
CD13 induces autophagy to promote hepatocellular carcinoma cell chemoresistance through the P38/Hsp27/CREB/ATG7 pathway

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Running Title Page:

a) **The running title:** CD13 promotes HCC cell chemoresistance

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c) **Abbreviations:** HCC, heptaocellular carcinoma; 5FU, 5-fluorouracil; PBS, Phosphate-buffered saline; MAPK, mitogen-activated protein kinase; Hsp27, heat shock protein 27; CREB, cAMP response element-binding protein; p-P38, phospho-P38; p-Hsp27, phospho-Hsp27; p-CREB, phospho-CREB; ATG7, autophagy related 7.

d) Other

Abstract

The chemoresistance of hepatocellular carcinoma is a serious problem that directly hinders the effect of chemotherapeutic agents. We previously reported that CD13 inhibition can enhance the cytotoxic efficacy of chemotherapy agents. In the present study, we utilize liver cancer cells to explore the molecular mechanism accounting for the relationship between CD13 and chemoresistance. We demonstrate that CD13 over-expression activates the P38/Hsp27/CREB signaling pathway to limit the efficacy of cytotoxic agents. Moreover, blockade of P38 or CREB sensitizes HCC cells to 5FU. Then, we reveal that CREB binds to the *ATG7* promoter to induce autophagy and promote HCC cell chemoresistance. CD13 inhibition also down-regulates the expression of *ATG7*, autophagy, and tumor cell growth *in vivo*. Overall, the combination of a CD13 inhibitor and chemotherapeutic agents may be a potential strategy for overcoming drug resistance in HCC.

Significance statement: Our study demonstrates that CD13 promotes HCC cell chemoresistance via the P38/Hsp27/CREB pathway. CREB regulates *ATG7* transcription and expression to induce autophagy. Our results collectively suggest that CD13 may serve as a potential target for overcoming HCC resistance.

1. Introduction

Hepatocellular carcinoma (HCC), also known as malignant hepatoma, is characterized by high morbidity and mortality rates and one of the most common malignancies worldwide (Xu et al., 2012; Guo et al., 2017). Recent developments in treatment methods for HCC, including surgical resection and chemotherapy, have shown considerable efficacy (Wei et al., 2014; Zhang et al., 2015). Surgical resection is mainly applicable to early-stage patients, while chemotherapy is a crucial strategy for advanced patients (Raza and Sood, 2014). However, the rates of intrahepatic recurrence and mortality remain high because of the occurrence of resistance to chemotherapeutic drugs (Yamashita et al., 2016). Therefore, examining the mechanism of drug resistance and exploring new therapeutic targets that could reverse the drug resistance of HCC cell may provide novel strategies to treat the disease.

Aminopeptidase N (APN, EC 3.4.11.2), also referred to as CD13, is a type II zinc-binding transmembrane metallopeptidase with the structure of a non-covalent bound homodimer found on the exterior of cellular membranes (Mina-Osorio, 2008). CD13 is highly expressed in various types of tumors, including renal carcinoma, lung cancer, colon cancer, and prostate cancer (Ishii et al., 2001; Hashida et al., 2002; Sun et al., 2015). CD13 plays a vital role in tumor angiogenesis, invasion, and metastasis (Sato, 2003). A recent study demonstrates that the enzyme acts as a marker of semi-quiescent cancer stem cells (CSCs) in human liver cancer (Haraguchi et al., 2010). In an *in vivo* transplantation model of mice, the volume of solid tumors significantly decreased after combination treatment with 5-fluorouracil (5FU) and ubenimex, a CD13 inhibitor (Nagano et al., 2012). We previously reported that

ubenimex and neutralizing antibody could enhance the antitumor effects of 5FU and other cytotoxic agents on HCC cell lines by up-regulating the level of intracellular reactive oxygen species (ROS). Whereas CD13 over-expression could strengthen the resistance of PLC/PRF/5 cells to chemotherapeutic drugs, knockdown of CD13 remarkably increases the sensitivity of PLC/PRF/5 cells to anti-tumor drugs (Zhang et al., 2018).

Autophagy is an evolutionarily conserved “self-degradative” process by which cells clear long-lived or misfolded proteins, protein aggregates, and damaged organelles to maintain cellular homeostasis under low-oxygen, nutrient-deprived, and stressed conditions (Chen et al., 2011; Levy et al., 2017; Hu et al., 2019). Some studies show that autophagy functions as a tumor suppressor in the early stages of tumor initiation. Autophagy has also been noted to facilitate cancer cell survival at advanced stages of tumor development, which indicates that autophagy plays a dual role in cancer cells (Huang et al., 2018). Data also demonstrate that autophagy induction promotes chemoresistance to facilitate cell survival, which suggests that suppression of autophagy may enhance the sensitivity of cancer cells to chemotherapeutic drugs (O'Donovan et al., 2011; Pan et al., 2014).

Accumulating evidence indicates that the contribution of CD13 is closely related to the resistance of HCC cell to chemotherapeutic drugs, but the relevant molecular mechanism remains unknown. In the present study, we demonstrate that CD13 activates P38/Hsp27/CREB-mediated ATG7 transcription and expression to induce autophagy and promote the chemoresistance of HCC cell.

2. Materials and methods

2.1 Cell culture

The human hepatoma cell lines PLC/PRF/5 and Huh7 were obtained from the Cell Bank of Shanghai (Shanghai, China). We constructed stable CD13 overexpression (PLC/PRF/5/CD13) and knockdown (PLC/PRF/5/CD13 shRNA) PLC/PRF/5 cells by using the lentiviral vector (Genechem, China) and puromycin screening (Zhang et al., 2018). The obtained PLC/PRF/5 and Huh7 cell lines were respectively maintained in modified Eagle's medium and Dulbecco's modified Eagle's medium respectively supplemented with 10% fetal bovine serum and penicillin-streptomycin in a humidified incubator under of 5% CO₂ at 37 °C.

