Cinobufacini inhibits epithelial-mesenchymal transition of human hepatocellular carcinoma cells through c-Met/ERK signaling pathway

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide with over 740,000 new cases per year and the second leading cause of cancer-related deaths worldwide (1,2). At present, resection and transplantation are still the available curative treatments, but are hampered because of their high recurrence rates and development of metastasis (3). Epithelial-mesenchymal transition (EMT) is an important biological process in the progression of malignant tumors. The cancer cells undergoing EMT lose their adhesion molecules such as E-cadherin, and gain mesenchymal cell markers, such as N-cadherin, Vimentin, alpha smooth muscle actin (α-SMA), fibronectin, and collagen I, which will enhance cells migration and invasion (4). These characteristic markers are modulated transcriptionally by several key transcription factors including Snail, Slug, Zeb, Twist and so forth (5). Accumulating data suggested that EMT was identified as the important initiation step for HCC metastasis (5). Therefore, to reduce the morbidity...
and mortality rates of HCC, prevention of EMT is important for the inhibition of tumor metastasis and the molecular targets in the pathway of EMT have received great attention in HCC treatment.

c-Met, also called tyrosine-protein kinase Met or hepatocyte growth factor (HGF) receptor, is widely expressed by epithelial cells, endothelial cells, neurons, hepatocytes, and hematopoietic cells (7). HGF, the known ligand of c-Met, induces c-Met dimerization and activation, leading to stimulation of multiple downstream signaling pathways, including the MEK/ERK and PI-3K pathways, STAT3, RAC1, and the NF-KB pathway (8). This plays several causal roles in cancer progression, including the induction of EMT (9). Inhibitors of c-Met/HGF signaling have demonstrated antitumor potential in preclinical and clinical models of HCC (10). c-Met inhibitors have shown signs of efficacy in the treatment of HCC, particularly against c-Met positive tumors (11). Our previous basic studies showed that c-Met inhibitor SU11274 could neutralize the activation of HCC cell growth resulting from the addition of des-γ-carboxyprothrombin (DCP) by inhibiting the phosphorylation of c-Met and ERK (12). Moreover, clinical studies indicated that some encouraging phase II data on two c-MET inhibitors, tivantinib and cabozantinib, has led to phase III trials (13). Taken together, c-Met is a therapeutically relevant target in HCC, with important roles in tumor proliferation, motility, and invasion. It should be of significance to further investigate the mechanisms of c-Met on metastasis and EMT in HCC cells, which will be better for HCC treatment.

In recent years, many traditional Chinese medicines have been shown to have potent anti-cancer effects and have attracted considerable interest as potential candidates for the development of novel cancer therapeutics (14). Cinobufacini is a watersoluble extract from the skins and parotid venom glands of the toad Bufo bufo gargarizans Cantor, was obtained from Anhui Jinchuan Biochemical Co., Ltd., Anhui, China. The extraction process was as follows: Bufo bufo gargarizans Cantor skins (20 g) were boiled with distilled water twice, and the resulting decoction was mixed and filtered using filter paper. Then, the filtered solution was collected, concentrated, lyophilized, and extracted two more times with ethanol. Finally, the decoction was concentrated to 1mL. In this study, various concentrations of cinobufacini were prepared by diluting the stock solution (20 g/mL) with serum-free DMEM medium.

2. Materials and Methods

2.1. Preparation of cinobufacini

The detailed preparation process of cinobufacini was described in previous studies (19). Cinobufacini, an aqueous extract from the skin and parotid venom glands of the toad Bufo bufo gargarizans Cantor, was purchased from Anhui Jinchuan Biochemical Co., Ltd., Anhui, China. The extraction process was as follows: Bufo bufo gargarizans Cantor skins (20 g) were boiled with distilled water twice, and the resulting decoction was mixed and filtered using filter paper. Then, the filtered solution was collected, concentrated, lyophilized, and extracted two more times with ethanol. Finally, the decoction was concentrated to 1mL. In this study, various concentrations of cinobufacini were prepared by diluting the stock solution (20 g/mL) with serum-free DMEM medium.

2.2. Cells

The HCC cell line HepG2 was purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). HepG2 cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD, USA), 100 U/mL of penicillin and 100 g/mL of streptomycin in a humidified atmosphere with 5% CO₂ in air at 37°C.

2.3. Cell viability assay

Cells in the logarithmic growth phase were plated at a density of 6×10² cells/mL in 96-well plates. Twenty-four hours later, the cells were incubated with various concentrations of cinobufacini (0, 0.005, 0.01, 0.05, 0.1, and 0.5 mg/mL). At times of 24, 48, and 72 h after addition of cinobufacini, cell viability was analyzed using a cell counting kit-8 (CCK-8) protocol ((Dojindo Molecular Technologies Inc. Shanghai, China). The absorbance value (OD) of each well was measured at 450 nm. All experiments were performed at least in triplicate on three separate occasions.

