

The combination of ulinastatin and 5-fluorouracil synergistically inhibits hepatocellular carcinoma growth

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Abstract

Objective: Chemoresistance is a major problem during hepatocellular carcinoma (HCC) treatment; thus, finding novel chemosensitizers and elucidating the underlying mechanisms that contribute to chemoresistance in HCC is critical.

Methods: Cell viability assays were used to detect the combined effects of ulinastatin (UTI) and 5-fluorouracil (5-FU) on the proliferation of HCC cells. RT-qPCR, western blot, sphere formation, and aldehyde dehydrogenase I (ALDH1) activity assays were used to examine UTI-mediated effects on HCC cell stemness and related mechanisms.

Results: We constructed 5-FU-resistant HCC cell lines and found that their stemness was higher than parental cells, as evidenced by increased sphere-formation ability, ALDH1 activity, and expression of stemness regulatory genes. While UTI had no effect on the viability of HCC cells, it significantly reduced the stemness of 5-FU-resistant HCC cells, which was determined by decreased sphere-formation capacity, ALDH1 activity, and expression of stemness-related genes. Furthermore, UTI attenuated 5-FU resistance in 5-FU-resistant HCC cells and enhanced the 5-FU sensitivity of parental cells. Mechanistic studies revealed that UTI suppressed the Wnt/ β -catenin pathway, which was responsible for the activity of UTI on the stemness of HCC cells.

Conclusions: UTI enhanced the 5-FU sensitivity of HCC cells by attenuating their stemness via inhibiting Wnt/ β -catenin signaling.

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Keywords

Ulinastatin, 5-fluorouracil, chemoresistance, hepatocellular carcinoma, stemness, Wnt/ β -catenin

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Introduction

Chemoresistance is a common cause of tumor recurrence and metastasis; thus, exploring solutions to chemoresistance is key to improving the efficacy of chemotherapy.¹ Recently, researchers have found that a rare population of cancer stem cells (CSCs) has a strong ability to form tumors.¹ These CSCs are insensitive to conventional chemotherapies and are a main reason for chemoresistance in cancer patients.² It has been confirmed that CSCs exist in prostate cancer,³ renal cancer,⁴ pancreatic cancer⁵ and hepatocellular carcinoma (HCC).⁶ Finding an effective way to inhibit CSC progression or induce their differentiation is an effective means to solve chemoresistance in cancer.

Ulinastatin (UTI) is a protease inhibitor that inhibits trypsin, mercaptase, fibrinolytic enzyme, alpha chymotrypsin, hyaluronidase, granulocyte elastase, and other enzymes.⁷ Moreover, UTI inhibits the release of lysosomal enzymes, the production of myocardial inhibitors, the release of inflammatory mediators, and scavenges oxygen free radicals by stabilizing lysosomal membranes.⁸ UTI is clinically used as an adjuvant drug for acute pancreatitis, circulatory failure, and chronic recurrent pancreatitis.⁹ Recent studies have found that UTI plays important roles in tumor progression. For example, UTI suppressed the proliferation and motility of gastric cancer cells partially by down-regulating the expression of urokinase-type plasminogen activator (uPA),^{10,11} UTI has protective effects in non-small cell lung cancer patients after radiation therapy,¹²

and UTI reduces the metastasis of colon cancer by inactivating MMP9 through the antifibrinolytic pathway.¹³ Additionally, clinical evidence indicates that UTI synergizes with other drugs, including methylprednisolone to reduce tumor cell metastasis after surgery.¹⁴ Finally, the combination of UTI and curcumin had a greater inhibitory effect against colorectal cancer liver metastases,¹³ and UTI synergized with docetaxel to block the invasion and metastasis of breast cancer cells.¹⁵ However, the effects of UTI on the chemoresistance of HCC cells and the underlying mechanisms have not been revealed.

In this study, we investigated the effects of UTI on 5-FU resistance and sensitivity in HCC cells and found that UTI enhanced 5-FU sensitivity and attenuated 5-FU resistance. Mechanistically, we found that UTI reduced the stemness of 5-FU-resistant HCC cells, which displayed increased stemness compared with parental HCC cells. This activity of UTI was found to be partially mediated by inhibiting the Wnt/ β -catenin pathway.

Material and methods

Ethics and consent statements

As this work does not report animal or human studies, ethics and consent statements are unnecessary.