2.2 Western blot analysis

Cells were washed with 1× PBS and lysed in RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, USA). Protein concentrations were determined using Bradford protein quantification and subjected to SDS-PAGE. The gel was cut according to the relevant markers, and target proteins were transferred onto polyvinylidene difluoride membranes (Millipore, USA). The target proteins were determined by western blot with their respective specific antibodies. β-actin or total protein was used as an internal control. Blots were visualized by an enhanced chemiluminescence kit (Millipore, USA). Densitometry for western blot was performed using AlphaEaseFC-v4.0.0 software. The following antibodies were used in this study: CD13 (dilution of 1:1200, Cat. No. sc-51522, Santa Cruz Biotechnology, China), P38 (dilution of 1:1000, Cat. No. sc-271120, Santa Cruz Biotechnology, China), p-P38 (dilution of 1:1000, Cat. No. sc-166182, Santa Cruz Biotechnology, China), Hsp27 (dilution of 1:1000, Cat. No. sc-13132, Santa Cruz Biotechnology, China), p-Hsp27 (dilution of 1:1000, Cat. No. sc-101700, Santa Cruz Biotechnology, China), CREB

(dilution of 1:1000, Cat. No. sc-240, Santa Cruz Biotechnology, China), p-CREB (dilution of 1:1000, Cat. No. sc-7978, Santa Cruz Biotechnology, China), ATG7 (dilution of 1:1000, Cat. No. 8558, Cell Signaling Technology, USA), LC3 (dilution of 1:1000, Cat. No. 3868, Cell Signaling Technology, USA), and β -actin (dilution of 1:1000, Cat. No. sc-1616, Santa Cruz Biotechnology, China).

2.3 MTT assay

Cell viability was evaluated by MTT assay. The PLC/PRF/5, Huh7, and PLC/PRF/5/5FU cells were seeded in 100 μ l of growth medium at a density of 5×10^3 cells well in 96-well plates. After an overnight incubation, the cells were treated with specific reagents. Following 48 h of treatment, the cells were added with MTT solution (Beijing Solarbio Science and Technology, China) and incubated for another 4 h at 37 °C. The supernatant were discarded, and 100 μ l of dimethyl sulfoxide was added to each well. The optical density (OD) of the solutions in the wells was measured at 570 and 630 nm using a multifunctional microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA). The inhibition rate was calculated via the formula $(OD_{\text{control}} - OD_{\text{tested}}) / OD_{\text{control}} \times 100\%$, where the OD is the mean value of three replicate wells. The IC_{50} values were determined using ORIGIN 8 software (OriginLab Corporation, Northampton, MA, USA).

2.4 Quantitative real-time PCR

Total RNA was extracted with Trizol reagent (Ambion, USA), and the RNA concentration was detected by a Nano Drop instrument. Single-strand cDNA was reverse-transcribed using a HiFiscript cDNA Synthesis Kit (CWBio, China) according to the manufacturer's protocol. The cDNA was then amplified with a SYBR Green

mixture (CWBIO, China) and gene-specific primers. The primers used for q-PCR were as follows: CD13 forward: 5'-GGGCAGGGAAGAGGGTGGAC-3'; reverse: 5'-AGGAAGGTGGCTGAGAGTGGATG-3'. ATG7 forward: 5'-CTGCCAGCTCGCTTAACATTG-3'; reverse: 5'-CTTGTTGAGGAGTACAGGGTTTT-3'. Finally, qRT-PCR was performed on an Authorized Thermal Cycler (LighterCycler480 Systems, USA) and the relative mRNA level of the target gene was measured via the $2^{-\Delta\Delta CT}$ method. GAPDH was used as an internal control, and each reaction was performed in triplicate.

2.5 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed using an EZ-Magna CHIPTMA/G Kit (Millipore, #17-10086, USA) according to the manufacturer's standard protocol. Briefly, 1×10^7 of PLC/PRF/5 and PLC/PRF/5/5FU cells were fixed in 1% formaldehyde and incubated for 10 min at 37 °C. Then unreacted formaldehyde was quenched by 10× glycine for 5 min at room temperature, and cells were collected with cold 1× PBS containing 1× protease inhibitor cocktail II. Cell and nuclear lysis buffers containing protease inhibitor cocktail II were added to each microfuge tube to isolate nuclei. Then, the cells were sonicated to create chromatin fragments of 200–500 bp. Aliquots of 50 µl were immunoprecipitated after supplementation with 1 µg of anti-CREB (Cell Signaling Technology, USA) or 1 µg of normal mouse IgG and 20 µl of fully resuspended protein A/G magnetic beads. The mixture was placed on a rotator at 4°C and incubated overnight. Each tube was added with elution buffer and incubated at 62 °C for 2 h with shaking and 95 °C for 10 min to reverse the

protein/DNA complexes. IgG was used as a negative control. Isolated RNA was assayed by 1.5% agarose gel electrophoresis and qRT-PCR. The *ATG7* promoter region (-2074 to +100) was divided into seven parts (Table 1).

2.6 stubRFP-sensGFP-LC3B system

The PLC/PRF/5 cells were infected with the stubRFP-sensGFP-LC3B lentivirus (Genechem, Cat # GRL2001, China) and screened by puromycin. The fluorescence of the construct depended on the difference in pH between the acidic autolysosome and the neutral autophagosome. The progression of autophagic flux was assessed by monitoring the red/green (yellow) or red fluorescence of the cells. The infected cells were treated with 5FU/217505 for 24 h and their fluorescence was detected by laser scanning confocal microscopy.

2.7 Nude mice xenograft model assay

Four week-old female nude mice were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China) and fed under specific pathogen-free conditions. PLC/PRF/5 cells were collected and resuspended at a density of 5×10^7 /ml with PBS. Then, 100 μ l of the cell suspension was subcutaneously injected into the mice. After tumor implantation, the mice were randomly divided into six groups ($n=6$) and administered PBS or 5FU (25 mg/kg/d) and ubenimex (74 mg/kg/d). Tumor volumes and body weights were monitored every 3 days. After 2 weeks, all mice were sacrificed and dissected, and tumor tissues were weighed. All animal experiments were approved by the Guidelines of the Animal Care and Use Committee of Weifang Medical University.