2.4. Quantitative real-time RT-PCR assay

Following previous studies, quantitative real-time RT-PCR was used to detect the mRNA expression of EMT
The anti-proliferation effect of cinobufacini on HCC cells HepG2 was estimated by CCK-8 assay. As shown in Figure 1, cinobufacini had a significant inhibitive effect on HepG2 cells. The viability of HepG2 cells treated by various concentrations of cinobufacini was detected at different times, respectively. After treatment with 0.1 mg/mL cinobufacini for 24 h, 48 h and 72 h, the growth inhibition rates of HepG2 cells were 36.8%, 49.5%, and 62.3% respectively. These results indicated that cinobufacini had a significant growth inhibiting effect on HepG2 cells in a dose- and time-dependent manner.

3.2. Effects of cinobufacini on EMT of hepatocellular carcinoma cells

To explore the effect of cinobufacini on EMT of hepatocellular carcinoma cells, EMT related markers E-cadherin, N-cadherin, and Vimentin were detected by Western blot analysis, and EMT transcription factors Snail and Twist were detected by quantitative real-time RT-PCR assay. As shown in Figure 2A, after treatment with or without cinobufacini (0.01, 0.05 and 0.1 mg/mL) for 24 h, 48 h and 72 h, the growth inhibition rates of HepG2 cells were 36.8%, 49.5%, and 62.3% respectively. These results indicated that cinobufacini had a significant growth inhibiting effect on HepG2 cells in a dose- and time-dependent manner.

2.5. Western blot analysis

After cells were treated with specified concentrations of cinobufacini for 24h, total cell lysates and cytosolic fractions were prepared as previously described (22). Thirty micrograms of total cellular proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE) and transferred onto polyvinylidene fluoride (PVDF) transfer membranes by Western blotting. The results were quantified using Image J (National Institutes of Health, Bethesda, MD, USA). The following antibodies were used: c-Met and MMP-9 (Proteintech, Wuhan, China); p-c-Met and MMP-2 (Abcom, UK); N-cadherin, Vimentin, GAPDH and β-actin (Servicebio, Wuhan, China); E-cadherin and MEK1/2 (Affinity Biosciences, Cincinnati, OH, USA); and ERK1/2 (Cell Signaling Technology, Boston, MA, USA).

2.6. Statistical analysis

All experiments were performed in triplicate and the results were analyzed by ANOVA (one-way analysis of variance) using GraphPad Prism 4, followed by Student’s t-test using Microsoft Office Excel software. p < 0.05 was indicative of significant difference.

3. Results

3.1. Effects of cinobufacini on the proliferation of hepatocellular carcinoma cells

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had a significant effect on inhibiting of EMT of HepG2 cells.

3.3. Cinobufacini inhibits EMT of hepatocellular carcinoma cells through c-Met /ERK signaling pathway in hepG2 cells

c-Met was reported to play important roles in cancer progression, including the induction of EMT (9). MEK/ERK is one of the definite downstream targets of c-Met receptor. Inhibition of c-Met/ERK signaling pathway has proven to be an efficient way to attenuate tumor proliferation, motility, and invasion in HCC (11,12). Here we investigated the effect and mechanism of Cinobufacini on EMT of hepatocellular carcinoma cells by Western blot analysis. We found that c-Met/ERK signaling pathway was inhibited after treatment with cinobufacini. As shown in Figure 3A, the protein expression of phosphorylated-c-Met (p-c-Met) gradually decreased in a dose-dependent manner with cinobufacini, while the expression of c-Met did not change significantly with the addition of cinobufacini. Furthermore, the protein expression of MEK1/2 and ERK1/2, the downstream kinase of the signal transduction pathway activated by c-Met, also decreased in a dose-dependent manner with cinobufacini. These results suggest that cinobufacini could inhibit EMT of hepatocellular carcinoma cells through c-Met/ERK signaling pathway.

3.4. Cinobufacini inhibits cell migration and invasion by regulating MMP-2 and MMP-9 in hepatocellular carcinoma cells

MMP-2 and MMP-9 are factors, which have been proven to play significant function in tumor metastasis (23). Thus, the expression of MMP-2 and MMP-9 were examined to further determine the mechanism by which cinobufacini inhibited migration and invasion in hepatocellular carcinoma cells. Here the protein expression of MMP-2 and MMP-9 were detected by Western blot analysis. As shown in Figure 3B, after treatment with or without cinobufacini (0.01, 0.05 and 0.1 mg/mL) for 24 h, the expression of E-cadherin was decreased.

