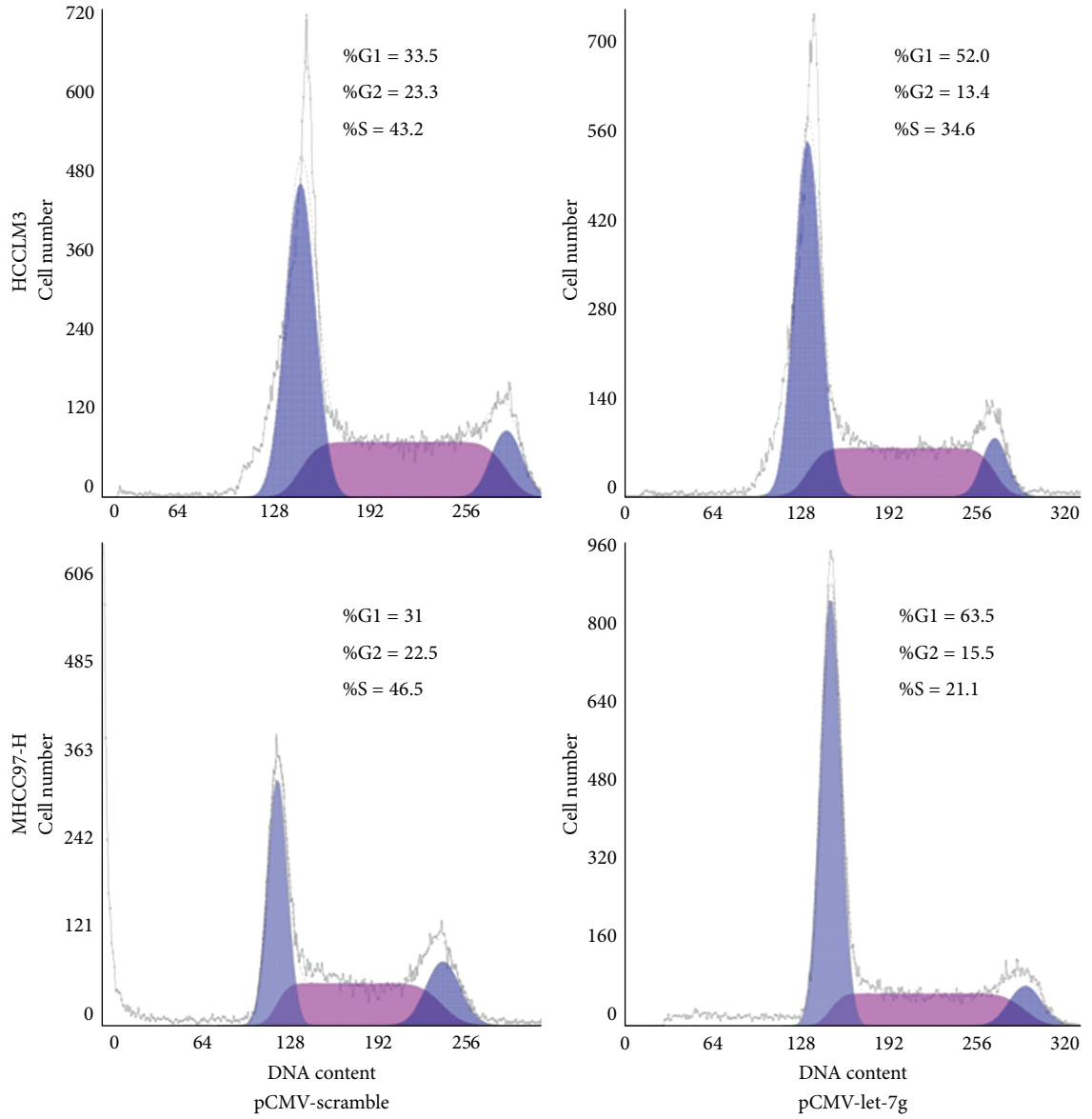


(a)