2.8 Statistical analysis

Data are presented as mean \pm SD and were analyzed using GraphPad Prism 6.0 statistical software. Student's *t*-test was used to analyze differences in the data between two groups. Multiple groups of data were compared by one-way ANOVA followed by Dunnett's test or two-way ANOVA followed by Bonferroni's test. $P \leq 0.05$ was considered to indicate statistical significance.

3. Results

3.1 CD13 overexpression results in HCC cell chemoresistance

To investigate the role of CD13 in cell resistance to cytotoxic agents, we first constructed the resistant cell line PLC/PRF/5/5FU by exposing PLC/PRF/5 cells to increasing gradient concentrations of 5FU over 4 months. MTT assay showed that the IC_{50} of the PLC/PRF/5/5FU cells was significantly higher than that of parental cells, thus suggesting that the desired resistant HCC cells had been successfully established (Figure 1A). Western blot and qPCR were then employed to detect the expression of CD13 in PLC/PRF/5 and the PLC/PRF/5/5FU cells. As shown in Figures 1B and C, CD13 expression was up-regulated in the resistant cells. SiRNA targeting CD13 was employed to confirm CD13 antibody really bind with CD13 antigen (Supplemental Figures 1A and B). In our previous study, CD13 over-expression or knockdown in PLC/PRF/5 cells was achieved by lentivirus infection supplemented with puromycin screening. The data indicated that CD13 over-expression induces HCC cell resistance to 5FU, GEM, cis-DDP, and PTX, whereas CD13 knockdown sensitizes cells to cytotoxic agents. The CD13 inhibitor ubenimex enhances the cytotoxicity of chemotherapeutic agents to HCC cell (Zhang et al., 2018). Overall, these results demonstrate that CD13 over-expression may contribute to HCC cell resistance to chemotherapeutic drugs.

3.2 Inhibition of the P38/Hsp27/CREB signaling pathway sensitizes HCC cell to 5FU

The molecular mechanism through which CD13 induces HCC cell resistance remains unknown. Hence, protein chip assay was employed to analyze the signaling pathway proteins phosphorylation in PLC/PRF/5 and PLC/PRF/5/5FU cells. As shown in Figure 2A, the protein phosphorylation expression levels of P38 protein kinase (P38), heat shock protein 27 (Hsp27), and cAMP responsive element binding protein (CREB) were up-regulated in resistant cells. In particular, the phosphorylation level of p-Hsp27 obviously increased. The data indicate that P38, Hsp27, and CREB may participate in the resistance of HCC cell to 5FU. We investigated whether 5FU could up-regulate the phosphorylation of P38, Hsp27, and CREB in PLC/PRF/5 cells. As shown in Figure 2B, 5FU could activate this pathway in a time-dependent manner. To determine whether the P38/Hsp27/CREB pathway is activated in PLC/PRF/5/5FU resistant cells, we measures the phosphorylation of these proteins and found elevated p-P38, p-Hsp27, and p-CREB expression levels in resistant cells compared with parental cells. These results suggest that the P38/Hsp27/CREB pathway is positively correlated with 5FU-induced HCC cell resistance (Figure 2C). We supposed that P38, Hsp27, and CREB activation are triggered by 5FU induction to allow HCC cell to develop resistance. We examined this possibility by employing the P38 inhibitor SB202190 and the CREB inhibitor 217505 to detect their combined effect with 5FU on HCC cell. MTT assay showed that inhibition of P38 by SB202190 decreases the cell

viability in a dose-dependent manner after 5FU treatment of PLC/PRF/5, Huh7, and PLC/PRF/5/5FU cells. This result suggests that P38 inhibition could contribute to the reversion of 5FU resistance (Figures 2D–2F). Interestingly, similar results were obtained after treatment of the cells with 217505 (Figures 2G–2I). Overall, these data suggest that blocking the P38/Hsp27/CREB pathway can help reverse HCC cell resistance.

3.3 CD13 promotes HCC cell resistance to 5FU by activating P38/Hsp27/CREB signaling

A previous study indicated that CD13 and the P38/Hsp27/CREB pathway are involved in the drug resistance of liver cancer cell. Thus, we considered the possibility that CD13 activates the P38/Hsp27/CREB pathway to induce the drug resistance of HCC cell. To explore this possibility, we detected the effects of ubenimex on the P38/Hsp27/CREB pathway. As expected, ubenimex could down-regulated p-P38, p-Hsp27, and p-CREB expression levels in HCC cells (Figures 3A, 3B). To confirm the relationship between CD13 and P38/Hsp27/CREB pathway, we constructed PLC/PRF/5 cells exhibiting stable CD13 over-expression or knockdown. As expected, the western blot results showed that the phosphorylation levels of P38, Hsp27, and CREB are up-regulated in PLC/PRF/5/CD13 cells compared with those in parental cells. In addition, knockdown of CD13 down-regulated phosphorylation levels, which suggests that CD13 functions upstream of the P38/Hsp27/CREB pathway to facilitate HCC cell resistance (Figure 3C). A recent study indicated that P38 could regulate the expression and phosphorylation level of Hsp27 (Guay et al., 1997; He et al., 2012). We examined the relationship between P38/Hsp27 and CREB by treating PLC/PRF/5

and Huh7 cells with SB202190. Western blot results suggested that the expression of p-Hsp27 and p-CREB is down-regulated after SB202190 treatment in a dose-dependent manner, thereby indicating that CREB is located downstream of P38/Hsp27 (Figures 3D, 3E). These results reveal that the CD13-stimulated P38/Hsp27/CREB cascade pathway accounts for HCC cell resistance.