4. Discussion

Cinobufacini, a well-known traditional Chinese medicine, is widely used to treat a variety of cancers in clinical cancer therapy in China. Its therapeutic effect is especially pronounced in liver cancer (15). The antitumor effects of cinobufacini mainly include inhibiting cancer cell proliferation and differentiation, inducing apoptosis, and enhancing immune responses against cancer (24). Recently, an experimental study
showed cinobufacini could prevent cell migration and invasion of human breast carcinoma MDA-MB-231 cells into a model stromal tissue, which indicated cinobufacini might possess cancer cell migration preventing activity in addition to cell toxicity such as apoptosis-inducing activity (25). However, there are few studies about the effects of cinobufacini on inhibiting HCC cells invasion and metastasis. Here, we investigated the effects and mechanisms of cinobufacini on inhibiting HepG2 cells migration and invasion. We found that cinobufacini could prevent HepG2 cells invasion and metastasis via inhibiting EMT through c-Met/ERK signaling pathway.

Previous studies demonstrated that EMT activation in cancer cells contributed to tumor invasion and metastasis in various types of cancer, including HCC, resulting in aggressive cancer progression. EMT is the conversion of epithelial cells to mesenchymal cells, in which cells undergo physiological or pathological changes including the loss of cell polarity and cell-cell adhesion as well as the acquisition of migratory and invasive properties. The classical EMT is featured with the loss of epithelial markers, such as E-cadherin and the up-regulation of mesenchymal markers, such as Vimentin and N-cadherin. These characteristic markers are modulated transcriptionally by several key transcription factors including Snail and Twist (5). Snail is a zinc-finger transcription factor that is known as an essential player in the aggressive phenotype of the EMT. Snail binds to the E-boxes of the human E-cadherin promoter and represses E-cadherin expression (26). Recently, the basic helix-loop-helix transcription factor Twist has been added to the list of developmental genes with a key role in E-cadherin repression and EMT induction (27). Lee et al. found that overexpression of Twist was correlated with HCC metastasis and its expression was negatively correlated with E-cadherin expression by tissue microarray (28). Moreover, Twist was able to suppress E-cadherin expression and induce EMT changes in metastatic HCC cell lines, which was correlated with increased HCC cell invasiveness. These findings indicated that EMT was associated with HCC metastasis and therefore inhibition of EMT might be a novel approach for HCC treatment. Here we found that after treatment with or without cinobufacini for 24 h, there were dose-dependent increases in E-cadherin protein expression and decreases in N-cadherin and Vimentin protein expression in HepG2 cells. Moreover, the mRNA expression of Snail and Twist were decreased after treatment with or without cinobufacini. Our findings indicated that cinobufacini could prevent HepG2 cells invasion and metastasis by inhibiting EMT with the expression of EMT and transcription factors reversed.

The phosphorylation of c-Met and subsequent activation of various downstream signal transduction pathways may play a significant role in the progression of HCC including cell proliferation and invasion (12). Once c-Met binds to its ligand HGF, it is dimerized and activated, leading to stimulation of multiple downstream signaling pathways, including the MEK/ERK and PI-3K pathways, STAT3, RAC1, and the NF-kB pathway (8). The ERK pathway has significant roles in cell proliferation, cell survival and metastasis. Deregulation of the ERK pathway has been implicated in multiple types of human cancers, including HCC (29). Previous studies have established a critical role for receptor tyrosine kinase signaling and ERK pathway in EMT (30). In HepG2 and Huh7 HCC cell lines, HGF-induced EMT phenotypes are significantly inhibited by the Raf inhibitor sorafenib and the MEK inhibitor U0126, but not the PI3K inhibitor wortmannin (31). This finding suggests that the Raf-ERK-MAPK pathway mediates the signal between c-MET activation and EMT induction in HCC cell lines. Therefore, c-Met/ERK signaling pathway might be an important pathway in EMT of HCC cells and inhibiting this pathway might be significant for preventing EMT and migration and invasion. Here we found that the phosphorylation of c-Met was inhibited by cinobufacini. Furthermore, the
expression of MEK1/2 and ERK1/2, the downstream kinase of the signal transduction pathway activated by c-Met, also decreased in a dose-dependent manner with cinobufacini. These findings suggested that cinobufacini could inhibit EMT of HCC cells through c-Met/ERK signaling pathway.

MMPs are major proteolytic enzymes that are important in the degradation of the extracellular matrix thus influencing distinct cellular functions (32). MMPs contribute to the regulation of cancer cell invasion and tumor metastasis. Expression of various MMPs including MMP-1, MMP-2, MMP-9, MMP-12 and MMP-14 were implicated in regulating HCC tumor progression and prognosis. Here we found that after treatment with cinobufacini, there was a significant decrease in MMP-2 and MMP-9 expressions in HepG2 cells. These results indicated that cinobufacini could inhibit cell invasion and metastasis by regulating MMP-2 and MMP-9 in HCC cells.

5. Conclusion

In conclusion, the current study suggested cinobufacini could prevent HepG2 cells invasion and metastasis by inhibiting EMT through c-Met/ERK signaling pathway, which might provide experimental evidence for cinobufacini treatment of HCC (Figure 4).

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References


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