Cell culture and reagents

The HCC cell line Huh7 was purchased from Sino Biological (Beijing, China) and cultured in DMEM (Thermo Fisher

Scientific, Waltham, MA, USA) with 15% fetal bovine serum (FBS; Sciencell, Carlsbad, CA, USA) at 37°C in 5% CO₂. To construct 5-FU-resistant HCC cells (Huh7-FT), parental Huh7 cells in logarithmic growth phase were inoculated into culture flasks at a concentration of 5×10^5 cells/mL. After cells adhered to the plate, the medium was replaced with DMEM containing 2×10^3 ng/mL 5-FU for further culture. After 3 to 5 days, the supernatant and suspended dead cells were discarded and the medium was replaced with the same concentration of 5-FU for another 3 to 5 days. After this incubation, the medium was replaced with DMEM containing 2× the 5-FU concentration. This cycle was repeated for 4 months until the cells could grow steadily in DMEM containing 2×10^5 ng/mL 5-FU. These cells were named Huh7-FT and were maintained in DMEM containing 2×10^4 ng/mL 5-FU to sustain 5-FU resistance. UTI was purchased from Techpool Bio-pharma (Guangdong, China), and UTI concentrations of 50, 100, and 200 µg/mL were used in this study. SKL2001 was purchased from Cayman Chemical (Ann Arbor, MI, USA) and was used at a concentration of 20 µM.

Cell viability assay

Cells were inoculated into 96-well plates at 3000 cells/well in 200 µL of medium. After adherence, the cells were treated with different drug combinations for 48 hours, and then IC₅₀ values were calculated. For cell proliferation analysis, cells were treated with drug combinations for 24, 48, and 72 hours. MTT solution at a concentration of 5 mg/mL was added into each well and incubated for 4 hours in the dark. The MTT solution was discarded and 150 µL of dimethyl sulfoxide was added into each well, shaken on a shaking bed, placed on an enzyme label, and then absorbance

values were measured at 490 nm. All assays were performed in triplicate.

Real-time quantitative PCR (RT-qPCR)

After total RNA was extracted with TRIzol reagent (Transgen, Beijing, China), a RT reverse transcription kit (Transgen) was used to reverse transcribe RNA into cDNA, and then RT-qPCR was performed on an ABI 7500 Fast PCR instrument. The reaction conditions were 95°C for 5 minutes, and then 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 75°C for 30 seconds. Relative expression data were analyzed by the $2^{-\Delta\Delta CT}$ method.

Western blotting

Total cellular protein was extracted using a protein extraction kit (Yifeixue, Nanjing, China) 48 hours after drug treatment, and protein concentrations were measured using the BCA assay (Yifeixue). For western blotting, 30 µg of total protein was used for electrophoresis, and then this was transferred onto PVDF membranes (Merck, Kenilworth, NJ, USA) by the semi-dry method. Following incubation with 5% skimmed-milk for 1 hour, membranes were incubated with primary antibodies at 4°C overnight, and then incubated with horseradish peroxidase-labeled secondary antibodies at room temperature. The Bio-Rad imaging system was used for exposure with the ECL chemiluminescence reaction kit (Yifeixue, Nanjing, China).

Sphere formation assay

Sphere formation assays were performed in sphere-culture medium (DMEM/F12 supplemented with 1% N2, 2% B27, 20 ng/mL fibroblast growth factor, and 20 ng/mL epidermal growth factor). After digestion with trypsinase, the cells were centrifuged for 5 minutes, and the supernatant was absorbed and suspended into

sphere-culture medium (repeated twice). The cells were then seeded in a 24-well plate with ultra-low attachment. Each group had three wells, and the medium was replaced every 3 days. After 10 to 15 days culture, the spheres were imaged under a microscope and counted.

ALDH1 activity assay

ALDH1 activity was measured using an ALDH1 activity detection kit (Cat # BC0755, Solarbio, Beijing, China) according to the manufacturer's instructions.

Statistical analysis

IBM SPSS Statistics for Windows, version 19.0 (IBM Corp., Armonk, NY, USA) was used to analyze all data. Data are presented as the mean \pm SD. Variance analysis was used for comparisons among groups, and the LSD-t test was used for comparisons between two groups. The t test was also used for comparison between two groups where applicable. $P < 0.05$ was used to define statistically significant results.