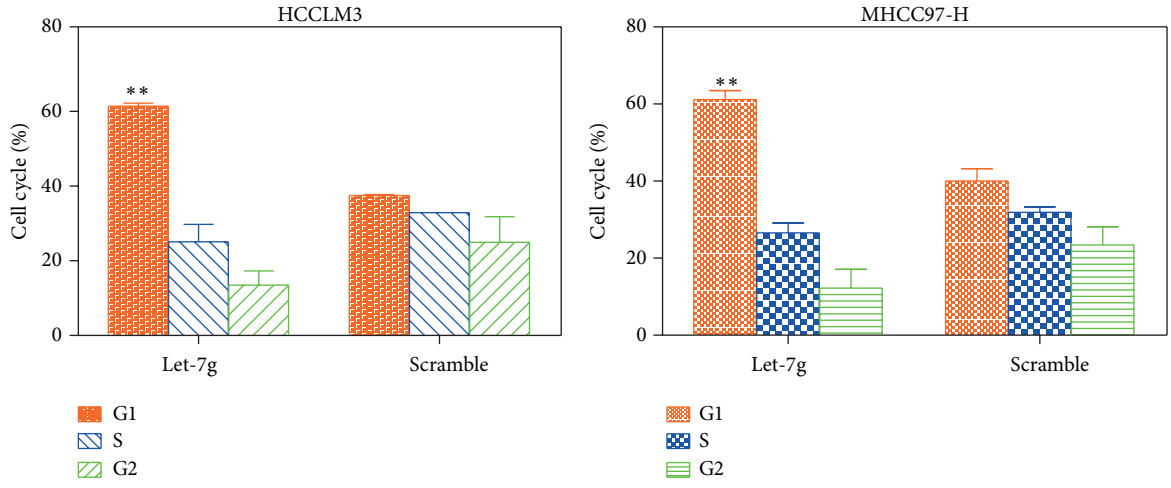


(b)

Figure 6: Continued.

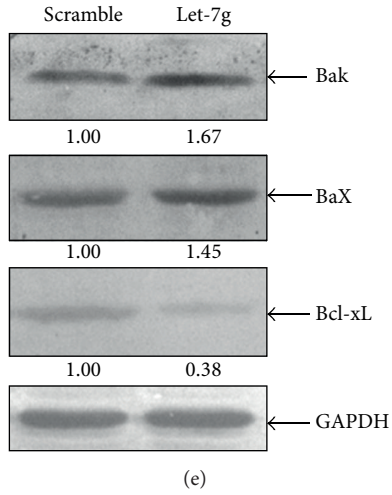


(c)



(d)

Figure 6: Continued.



(e)

Figure 6: Reexpression of let-7g induced cell apoptosis and changes of cell cycle. (a) Qualification assay of cell apoptosis of MHCC97-H and HCCLM3 after reexpression of let-7g. (b) Quantitation assay of cell apoptosis of MHCC97-H and HCCLM3 after re-expression of let-7g ($^{**}P < 0.01$, Student's t-test). (c) Qualification assay of cell cycle of MHCC97-H and HCCLM3 after reexpression of let-7g. (d) Quantitation assay of cell cycle of MHCC97-H and HCCLM3 after reexpression of let-7g ($^{**}P < 0.01$, Student's t-test). (e) Biochemical analysis of proteins that are reported to be involved in the regulation of cell cycle. The representative results out of three times experiments were shown here. 80 μg total protein was loaded per lane when performing Western-blotting.

