Original Article

DOI: 10.5582/bst.2018.01082

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

Fanghua Qi[1], Jinjing Wang[2], Lin Zhao[1], Pingping Cai[1], Wei Tang[3], Zhixue Wang[1,*]

[1] *Department of Traditional Chinese Medicine, Shandong Provincial Hospital affiliated to Shandong University, Ji'nan, China;*
[2] *Shandong University of Traditional Chinese Medicine, Ji'nan, China;*
[3] *Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, the University of Tokyo, Tokyo, Japan.*

Summary **Cinobufacini, an aqueous extract from the skins and parotid venom glands of the toad *Bufo bufo gargarizans* Cantor, is a well known traditional Chinese medicine widely used in clinical cancer therapy in China. Its therapeutic effect is especially pronounced in liver cancer. However, the precise mechanisms induced by cinobufacini in human hepatocellular carcinoma (HCC) cells are still not very clear. Here, we investigated the effects and mechanisms of cinobufacini on inhibiting HepG2 cells invasion and metastasis. Epithelial-mesenchymal transition (EMT) is identified as an important initiation step for HCC metastasis. After the HepG2 cells were treated with different concentrations of cinobufacini, the expression of EMT related E-cadherin was increased while N-cadherin and Vimentin were decreased, and the expression of EMT related transcription factors Snail and Twist were decreased. Moreover, the phosphorylation of c-Met was inhibited by cinobufacini, and 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. In addition, after the cells were treated with different concentrations of cinobufacini, there was a significant decrease in MMP-2 and MMP-9 expression in HepG2 cells. In conclusion, the current study suggested cinobufacini could prevent HepG2 cells migration and invasion *via* inhibiting EMT through c-Met/ERK signaling pathway, which might provide experimental evidence for cinobufacini treatment of HCC.**

Keywords: Cinobufacini, hepatocellular carcinoma (HCC), invasion and metastasis, epithelial-mesenchymal transition (EMT), c-Met/ERK signaling pathway

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

Released online in J-STAGE as advance publication May 22, 2018.

**Address correspondence to:*
Dr. Zhixue Wang, Department of Traditional Chinese Medicine, Shandong Provincial Hospital affiliated to Shandong University, No. 324, Jingwuweiqi Road, Ji'nan 250021, Shandong, China.
E-mail: wangzhixue_666@sina.com

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 water-soluble extract from the skins and parotid venom glands of the toad *Bufo bufo gargarizans* Cantor. It composes a variety of bufadienolide cardiotonic steroids, such as bufalin, cinobufagin, resibufogenin, and telocinobufagin, which are the major active components (*15*). In 2005, cinobufacini was approved by the Chinese State Food and Drug Administration (SFDA) and then wildly accepted to treat patients with cancers at oncologic clinics in China (*16*). Clinical studies have suggested that cinobufacini used alone or in combination with other chemotherapeutic agents had significant activity against cancers, such as HCC, pancreatic cancer, non-small cell lung cancer, and gallbladder carcinoma (*17,18*). Experimental studies have indicated that cinobufacini has a significant apoptosis-inducing effect on a number of cancer cells. Our previous studies suggested that cinobufacini leads to apoptosis of HCC cells in a dose-dependent manner through the mitochondria- and Fas-mediated pathways (*19,20*).

According to previous studies, cinobufacini plays significant roles on HCC treatment. However, there are few studies on the effect and mechanism of cinobufacini on metastasis and EMT in HCC cells. Therefore, the aim of the present study was to clarify the possible signal pathway and related molecular mechanisms by which cinobufacini inhibited EMT in the HCC cell line HepG2.

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 obtained from Anhui Jinchan 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% CO_2 in air at 37°C.