Results

5-FU-resistant HCC cells exhibited greater stemness than parental cells

To explore the underlying mechanisms that contribute to 5-FU resistance in HCC cells, we initially constructed 5-FU-resistant HCC cells (Huh7-FT). The resistance index was confirmed by examining the IC_{50} value of 5-FU (Figure 1a and 1b). As CSCs result in chemoresistance, the stemness of Huh7-FT and parental Huh7 cells was compared. These assays demonstrated that Huh7-FT cells displayed a stronger sphere-formation capacity, which was characterized by increased sphere size and number (Figure 1c and 1d). Additionally, ALDH1 activity was significantly higher

in Huh7-FT cells than in Huh7 cells (Figure 1e). Furthermore, the expression of the stemness regulators ABCG2 and Oct4 were higher in Huh7-FT cells than in Huh7 cells (Figure 1f and 1g). These results suggested that Huh7-FT cells had increased stemness compared with parental Huh7 cells.

UTI attenuated the stemness of 5-FU-resistant HCC cells

We next investigated whether UTI could attenuate the stemness of Huh7-FT cells. The results showed that UTI attenuated the sphere-formation ability of Huh7-FT cells, which was evident by decreased sphere size and number (Figure 2a and 2b). Additionally, UTI reduced ALDH1 activity in Huh7-FT cells (Figure 2c). Furthermore, the expression of stemness regulators was decreased by UTI treatment (Figure 2d and 2e). However, UTI had no effect on the proliferation of Huh7 or Huh7-FT cells (Figure 2f and 2g).

UTI attenuated the 5-FU resistance of 5-FU-resistant HCC cells

Given that UTI reduced the stemness of Huh7-FT cells, we next examined whether it also attenuated their 5-FU resistance. As expected, UTI enhanced the 5-FU sensitivity of Huh7 cells (Figure 3a). Additionally, the 5-FU resistance of Huh7-FT cells was attenuated by UTI treatment (Figure 3b). Consistent results were obtained upon detecting the expression of the apoptotic executors cleaved caspase 3 and cleaved PARP (Figure 3c and 3d). Thus, these results demonstrated that UTI attenuated the chemoresistance of 5-FU-resistant HCC cells by regulating their stemness.