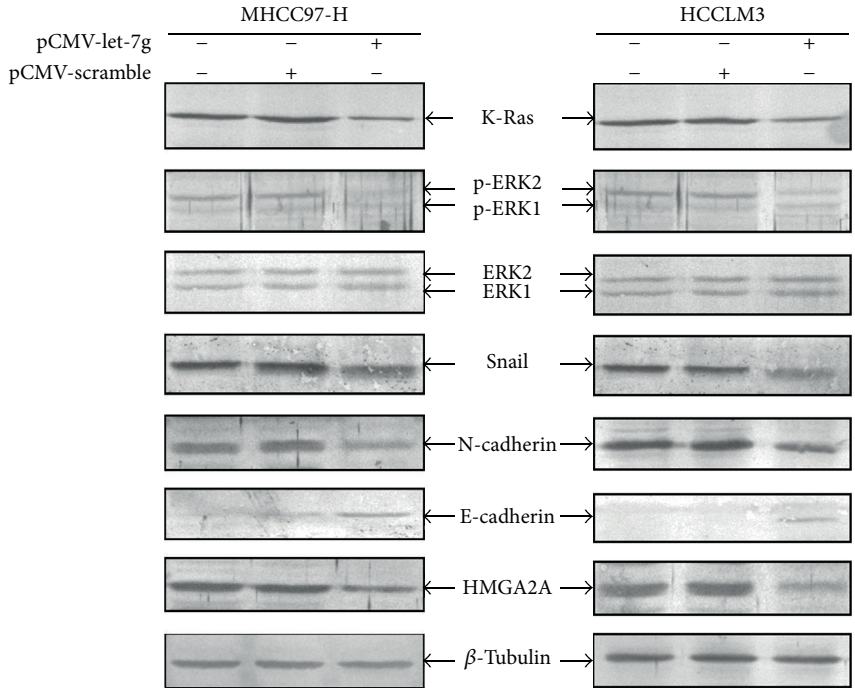


Figure 7: Reexpression of let-7g regulated EMT through downregulating the K-Ras/ERK1/2 signaling pathway. Western-blot analysis of K-Ras, p-ERK1/2, ERK1/2, Snail, E-cadherin, N-cadherin, and HMGA2A after transfection with let-7g precursor and scramble sequence control plasmids for 72 hours. β-Tubulin was as loading control. 80 μg total protein was loaded per lane, which was separated by 10% SDS-PAGE followed by visualization with WesternBreeze kit (Invitrogen, USA).

might lead to initial suppression of tumor growth before the 10th day but let-7g-resistant tumors would eventually and inevitably emerge. The active effect of let-7g may be related to the drug infiltration and microenvironment change of the tumors [26]. In spite of the limitation of the results, our study indicates that let-7g could still be used as an ideal therapeutic agent *in vivo* in the earlier stage therapy of HCC.

In our present study, we have employed 40 cases of HCC tissues and their controls, although quite limited, observing that low expression of let-7g was significantly associated with

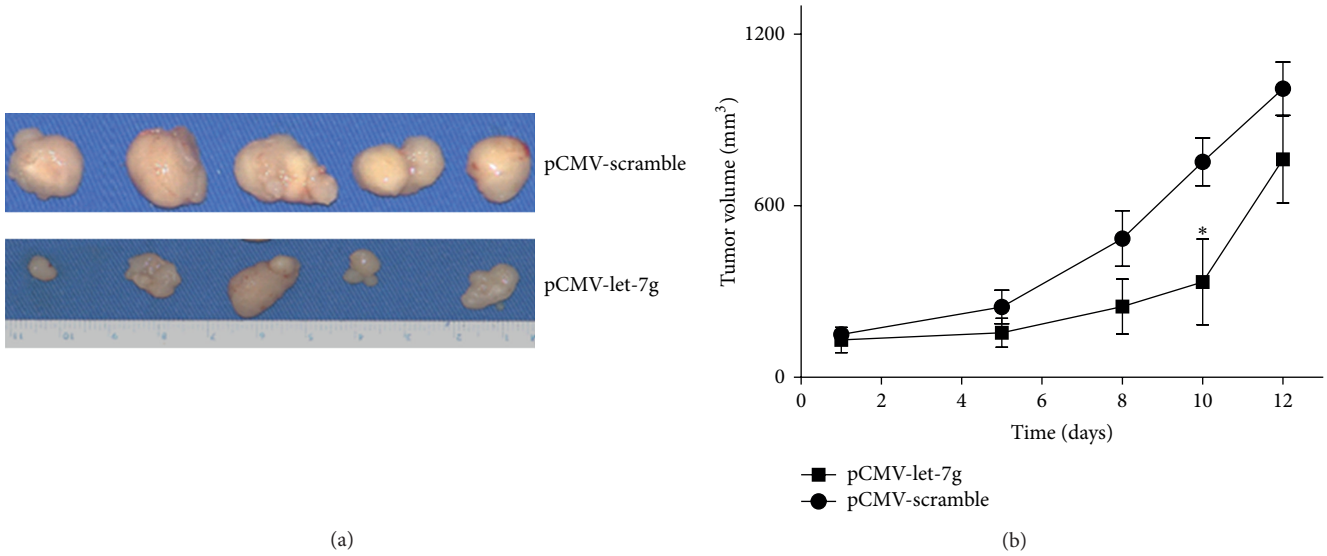


Figure 8: Reexpression of let-7g inhibited the tumorigenesis of HCC in xenografted nude mice. Every 2 days intratumoral injection with let-7g precursor plasmid significantly reduced the tumor volume at the 10th day as compared with control plasmid group. (a) Representative image of tumors resected from the two groups. (b) Quantitative evaluation of tumor size at different therapeutic times ($^{*}P < 0.05$, Student's t-test).