2.3. *Cell viability assay*

Cells in the logarithmic growth phase were plated at a density of 6×10^4 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

Table 1. Specific primer sequences for Snail, Twist and GAPDH

Gene	Forward primer (From 5' to 3')	Reverse primer (From 5' to 3')
Snail	GCTCCCTCTTCCTCTCCATACC	AAGTCCTGTGGGGCTGATGT
Twist	CAGCTACGCCTTCTCGGTCT	CTGTCCATTTTCTCCTTCTCTGG
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

related transcription factors Snail and Twist (*21*). First, after cells were treated with specified concentrations of cinobufacini for 24h, total RNA was prepared by using TRIzol reagent (Grand Island, NY, USA) according to the manufacturer's instructions. (Grand Island, NY, USA). In the current study, relative mRNA expression levels for the target genes were determined by using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a normalization control. Specific primer sequences of the target genes and GAPDH were designed by TaKaRa (TaKaRa Bio Inc., Dalian, China) as shown in Table 1. Afterwards, according to the protocol of SYBR Premix Ex Taq^{TM} kit (TaKaRa Bio Inc., Dalian, China), amplification of target genes and GAPDH were conducted using ABI Prism 7500 Detection System (Applied Biosystems, Inc., USA). Analysis of relative genes expression was performed by the comparative $2^{-\Delta\Delta CT}$ method.

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*

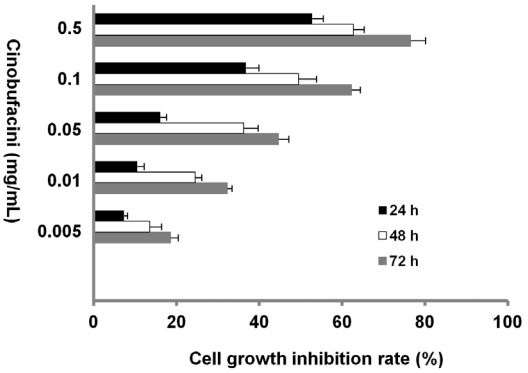

Figure 1. Growth inhibition effects of cinobufacini on HepG2 cells. Cells were treated with different concentrations of cinobufacini (0, 0.005, 0.01, 0.05, 0.1, and 0.5 mg/mL) for 24 h, 48 h and 72 h, and the cell viability was determined by CCK-8 assay. The results are expressed as percentage of cell growth relative to untreated control cells

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, 48h and 72h, 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, 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 expressions of Snail and Twist were decreased after treatment with or without cinobufacini (0.01, 0.05 and 0.1 mg/mL) for 24 h (Figure 2B). These results indicated that cinobufacini

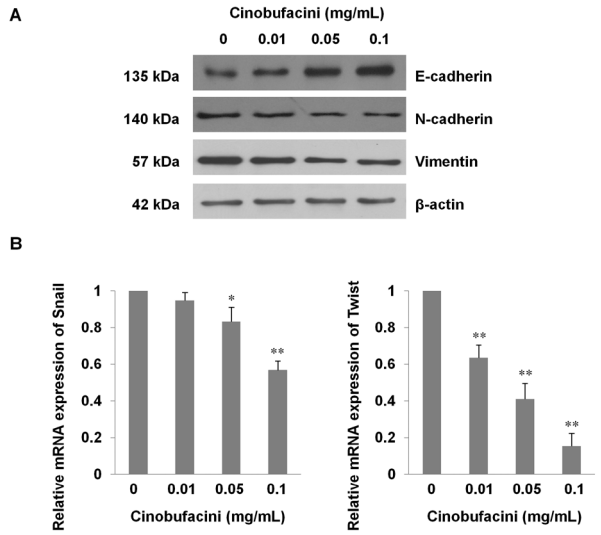

Figure 2. Effects of cinobufacini on EMT of hepatocellular carcinoma cells. (A) The protein expressions of EMT related markers E-cadherin, N-cadherin and Vimentin were detected by Western blot analysis. After the HepG2 cells were treated with different concentrations of cinobufacini (0, 0.01, 0.05, and 0.1 mg/mL) for 24 h, the expression of E-cadherin was increased while N-cadherin and Vimentin were decreased. **(B)** The mRNA expression of EMT transcription factors Snail and Twist were detected by quantitative real-time RT-PCR assay. After the HepG2 cells were treated with different concentrations of cinobufacini (0, 0.01, 0.05, and 0.1 mg/mL) for 24 h, the mRNA expression of Snail and Twist were decreased. *$p < 0.05$, **$p < 0.01$ *vs*. untreated controls.