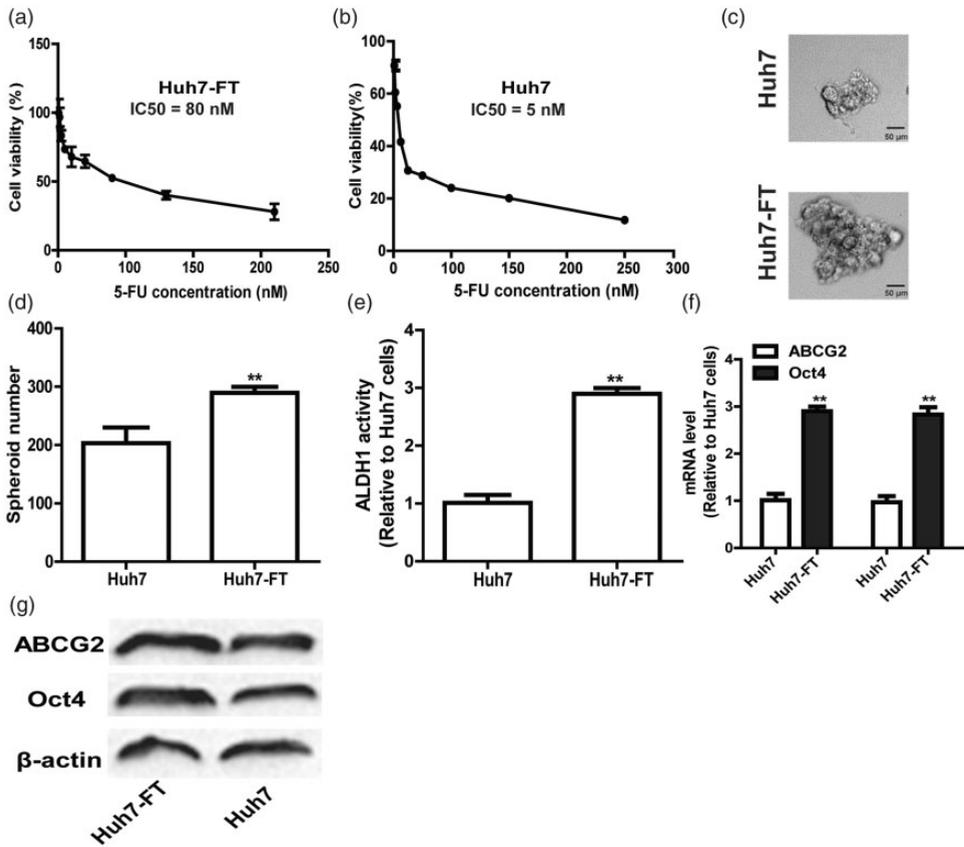


Figure 1. 5-FU-resistant HCC cells exhibited increased stemness compared with parental cells. (a and b) The IC₅₀ values of 5-FU in Huh7-FT and Huh7 cells. (c and d) Sphere-formation ability of Huh7-FT and Huh7 cells. (e) ALDH1 activity was determined in Huh7 and Huh7-FT cells. (f and g) The expression of stemness regulators were measured in Huh7 and Huh7-FT cells. **P*<0.01. 5-FU, 5-fluorouracil; HCC, hepatocellular carcinoma; ALDH1, aldehyde dehydrogenase.

UTI suppressed Wnt/ β -catenin signaling in Huh7-FT cells

We further investigated the underlying mechanisms that contributed to UTI-mediated effects. The Wnt/ β -catenin pathway plays important regulatory roles in maintaining CSCs; thus, we evaluated the effects of UTI on this pathway. As shown in Figure 4a and 4b, the expression of Wnt3a and β -catenin were decreased by UTI treatment in Huh7-FT cells. Additionally, the expression of downstream

effectors of β -catenin, including Cyclin-D1, AXIN2 and c-Myc, were decreased in 5-FU-FT cells following UTI treatment (Figure 4c and 4d).

UTI attenuated the stemness and 5-FU resistance of 5-FU-resistant HCC cells via decreasing Wnt/ β -catenin signaling

Finally, we evaluated whether Wnt/ β -catenin signaling was responsible for the effects of UTI in Huh7-FT cells. As expected, reactivation of the Wnt/ β -catenin

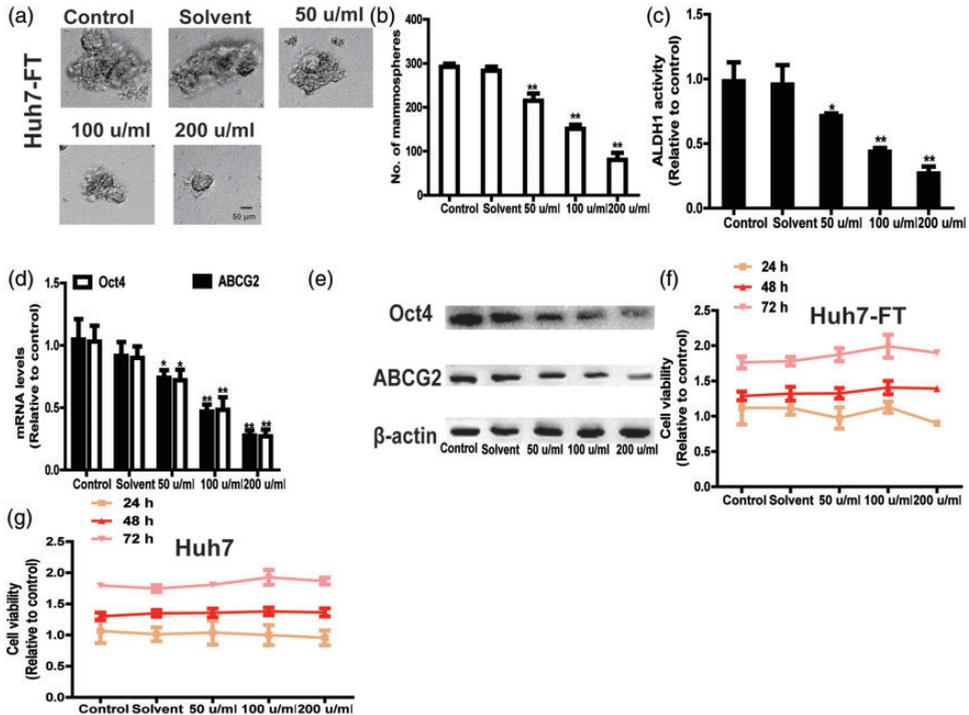


Figure 2. UTI attenuated the stemness of 5-FU-resistant HCC cells. (a and b) Sphere-formation ability was evaluated in Huh7-FT cells treated with different concentrations of UTI. (c) ALDH1 activity was measured in Huh7-FT cells treated with different concentrations of UTI. (d and e) The expression of stemness regulators was detected in Huh7-FT cells treated with different concentrations of UTI. (f and g) Cell viability was examined in the cells described in (a). * $P < 0.05$, ** $P < 0.01$. UTI, ulinastatin; 5-FU, 5-fluorouracil; HCC, hepatocellular carcinoma; ALDH1, aldehyde dehydrogenase.