3.4 CREB-mediated autophagy facilitates HCC cell resistance to 5FU via binding to the *ATG7* promoter region

Differences in mRNA level between PLC/PRF/5 and PLC/PRF/5/CD13 cells were analyzed by RNA transcriptome chip technology (Supplemental Figures 2A-2C) to explore the mechanism of HCC cell resistance further. We found that *ATG7* mRNA expression is significantly higher in PLC/PRF/5/CD13 cells than in parental cells. *ATG7* mRNA and protein levels were determined in PLC/PRF/5 and PLC/PRF/5/CD13 cells by qPCR and western blot respectively, data indicated that *ATG7* is obviously up-regulated in PLC/PRF/5/CD13 cells compared with that in parental cells (Figures 4A, 4B). CREB reportedly serves as a transcriptional factor that promotes gene transcription (Lamprecht, 1999). Given the role of CREB and its transcriptional activation of *ATG7*, we deduced that CREB may directly bind to the *ATG7* promoter region. We designed a series of primers flanking the *ATG7* promoter from -2074 to +100. As shown in Supplemental Figure 3, CREB could bind to the -1809 to -1412 promoter region of *ATG7*. Moreover, a significant increase in the binding activity of CREB to the *ATG7* promoter region was observed in PLC/PRF/5/5FU cells compared with that in parental cells (Figure 4C, 4D). Western blot results further showed that *ATG7* expression in resistant cells is higher than that in parental cells.

ATG7 is an E-1 enzyme that activates ubiquitin-like proteins, such as ATG12 and ATG8, and promotes their transfer to an E-2 enzyme, which is crucial for autophagosome formation in the canonical pathway (Zhu et al., 2019). Thus, we hypothesized that CREB-mediated autophagy may be involved in HCC cell resistance. Protein expression associated with autophagy was detected by western blot, and results showed that microtubule-associated protein light chain 3 II (MAP1LC3-II) expression is down-regulated whereas sequestosome 1 (SQSTM1/p62) expression is up-regulated in resistant cells. This finding suggests that the autophagy process may facilitate HCC cell resistance (Figure 4E). The CREB inhibitor 217505 was then employed to determine whether CREB is required for the expression of ATG7 and autophagy. The data indicated that ATG7 expression and autophagy are up-regulated by 5FU treatment, but neutralized by 217505 treatment in PLC/PRF/5 and Huh7 cells (Figures 4F, 4G). PLC/PRF/5 cells were transfected with the stubRFP-sensGFP-LC3B lentivirus to mark LC3B-II and explore whether CREB mediates autophagy. The infected cells were treated with 5FU or 217505 to detect the number of autophagosomes and autolysosomes formed. The results demonstrated that 5FU could induce the autophagy process and that this effect could be neutralized by 217505. These results indicate that CREB participates in autophagy (Figure 4H). We confirmed the relationship between autophagy and resistance by MTT assay. PLC/PRF/5 and PLC/PRF/5/5FU cells were treated with the autophagy inhibitor 3-methyladenine (3-MA) and 5FU. The data indicated that 3-MA could similarly sensitize resistant and parental cells to 5FU (Figure 4I). These results indicate that CREB-mediated ATG7 expression and autophagy could promote HCC cell resistance to 5FU.

3.5 Combination of ubenimex and 5FU inhibits nude mice tumor growth *in vivo*

In this study, nude mice bearing tumor cells were utilized to confirm whether inhibition of CD13 enhances the anti-tumor effect of 5FU *in vivo*. Approximately, 1×10^7 PLC/PRF/5 cells were administrated into the right armpit of the nude mice. The results obtained showed that the combination of 5FU and ubenimex markedly inhibits the growth of tumor tissue compared with 5FU or ubenimex alone (Figures 5A–5D). We then detected protein expression in the tumor tissues. Western blot indicated that whereas 5FU could induce the expression of CD13, p-CREB, ATG7, and autophagy, ubenimex could suppress this effect (Figure 5E). Taken together, the data prove that CD13 over-expression could induce autophagy to promote HCC cell resistance to 5FU *in vivo*.

4. Discussion

HCC, which is characterized by a high degree of malignancy and poor prognosis, is the fifth most frequently occurring cancer worldwide (El-Serag and Mason, 1999; Mlynarsky et al., 2015). While anti-cancer chemotherapeutic drugs for HCC have shown considerable efficacy in terms of prolonging life expectancy, chemoresistance is still often encountered. Chemoresistance reduces the effect of anticancer drugs, and results in failure of HCC therapy (Fu et al., 2018). Thus, exploring the molecular mechanism underlying the development of HCC cell chemoresistance is an important endeavor. In this study, we found that CD13 inhibition could increase the sensitivity of HCC cell to 5FU and reverse their chemoresistance, thereby indicating that CD13 is a potential therapeutic target. Our results further showed that CD13 promotes the expression of ATG7 by activating the P38/Hsp27/CREB signaling pathway to induce autophagy leading to HCC cell resistance.

CD13, a biomarker of human liver CSCs, is related to cancer multidrug resistance (MDR), recurrence, and metastasis (Haraguchi et al., 2010). In this study, the resistant cell line PLC/PRF/5/5FU was employed to detect the relationship between CD13 and resistance. Western blot results showed that the expression of CD13 is highly elevated in resistant cells compared with that in parental cells. Moreover, our previous study revealed that ubenimex and neutralizing antibody could enhance the sensitivity of tumor cells to cytotoxic agents. CD13 over-expression or knockdown affects the sensitivity of cells to cytotoxic agents (Zhang et al., 2018). The compound BC-02, which could decompose into 5FU and ubenimex, showed significant suppression of the self-renewal and malignant proliferation of liver CSCs compared with the control, ubenimex, 5FU and even 5FU + ubenimex treatments, thus suggesting that the combination of 5FU and a CD13 inhibitor could reverse tumor cell resistance (Dou et al., 2017). The APN inhibitor 4cc also synergizes the antitumor effects of 5FU in human liver cancer cells via ROS-mediated drug resistance inhibition and concurrent activation of the mitochondrial pathway during apoptosis (Sun et al., 2015). Overall, we deduced that CD13 may contribute to chemoresistance of HCC cell to 5FU.