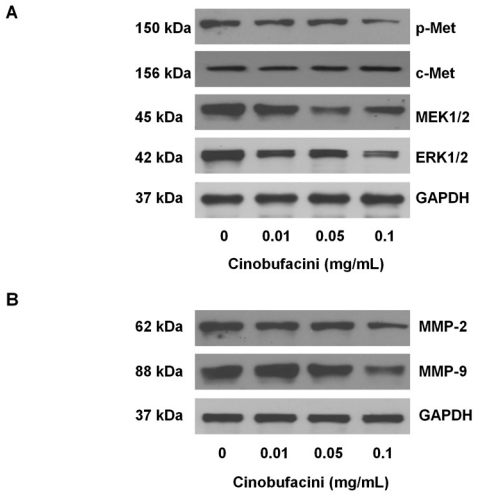

Figure 3. Cinobufacini inhibits invasion and metastasis of HCC cells through c-Met /ERK signaling pathway in hepG2 cells. (A) The protein expression of p-c-Met, c-Met, MEK1/2 and ERK1/2 were detected by Western blot analysis. The expression of p-c-Met gradually decreased in a dose-dependent manner with cinobufacini, while the expression of c-Met did not change significantly. The protein expression of MEK1/2 and ERK1/2 decreased in a dose-dependent manner with cinobufacini. **(B)** The protein expression of MMP-2 and MMP-9 were detected by Western blot analysis. After the HepG2 cells were treated with different concentrations of cinobufacini (0, 0.01, 0.05, and 0.1 mg/mL) for 24 h, the expression of E-cadherin was decreased.

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 protein 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, there were dose-dependent decreases in MMP-2 and MMP-9 protein expression in HepG2 cells. These results indicated that cinobufacini could inhibit cell invasion and metastasis by regulating MMP-2 and MMP-9 in hepatocellular carcinoma cells.

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 Ecadherin 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

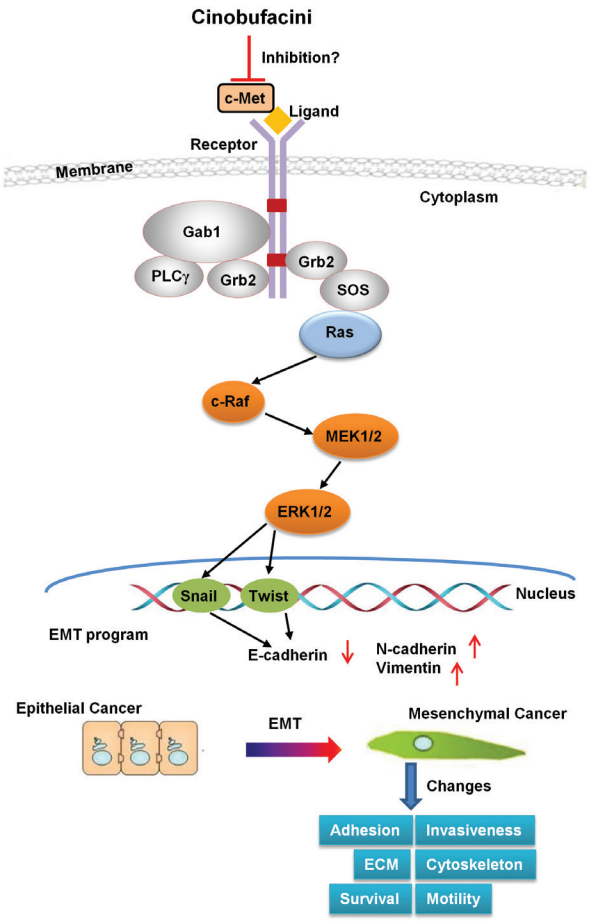

Figure 4. Cinobufacini might prevent HCC cells invasion and metastasis *via* inhibiting EMT through c-Met/ERK signaling pathway.

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).

Acknowledgments

This study was funded by Shandong Provincial Natural Science Foundation, China (No. ZR2015HQ015) and National Natural Science Foundation of China (No. 81603449).