pathway using the activator SKL2001 attenuated the decrease in sphere-formation ability following UTI treatment (Figure 5a and 5b). Additionally, the UTI-induced decrease in ALDH1 activity was rescued by SKL2001 (Figure 5c). Furthermore, the decreased expression of stemness regulators that was induced by UTI was partially reversed by SKL2001 (Figure 5d and 5e). Finally, SKL2001 rescued the effects of UTI on the 5-FU resistance and sensitivity of HCC cells (Figure 5f and 5g). Collectively, these results demonstrated that UTI enhanced the 5-FU sensitivity of parental Huh7 cells and attenuated the 5-FU resistance Huh7-FT cells by inhibiting Wnt/ β -catenin

signaling, which reduced the stemness of HCC cells.

Discussion

HCC is one of the five most common cancers and a leading cause of cancer-related death. While traditional methods have made some progress in the treatment of early-stage HCC, recurrence, metastasis, and drug resistance of HCC have not been solved. These barriers to treatment can all be explained by CSCs, and HCC is thought to originate from hepatocellular CSCs. In this work, we found that UTI could specifically attenuate HCC cell stemness but had no effects on cell viability. This work

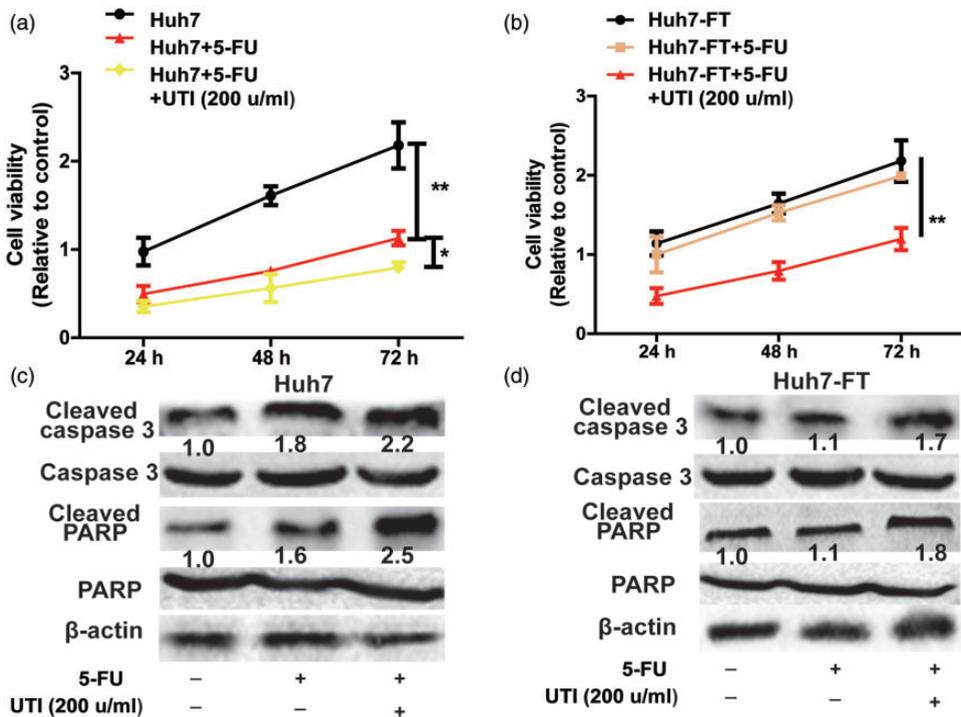


Figure 3. UTI attenuated the 5-FU resistance of 5-FU-resistant HCC cells. (a and b) Cell viability was determined in Huh7 and Huh7-FT cells treated with 5-FU with and without UTI. (c and d) Levels of the apoptotic executors cleaved caspase 3 and cleaved PARP were examined in the cells described in (a). ** $P < 0.01$. UTI, ulinastatin; 5-FU, 5-fluorouracil; HCC, hepatocellular carcinoma.

is the first to demonstrate the effects of UTI on CSCs.