To verify the molecular mechanism through which CD13 participates in HCC cell resistance, we employed protein chip technology data to show that the phosphorylation levels of P38, Hsp27, and CREB are obviously up-regulated in resistant cells compared with those in parental cells. P38 protein kinase is a member of the mitogen-activated protein kinase (MAPK) family (Rincon and Davis, 2009). P38 MAPK is an important signaling pathway involved in the growth, survival, differentiation, and inflammatory responses of cells, and can be activated by a variety

of cellular stresses, including inflammatory cytokines, endotoxins, ultraviolet light, and growth factors. The existence of a new mechanism of resistance to camptothecin-related drugs has been reported in this mechanism, MAPK14/p38 α is activated upon SN38 induction and triggers survival promoting autophagy to protect tumor cells against the cytotoxic effects of drugs (Paillas et al., 2012; Hui et al., 2014; Yu et al., 2014; Zhou et al., 2019). Hsp27, a member of the small heat shock protein (Hsp) family, is induced by stress and protects against heat shock, hypertonic stress, oxidative stress, and other forms of cellular injury in numerous cell types (Xu et al., 2013). Over-expression of Hsp27 has been associated with poor prognosis for astrocytic brain tumor (Golembieski et al., 2008), breast cancer (Kang et al., 2008), ovarian carcinoma (Arts et al., 1999), and HCC (Yang et al., 2010). A previous study demonstrated that Hsp27 expression is a prognostic marker involved in the histologic grade and survival of patients with HCC (King et al., 2000; Feng et al., 2005). Yang et al. revealed that miR-17-5p promotes the migration of human HCC cell through the P38 mitogen-activated protein kinase-Hsp27 pathway, which suggests that Hsp27 may function downstream of P38 (Guay et al., 1997; Xu et al., 2013). Taken together, our findings suggest that P38/Hsp27 is associated with HCC cell resistance.

CREB is a nuclear transcriptional factor that regulates target gene transcription activity associated with cell survival and death (Mayr and Montminy, 2001; Wang et al., 2013a; Wang et al., 2013b). It is regulated by multiple protein kinases and protein phosphatases. Over-expression and hyper-activation of CREB1 are often observed in acute myeloid leukemia and several human solid malignancies including breast, lung, ovary, and prostate carcinomas. Inhibition of CREB1 in several human cancer cell lines induces apoptosis and suppression of cell proliferation, indicating its critical

role as a proto-oncogene (Wang et al., 2013b). In the current study, we found that CREB inhibition increases the sensitivity of HCC cell to 5FU. Thus, we speculate that CD13 may regulate the activity of CREB to induce HCC cell resistance through the P38/Hsp27 pathway.

The genes of peptide neurotransmitters, neurotrophic factors, and even transcriptional factors in specific brain regions are downstream targets of CREB (Pandey, 2003; Pandey et al., 2005; Zhang et al., 2005). However, other target genes of CREB have not been reported. Thus, we speculate that CD13 activates CREB to promote downstream protein expression through the P38/Hsp27 pathway, and facilitate HCC cell resistance. Using CHIP method, we found that CREB binds to the -1809 to -1412 region of the *ATG7* promoter. The E1-like enzyme *ATG7* coordinates with the E2-like enzyme *ATG10* to mediate the conjugation of *ATG12* to *ATG5* and promote lipid phosphatidylethanolamine formation (Luo et al., 2016). *ATG7* deletion in mouse liver facilitates HCC development (Takamura et al., 2011). By contrast, inhibition of *ATG7* suppresses RAS-mediated breast tumor growth (Guo et al., 2011), and high *ATG7* expression indicates poor prognosis for breast cancer (Desai et al., 2013). Up-regulation of *ATG7* has been detected in many HCC samples and appears to facilitate tumor cell survival during metabolic stress (Luo et al., 2016). Our current study showed that CREB binds to the *ATG7* promoter region to induce autophagy. We also demonstrated that autophagy inhibition could enhance the cytotoxicity of 5FU toward HCC cell. Our *in vivo* nude mice xenograft model assay confirmed this conclusion.

ROS up-regulation is involved in CD13 suppression-induced cell death. That work mainly focused on ROS. In the present study, we observed that CD13 inhibition can

reverse HCC cell resistance by blocking CREB-mediated autophagy. However, because CREB is a transcriptional factor that may bind to other target genes influencing HCC cell resistance, we further research on this topic is warranted.

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None.

Authorship Contributions

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Footnotes

Grants

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Conflict of Interest

The authors declare that they have no conflict of interest, financial or otherwise in this study.

Table and figure legends

Figure 1. Establishment of a resistant HCC cell line and determination of the expression level of CD13. (A) IC₅₀ values were detected by MTT assay. Data are presented as mean ± SD (n=3). *P ≤ 0.05. (B) CD13 expression was determined using western blot. A representative immunoblot from three independent experiments giving similar results is shown, and β-actin is used as a normalizing protein. (C) CD13 mRNA levels were detected by qPCR. Data are represented as mean ± SD (n=3). *P ≤ 0.05. Statistical analysis was conducted with using Student's *t*-test.

Figure 2. P38/Hsp27/CREB pathway participates in HCC cell chemoresistance.

(A) Phosphorylation of MAPK signaling pathway-related proteins were examined by protein chip assay in PLC/PRF/5 and PLC/PRF/5/5FU cells. (B) PLC/PRF/5 cells were treated with 5FU. Immunoblot was performed to detect the phosphorylation of P38, Hsp27, and CREB. (C) The protein expression of p-P38, p-Hsp27, and p-CREB was determined by western blot in PLC/PRF/5 and PLC/PRF/5/5FU cells. A representative immunoblot from three independent experiments giving similar results is shown and total protein was used as an internal control. The viability of PLC/PRF/5 (D), Huh7 (E), and PLC/PRF/5/5FU (F) cells treated with the P38 inhibitor SB202190 and 5FU for 48 h was examined by MTT assay. PLC/PRF/5 (G), Huh7 (H), and PLC/PRF/5/5FU (I) cells were treated with the CREB inhibitor 217505 and 5FU for 48 h and cell viability was measured by MTT assay. Data are represented as mean ± SD

(n=3). *P ≤ 0.05, **P ≤ 0.01. Statistical analysis was conducted by one-way ANOVA followed by Dunnett's test (D–I).