poorer overall survival. The result should have been required to be confirmed with a great larger number of patients with HCC in multidisciplinary centers.

Put together, we found that restoration of let-7g can significantly inhibit the malignant behaviors of HCC cells *in vitro* in the way through downregulating the K-Ras/HMGA2A/Snail axis and suppressing HCC tumorigenesis *in vivo* at the earlier stage. What is more is that low expression of let-7g was significantly associated with inferior overall survival of patients with HCC. Our results suggest that let-7g could be used as an ideal therapeutic agent *in vivo* in the management of patients with earlier stage of HCC.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

The whole work was supported by Department of Hepatic Surgery, Eastern Hepatobiliary Hospital, Second Military Medical University.

References

[1] L. L. Wong, B. Hernandez, S. Kwee, C. L. Albright, G. Okimoto, and N. Tsai, "Healthcare disparities in Asians and Pacific Islanders with hepatocellular cancer," *American Journal of Surgery*, vol. 203, no. 6, pp. 726–732, 2012.

[2] Y. Saito, H. Suzuki, M. Matsuura et al., "MicroRNAs in hepatobiliary and pancreatic cancers," *Frontiers in Genetics*, vol. 2, article 66, 2011.

[3] Y.-C. Wang, Y.-L. Chen, R.-H. Yuan et al., "Lin-28B expression promotes transformation and invasion in human hepatocellular carcinoma," *Carcinogenesis*, vol. 31, no. 9, pp. 1516–1522, 2010.

[4] G. Xu, Y. Zhang, J. Wei et al., "MicroRNA-21 promotes hepatocellular carcinoma HepG2 cell proliferation through repression of mitogen-activated protein kinase-kinase 3," *BMC Cancer*, vol. 13, article 469, 2013.

[5] D. Mao, X. Tao, F. Gao, C. Fan, and D. Wu, "miR-224 functions as an onco-miRNA in hepatocellular carcinoma cells by activating AKT signaling," *Oncology Letters*, vol. 4, no. 3, pp. 483–488, 2012.

[6] W. Wang, H. Lin, L. Zhou et al., "MicroRNA-30a-3p inhibits tumor proliferation, invasiveness and metastasis and is downregulated in hepatocellular carcinoma," *European Journal of Surgical Oncology*, 2013.

[7] Y. Zhang, X. Guo, L. Xiong et al., "MicroRNA-101 suppresses SOX9-dependent tumorigenicity and promotes favorable prognosis of human hepatocellular carcinoma," *FEBS Letters*, vol. 586, no. 24, pp. 4402–4470, 2012.

[8] B. Boyerinas, S.-M. Park, A. Hau, A. E. Murmann, and M. E. Peter, "The role of let-7 in cell differentiation and cancer," *Endocrine-Related Cancer*, vol. 17, no. 1, pp. F19–F36, 2010.

[9] B. J. Reinhart, F. J. Slack, M. Basson et al., "The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans," *Nature*, vol. 403, no. 6772, pp. 901–906, 2000.

[10] J. Ji, L. Zhao, A. Budhu et al., "Let-7g targets collagen type I α2 and inhibits cell migration in hepatocellular carcinoma," *Journal of Hepatology*, vol. 52, no. 5, pp. 690–697, 2010.

[11] P. Qian, Z. Zuo, Z. Wu et al., "Pivotal role of reduced let-7g expression in breast cancer invasion and metastasis," *Cancer Research*, vol. 71, no. 20, pp. 6463–6474, 2011.