The Wnt/ β -catenin signaling has been confirmed to play important roles in tumor progression, especially in CSCs. Targeting Wnt/ β -catenin signaling has achieved significant effects on CSCs, and thus tumor progression. For example, secreted frizzled-related proteins can act as biphasic modulators of Wnt signaling-elicited CSC properties beyond extracellular control,¹⁶ XB130 restrains breast cancer stemness by inhibiting Wnt/ β -Catenin signaling,¹⁷ and MEK inhibition induces stem cell plasticity in colorectal cancer *via* activating Wnt signaling.¹⁸ Here, we showed that UTI decreased Wnt3a and β -Catenin expression in HCC cells and

that reactivation of Wnt/ β -Catenin signaling rescued the effects of UTI on HCC cell stemness and chemoresistance. These findings suggested that UTI inhibited Wnt/ β -Catenin signaling, which could be responsible for the UTI-mediated effects on HCC cell stemness. Additionally, reactivation of Wnt/ β -Catenin signaling partially, but not completely, reversed the UTI-mediated inhibition of stemness in HCC cells. A previous study showed that UTI reduced epirubicin resistance by inhibiting autophagy in HCC cells;¹⁹ thus, we speculate that there might other mechanisms that contribute to the UTI-mediated effects in HCC cells, such as the TGF- β ,²⁰ Notch,²¹ and Hedgehog²² signaling pathways, which

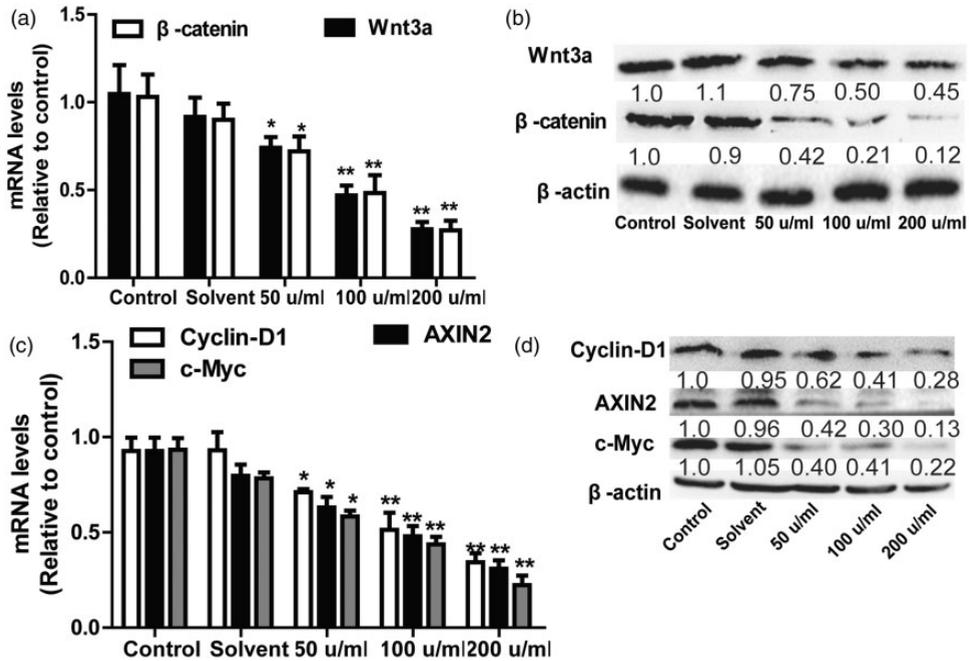


Figure 4. UTI suppressed Wnt/ β -catenin signaling in 5-FU-FT cells. (a and b) Wnt3a and β -catenin expression were detected in Huh7-FT cells treated with different concentrations of UTI. (c and d) The expression of downstream β -catenin effectors were examined in Huh7-FT cells treated with different concentrations of UTI. * $P < 0.01$. UTI, ulinastatin; 5-FU, 5-fluorouracil.

have also been shown to regulate critical processes in CSCs. These additional mechanisms should be explored in the future.

As HCC CSCs are involved in a complex mechanism that underlies malignant occurrence and progression, these cells could be the primary factor that leads to treatment failure. Given the complex regulatory environment of CSCs, the effects of targeting single molecules or pathways is limited. Although the previous studies have shown that UTI enhances the drug sensitivity of HCC cells, the effects of UTI alone on HCC cell viability are unclear. We found that UTI had no effects on HCC cell viability; we speculate that UTI may specifically kill CSCs in HCC, although more experiments are needed to confirm this speculation. Furthermore, the effects of UTI

on CSCs in other tumors should be investigated in future studies. Notably, this work only included *in vitro* experiments, although with different UTI concentrations. Future *in vivo* experiments should be performed to confirm these conclusion. Additionally, the potential dosage of UTI that can be combined with 5-FU will need to be confirmed with *in vivo* experiments.