Figure 3. P38/Hsp27/CREB pathway functions downstream of CD13. PLC/PRF/5 (A) and Huh7 (B) cells were treated with ubenimex for 30 h, and the phosphorylation of signaling pathway-related proteins was detected by western blot. (C) PLC/PRF/5 cells were infected with lentivirus. After puromycin screening, PLC/PRF/5 cells exhibiting stable CD13 over-expression or knockdown were obtained. The expression levels of p-P38, p-Hsp27, and p-CREB were detected by treatment of PLC/PRF/5 (D) and Huh7 (E) cells with the P38 inhibitor SB202190 for 24 h followed by western blot to measure protein phosphorylation. A representative immunoblot from three independent experiments giving similar results is shown and β-actin or total protein was used as a protein loading control.

Figure 4. Transcriptional factor CREB combines with the promoter of the autophagy-related protein ATG7. ATG7 mRNA (A) and protein (B) levels in PLC/PRF/5 and PLC/PRF/5/CD13 cells were analyzed by qPCR or western blot. ChIP assay covering the region of the ATG7 promoter from –1809 to –1412 was performed to measure the binding activity of CREB to the ATG7 promoter in PLC/PRF/5 and PLC/PRF/5/5FU cells (C). IgG was used as a negative control. Enrichment of the ATG7 promoters was quantified by qRT-PCR and the result is presented as a percentage relative to chromatin input (D). Data represent the mean ± SD of 3 independent experiments (*P < 0.05). (E) Protein expression levels of ATG7, LC3-II,

and p62 were detected by western blot in PLC/PRF/5 and PLC/PRF/5/CD13 cells. PLC/PRF/5 (F) and Huh7 (G) cells were pre-treated with the CREB inhibitor 217505 for 2 h and then with 5FU treatment for 24 h. Then, autophagy-related protein expression was examined by western blot. β -actin was used as a protein loading control. (H) PLC/PRF/5 cells were infected with the stubRFP-sensGFP-LC3B lentivirus, pre-treated with 217505 for 2 h and then treated with 5FU for 24 h. Quantification of autophagosome and autolysosome formation representing puncta staining sites per cell. Experiments were performed in triplicate (**P \leq 0.01 vs. the control, #P \leq 0.05, ##P \leq 0.01 vs 5FU). (I) PLC/PRF/5 and PLC/PRF/5/5FU cells were administered with 3-MA and 5FU for 48 h then subjected to MTT assay. Data are represented as mean \pm SD of three independent experiments.*P \leq 0.05, **P \leq 0.01 vs. PLC/PRF/5, #P \leq 0.05, ##P \leq 0.01 vs PLC/PRF/5/5FU. Statistical analysis was carried out using Student's *t*-test (A, D) and two-way ANOVA followed by Bonferroni's test (H–I).

Figure 5. Combination of ubenimex and 5FU inhibits the growth of tumor cells *in vivo*. PLC/PRF/5 cells were injected into the right armpit of nude mice. After tumor formation, the mice were randomly divided into four groups ($n=6$): Ctrl, 5FU (25 mg/kg/d), ubenimex (74 mg/kg/d), and ubenimex+5FU. The body weights and tumor volumes of the mice were monitored (D). After 2 weeks, all mice were sacrificed and dissected, and tumor tissue were weighed (A–C). (E) Protein expression in tumor samples of the Ctrl, 5FU, ubenimex, and ubenimex + 5FU groups was detected by

western blot. A representative immunoblot from three independent experiments giving similar results is shown and β -actin or total protein was used as a protein loading control. Data are represented as mean \pm SD. *P \leq 0.05, **P \leq 0.01 (B–C two-tailed *t*-test)

Table 1. Primer of ATG7 promoter sets used in this study.

Table 1

Region	Forward(5'-3')	Reverse(5'-3')
-2074—-1676	ATGACTCCTGGTTGCCTCCCTTG	TGCTCACACAAAGCCTGTTTGGT AG
-1809—-1412	GTCAGCAAAGGGTGGTGGGATT ATC	AGCAACTGAAGATCCGCAGAAG TG
-1502—-1105	GATCTTCAGTTGCTGCAAGCCAT C	TCCGCAGATGTTTCAATGGTTAG GG
-1227—-830	CCTTCTCTCCCACCTCTCTCACT TC	AGGCGGGCGGATCACGAG
-901—-504	GCCTCCCAAAGTGCTGTGATTAC	GCCTCCCAAAGTGCTGTGATTA C
-599—-202	GCAGTCATCGCTCTTGTTGTTAT G	GGGTGGCAGGTGTGGAGAG
-319—+78	GGGTGGCAGGTGTGGAGAG	TGGGAGGAACTTGAGTCGTGAG G

Figures:

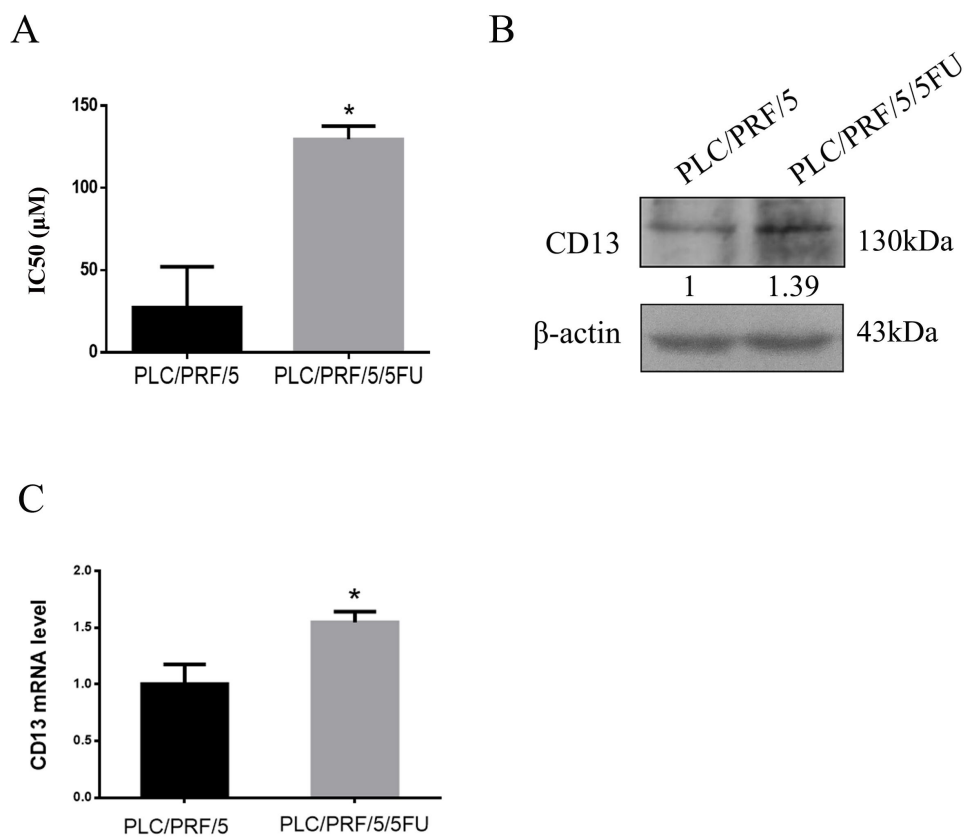


Figure 1

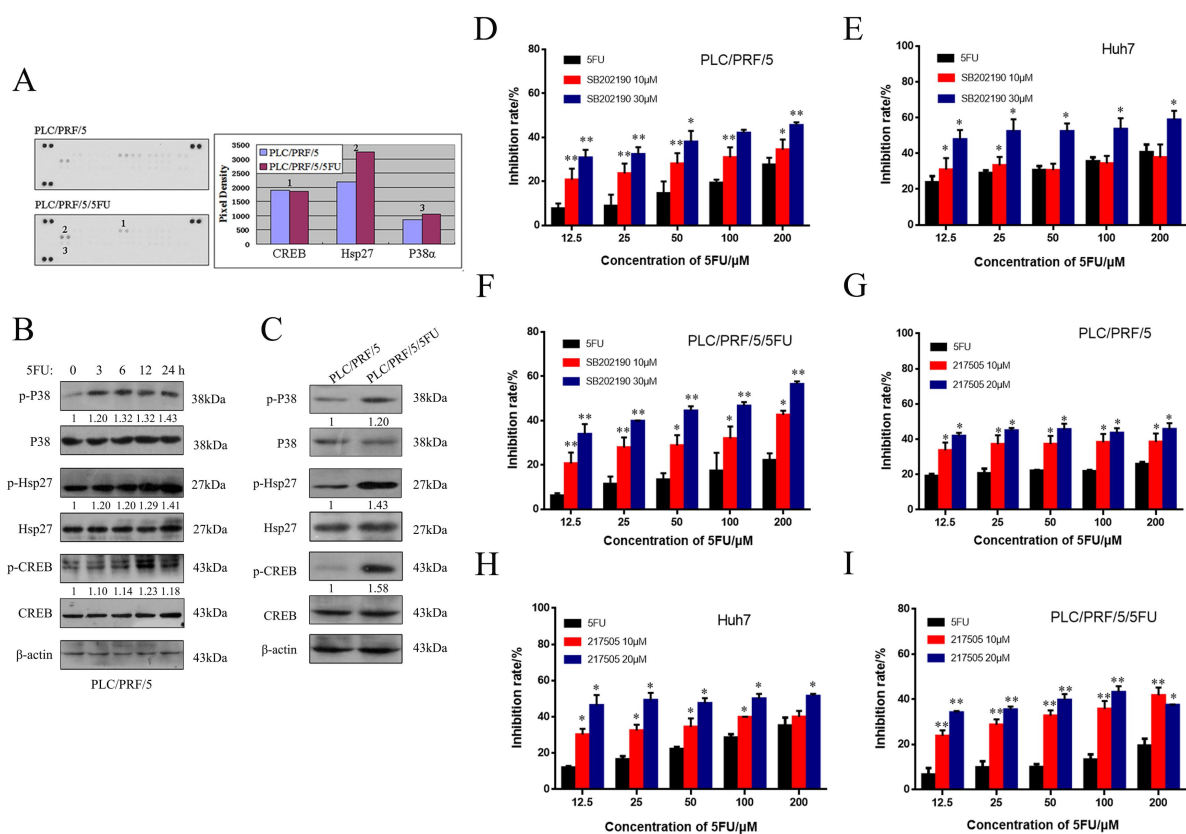


Figure 2