[12] S. Shimizu, T. Takehara, H. Hikita et al., "The let-7 family of microRNAs inhibits Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma," *Journal of Hepatology*, vol. 52, no. 5, pp. 698–704, 2010.

[13] H. Kamiyama, M. Kamiyama, S.-M. Hong et al., "In vivo and in vitro propagation of intraductal papillary mucinous neoplasms," *Laboratory Investigation*, vol. 90, no. 5, pp. 665–673, 2010.

[14] M. S. Kumar, S. J. Erkeland, R. E. Pester et al., "Suppression of non-small cell lung tumor development by the let-7 microRNA family," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 10, pp. 3903–3908, 2008.

[15] J. T. Whelan, S. E. Hollis, D. S. Cha, A. S. Asch, and M.-H. Lee, "Post-transcriptional regulation of the Ras-ERK/MAPK signaling pathway," *Journal of Cellular Physiology*, vol. 227, no. 3, pp. 1235–1241, 2012.

[16] Z. Paroo, X. Ye, S. Chen, and Q. Liu, "Phosphorylation of the human microRNA-generating complex mediates MAPK/Erk signaling," *Cell*, vol. 139, no. 1, pp. 112–122, 2009.

[17] S. Watanabe, Y. Ueda, S.-I. Akaboshi, Y. Hino, Y. Sekita, and M. Nakao, "HMGA2 maintains oncogenic RAS-induced epithelial-mesenchymal transition in human pancreatic cancer cells," *American Journal of Pathology*, vol. 174, no. 3, pp. 854–868, 2009.

[18] N. Zucchini-Pascal, L. Peyre, and R. Rahmani, "Crosstalk between beta-catenin and snail in the induction of epithelial to mesenchymal transition in hepatocarcinoma: role of the ERK1/2 pathway," *International Journal of Molecular Sciences*, vol. 14, no. 10, pp. 20768–20792, 2013.

[19] Y. Zhao, H.-L. Jia, H.-J. Zhou et al., "Identification of metastasis-related microRNAs of hepatocellular carcinoma in hepatocellular carcinoma cell lines by quantitative real time PCR," *Chinese Journal of Hepatology*, vol. 17, no. 7, pp. 526–530, 2009.

[20] F.-F. Lan, H. Wang, Y.-C. Chen et al., "Hsa-let-7g inhibits proliferation of hepatocellular carcinoma cells by downregulation of c-Myc and upregulation of p16INK4A," *International Journal of Cancer*, vol. 128, no. 2, pp. 319–331, 2011.

[21] F. van Zijl, S. Mall, G. Machat et al., "A human model of epithelial to mesenchymal transition to monitor drug efficacy in hepatocellular carcinoma progression," *Molecular Cancer Therapeutics*, vol. 10, no. 5, pp. 850–860, 2011.

[22] F. van Zijl, G. Zulehner, M. Petz et al., "Epithelial-mesenchymal transition in hepatocellular carcinoma," *Future Oncology*, vol. 5, no. 8, pp. 1169–1179, 2009.

[23] M. Liu, M. C. Casimiro, C. Wang et al., "$p21^{CIP1}$ attenuates Ras- and c-Myc-dependent breast tumor epithelial mesenchymal transition and cancer stem cell-like gene expression in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 45, pp. 19035–19039, 2009.

[24] E.-J. Tan, S. Thuault, L. Caja, T. Carletti, C.-H. Heldin, and A. Moustakas, "Regulation of transcription factor twist expression by the DNA architectural protein high mobility group A2 during epithelial-to-mesenchymal transition," *The Journal of Biological Chemistry*, vol. 287, no. 10, pp. 7134–7145, 2012.

[25] S. Thuault, E.-J. Tan, H. Peinado, A. Cano, C.-H. Heldin, and A. Moustakas, "HMGA2 and Smads co-regulate SNAIL1 expression during induction of epithelial-to-mesenchymal transition," *The Journal of Biological Chemistry*, vol. 283, no. 48, pp. 33437–33446, 2008.

[26] S. H. Beachy, M. Onozawa, Y. J. Chung et al., "Enforced expression of Lin28b leads to impaired T-cell development, release of inflammatory cytokines, and peripheral T-cell lymphoma," *Blood*, vol. 120, no. 5, pp. 1048–1059, 2012.