References

1. Song PP, Cai YL, Tang HW, Li C, Huang JW. The clinical management of hepatocellular carcinoma worldwide: A concise review and comparison of current guidelines from 2001 to 2017. BioSci Trends. 2017; 11:389-398.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015; 65:87-108.
3. Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet. 2012; 379:1245-1255.
4. Singh M, Yelle N, Venugopal C, Singh SK. EMT: Mechanisms and therapeutic implications. Pharmacol Ther. 2018; 182:80-94.
5. Nieto MA, Huang RY, Jackson RA, Thiery JP. EMT: 2016. Cell. 2016; 166:21-45.
6. Davis FM, Stewart TA, Thompson EW, Monteith GR. Targeting EMT in cancer: Opportunities for pharmacological intervention. Trends Pharmacol Sci. 2014; 35:479-488.
7. Fasolo A, Sessa C, Gianni L, Broggini M. Seminars in clinical pharmacology: An introduction to MET inhibitors for the medical oncologist. Ann Oncol. 2013; 24:14-20.
8. Giordano S, Columbano A. Met as a therapeutic target in HCC: Facts and hopes. J Hepatol. 2014; 60:442-452.
9. Eder JP, Vande Woude GF, Boerner SA, LoRusso PM. Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. Clin Cancer Res 2009; 15:2207-2214.
10. Bouattour M, Raymond E, Qin S, Cheng AL, Stammberger U, Locatelli G, Faivre S. Recent developments of c-Met as a therapeutic target in hepatocellular carcinoma. Hepatology. 2018; 67:1132-1149.
11. Bladt F, Friese-Hamim M, Ihling C, Wilm C, Blaukat A. The c-Met inhibitor MSC2156119J effectively inhibits tumor growth in liver cancer models. Cancers (Basel). 2014; 6:1736-1752.
12. Inagaki Y, Qi F, Gao J, Qu X, Hasegawa K, Sugawara Y, Tang W, Kokudo N. Effect of c-Met inhibitor SU11274 on hepatocellular carcinoma cell growth. Biosci Trends. 2011; 5:52-56.
13. Lee JJX, Chan JJ, Choo SP. Clinical Development of c-MET Inhibition in Hepatocellular Carcinoma. Diseases. 2015; 3:306-324.
14. Qi F, Zhao L, Zhou A, Zhang B, Li A, Wang Z, Han J. The advantages of using traditional Chinese medicine as an adjunctive therapy in the whole course of cancer treatment instead of only terminal stage of cancer. Biosci Trends. 2015; 9:16-34.
15. Qi F, Li A, Inagaki Y, Kokudo N, Tamura S, Nakata M, Tang W. Antitumor activity of extracts and compounds from the skin of the toad *Bufo bufo gargarizans* Cantor. Int Immunopharmacol. 2011; 11:342-349.
16. Qi F, Li A, Inagaki Y, Gao J, Li J, Kokudo N, Li XK, Tang W. Chinese herbal medicines as adjuvant treatment during chemo- or radio-therapy for cancer. Biosci Trends. 2010; 4:297-307.
17. Xia J, Inagaki Y, Gao J, Qi F, Song P, Han G, Sawakami T, Gao B, Luo C, Kokudo N, Hasegawa K, Sakamoto Y, Tang W. Combination of cinobufacini and doxorubicin increases apoptosis of hepatocellular carcinoma cells through the Fas- and mitochondria-mediated pathways. Am J Chin Med. 2017; 45(7):1537-1556.
18. Meng Z, Yang P, Shen Y, Bei W, Zhang Y, Ge Y, Newman RA, Cohen L, Liu L, Thornton B, Chang DZ, Liao Z, Kurzrock R. Pilot study of huachansu in patients with hepatocellular carcinoma, nonsmall-cell lung cancer, or pancreatic cancer. Cancer. 2009; 115:5309-5318.
19. Qi F, Li A, Zhao L, Xu H, Inagaki Y, Wang D, Cui X, Gao B, Kokudo N, Nakata M, Tang W. Cinobufacini, an aqueous extract from *Bufo bufo gargarizans* Cantor, induces apoptosis through a mitochondria-mediated pathway in human hepatocellular carcinoma cells. J Ethnopharmacol. 2010; 128:654-661.
20. Qi F, Li A, Inagaki Y, Xu H, Wang D, Cui X, Zhang L, Kokudo N, Du G, Tang W. Induction of apoptosis by cinobufacini preparation through mitochondria- and Fas-mediated caspase-dependent pathways in human hepatocellular carcinoma cells. Food Chem Toxicol. 2012; 50:295-302.
21. Yin Y, Qi F, Song Z, Zhang B, Teng J. Ferulic acid combined with astragaloside IV protects against vascular endothelial dysfunction in diabetic rats. Biosci Trends. 2014; 8:217-226.
22. Qi F, Inagaki Y, Gao B, Cui X, Xu H, Kokudo N, Li A, Tang W. Bufalin and cinobufagin induce apoptosis of human hepatocellular carcinoma cells *via* Fas- and mitochondria-mediated pathways. Cancer Sci. 2011; 102:951-958.
23. Daniele A, Abbate I, Oakley C, Casamassima P, Savino E,