In conclusion, UTI can inhibit the stemness of HCC cells, which enhances their 5-FU sensitivity and attenuates their 5-FU resistance. The mechanism of this activity may be related to Wnt/ β -Catenin signaling. Based on this synergism from the combination of UTI and 5-FU, this study suggests that UTI might be used as a chemosensitizer for 5-FU-resistant HCC.

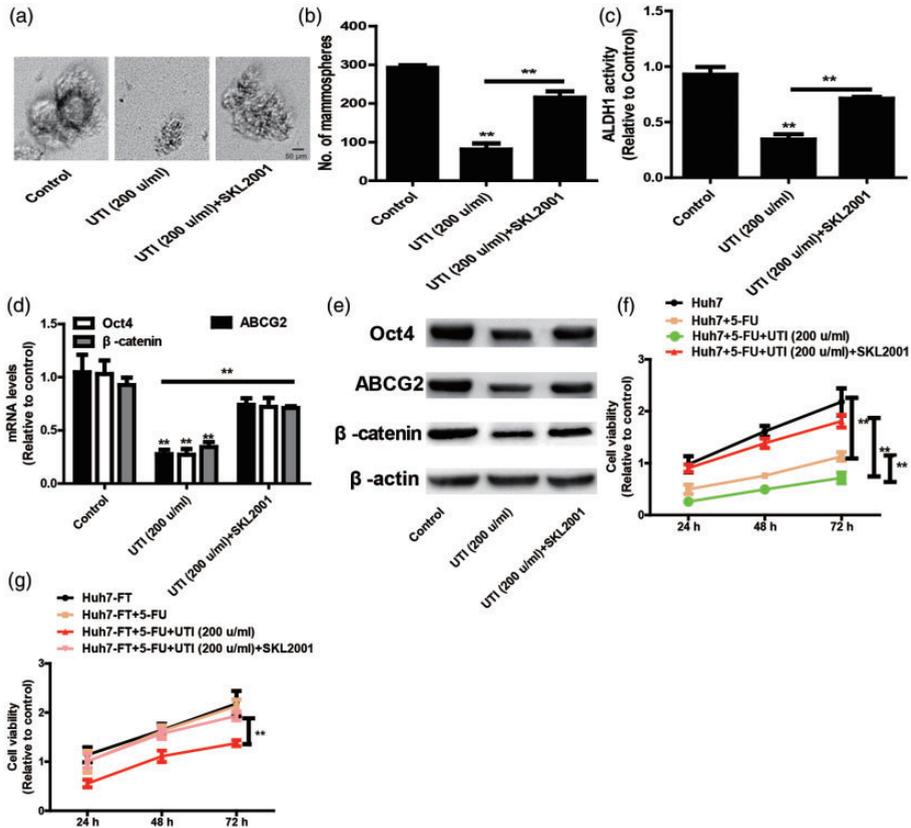


Figure 5. UTI attenuated the stemness and 5-FU resistance of 5-FU-resistant HCC cells by modulating Wnt/ β -catenin signaling. (a and b) Sphere-formation ability was evaluated in Huh7-FT cells treated with UTI with and without SKL2001. (c) ALDH1 activity was measured in Huh7-FT cells treated with UTI with and without SKL2001. (d and e) The expression of stemness regulators were examined in Huh7-FT cells treated with UTI with and without SKL2001. (f and g) The viability of Huh7 and Huh7-FT cells treated with 5-FU and UTI with and without SKL2001. ** $P < 0.01$. UTI, ulinastatin; 5-FU, 5-fluorouracil; HCC, hepatocellular carcinoma; ALDH1, aldehyde dehydrogenase.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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References

- Clarke MF. Clinical and therapeutic implications of cancer stem cells. *N Engl J Med* 2019; 380: 2237–2245.
- Yang M, Liu P and Huang P. Cancer stem cells, metabolism, and therapeutic significance. *Tumour Biol* 2016; 37: 5735–5742.
- Zhang Q, Huang H, Liu A, et al. Cell division cycle 20 (CDC20) drives prostate cancer progression via stabilization of beta-catenin in cancer stem-like cells. *EBioMedicine* 2019; 42: 397–407.

4. Xiao W, Gao Z, Duan Y, et al. Notch signaling plays a crucial role in cancer stem-like cells maintaining stemness and mediating chemotaxis in renal cell carcinoma. *J Exp Clin Cancer Res* 2017; 36: 41.
5. Yao W and Maitra A. Hear pancreatic cancer stem cells ROR. *Cell* 2019; 177: 516–518.
6. Zhu YJ, Zheng B, Luo GJ, et al. Circular RNAs negatively regulate cancer stem cells by physically binding FMRP against CCAR1 complex in hepatocellular carcinoma. *Theranostics* 2019; 9: 3526–3540.
7. Song Y, Miao S, Li Y, et al. Ulinastatin attenuates liver injury and inflammation in a cecal ligation and puncture induced sepsis mouse model. *J Cell Biochem* 2019; 120: 417–424.
8. Wang S, Cheng ZY, Chen XJ, et al. Ulinastatin protects rats with myocardial infarction by activating Nrf2/NOS pathway. *Eur Rev Med Pharmacol Sci* 2018; 22: 8990–8998.
9. Li S, Yang W, Zhou L, et al. Vascular permeability and hemodynamic effects of ulinastatin on organs affected by shock during early burn injury. *Am J Emerg Med* 2019; 37: 249–253.
10. Wang J, Chen X, Su L, et al. Suppressive effects on cell proliferation and motility in gastric cancer SGC-7901 cells by introducing ulinastatin in vitro. *Anticancer Drugs* 2016; 27: 651–659.
11. Luo J, Sun X, Gao F, et al. Effects of ulinastatin and docetaxel on breast cancer invasion and expression of uPA, uPAR and ERK. *J Exp Clin Cancer Res* 2011; 30: 71.
12. Bao P, Zhao W, Li Y, et al. Protective effect of ulinastatin in patients with non-small cell lung cancer after radiation therapy: a randomized, placebo-controlled study. *Med Oncol* 2015; 32: 405.
13. Shen F, Cai WS, Li JL, et al. Synergism from the combination of ulinastatin and curcumin offers greater inhibition against colorectal cancer liver metastases via modulating matrix metalloproteinase-9 and E-cadherin expression. *Onco Targets Ther* 2014; 7: 305–314.
14. Xu B, Li KP, Shen F, et al. Ulinastatin reduces cancer recurrence after resection of hepatic metastases from colon cancer by inhibiting MMP-9 activation via the anti-fibrinolytic pathway. *Biomed Res Int* 2013; 2013: 437950.
15. Gao F, Sun Z, Sun X, et al. Ulinastatin exerts synergistic effects with taxotere and inhibits invasion and metastasis of breast cancer by blocking angiogenesis and the epithelial-mesenchymal transition. *Cancer Biother Radiopharm* 2013; 28: 218–225.
16. Liang CJ, Wang ZW, Chang YW, et al. SFRPs are biphasic modulators of Wnt-signaling-elicited cancer stem cell properties beyond extracellular control. *Cell Rep* 2019; 28: 1511–1525.e5.
17. Xie T, Jiang C, Dai T, et al. Knockdown of XB130 restrains cancer stem cell-like phenotype through inhibition of Wnt/beta-Catenin signaling in breast cancer. *Mol Carcinog* 2019; 58: 1832–1845.
18. Zhan T, Ambrosi G, Wandmacher AM, et al. MEK inhibitors activate Wnt signaling and induce stem cell plasticity in colorectal cancer. *Nat Commun* 2019; 10: 2197.
19. Song B, Bian Q, Shao CH, et al. Ulinastatin reduces the resistance of liver cancer cells to epirubicin by inhibiting autophagy. *PLoS One* 2015; 10: e0120694.
20. Zhang B, Ye H, Ren X, et al. Macrophage-expressed CD51 promotes cancer stem cell properties via the TGF-beta1/smad2/3 axis in pancreatic cancer. *Cancer Lett* 2019; 459: 204–215.
21. Baker A, Wyatt D, Bocchetta M, et al. Notch-1-PTEN-ERK1/2 signaling axis promotes HER2+ breast cancer cell proliferation and stem cell survival. *Oncogene* 2018; 37: 4489–4504.
22. Sharma A, De R, Javed S, et al. Sonic hedgehog pathway activation regulates cervical cancer stem cell characteristics during epithelial to mesenchymal transition. *J Cell Physiol* 2019. DOI: 10.1002/jcp.28231.