Casamassima A, Sciortino G, Fazio V, Gadaleta-Caldarola G, Catino A, Giotta F, De Luca R, Divella R. Clinical and prognostic role of matrix metalloproteinase-2, -9 and their inhibitors in breast cancer and liver diseases: A review. Int J Biochem Cell Biol. 2016; 77(Pt A):91-101.
24. Wei X, Si N, Zhang Y, Zhao H, Yang J, Wang H, Wang L, Han L, Bian B. Evaluation of bufadienolides as the main antitumor components in cinobufacini injection for liver and gastric cancer therapy. PLoS One. 2017; 12:e0169141.
25. Nakata M, Mori S, Kamoshida Y, Kawaguchi S, Fujita-Yamaguchi Y, Gao B, Tang W. Toad skin extract cinobufatini inhibits migration of human breast carcinoma MDA-MB-231 cells into a model stromal tissue. Biosci Trends. 2015; 9:266-269.
26. Zucchini-Pascal N, Peyre L, Rahmani R. Crosstalk between beta-catenin and snail in the induction of epithelial to mesenchymal transition in hepatocarcinoma: Role of the ERK1/2 pathway. Int J Mol Sci. 2013; 14:20768-20792.
27. Zou H, Feng X, Cao JG. Twist in hepatocellular carcinoma: Pathophysiology and therapeutics. Hepatol Int. 2015; 9:399-405.
28. Lee TK, Poon RT, Yuen AP, Ling MT, Kwok WK, Wang XH, Wong YC, Guan XY, Man K, Chau KL, Fan ST. Twist overexpression correlates with hepatocellular carcinoma metastasis through induction of epithelial-mesenchymal transition. Clin Cancer Res. 2006; 12:5369-5376.
29. Roux PP, Blenis J. ERK and p38 MAPK-activated protein kinases: A family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev. 2004; 68:320-344.
30. McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B, Terrian DM, Milella M, Tafuri A, Stivala F, Libra M, Basecke J, Evangelisti C, Martelli AM, Franklin RA. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta. 2007; 1773:1263-1284.
31. Nagai T, Arao T, Furuta K, Sakai K, Kudo K, Kaneda H, Tamura D, Aomatsu K, Kimura H, Fujita Y, Matsumoto K, Saijo N, Kudo M, Nishio K. Sorafenib inhibits the hepatocyte growth factor-mediated epithelial mesenchymal transition in hepatocellular carcinoma. Mol Cancer Ther. 2011; 10:169-177.
32. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2002; 2:161-174.
33. Mckenna GJ, Chen Y, Smith RM, Meneghetti A, Ong C, McMaster R, Scudamore CH, Chung SW. A role for matrix metalloproteinases and tumor host interaction in hepatocellular carcinomas. Am J Sur. 2002; 183:588-594.

(Received March 21, 2018; Revised April 28, 2018; Accepted May 10, 2018)