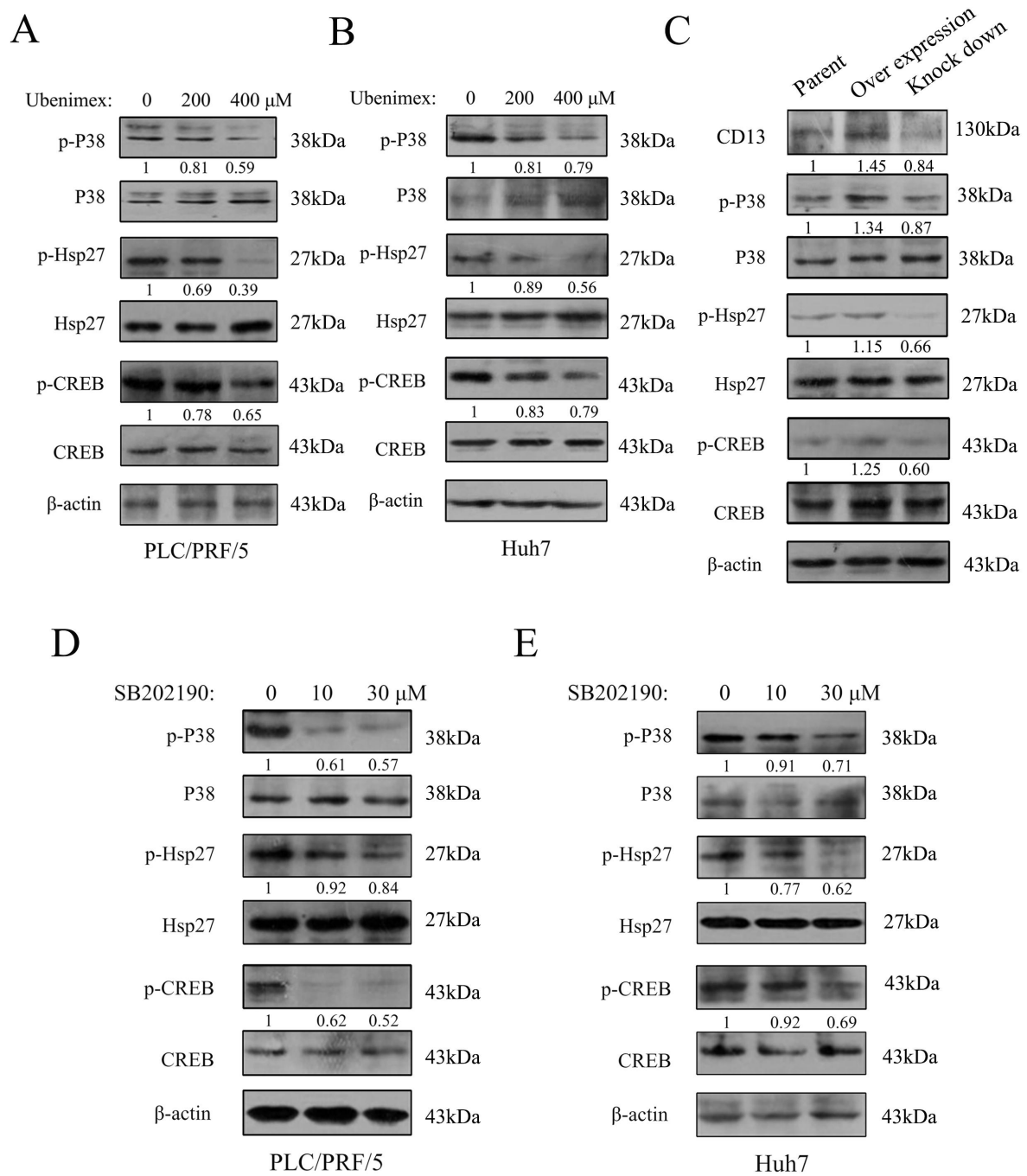


Figure 3

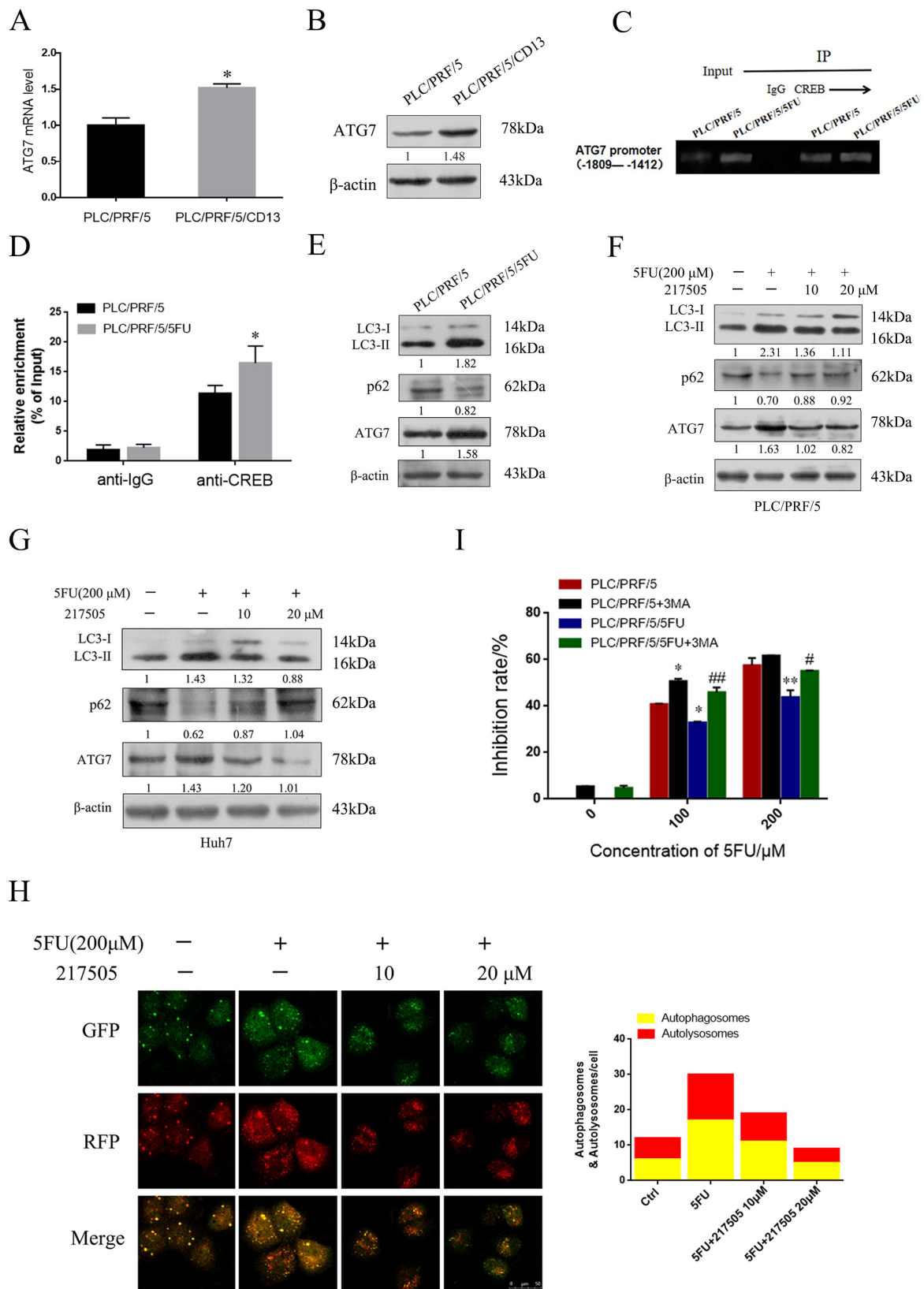


Figure 4

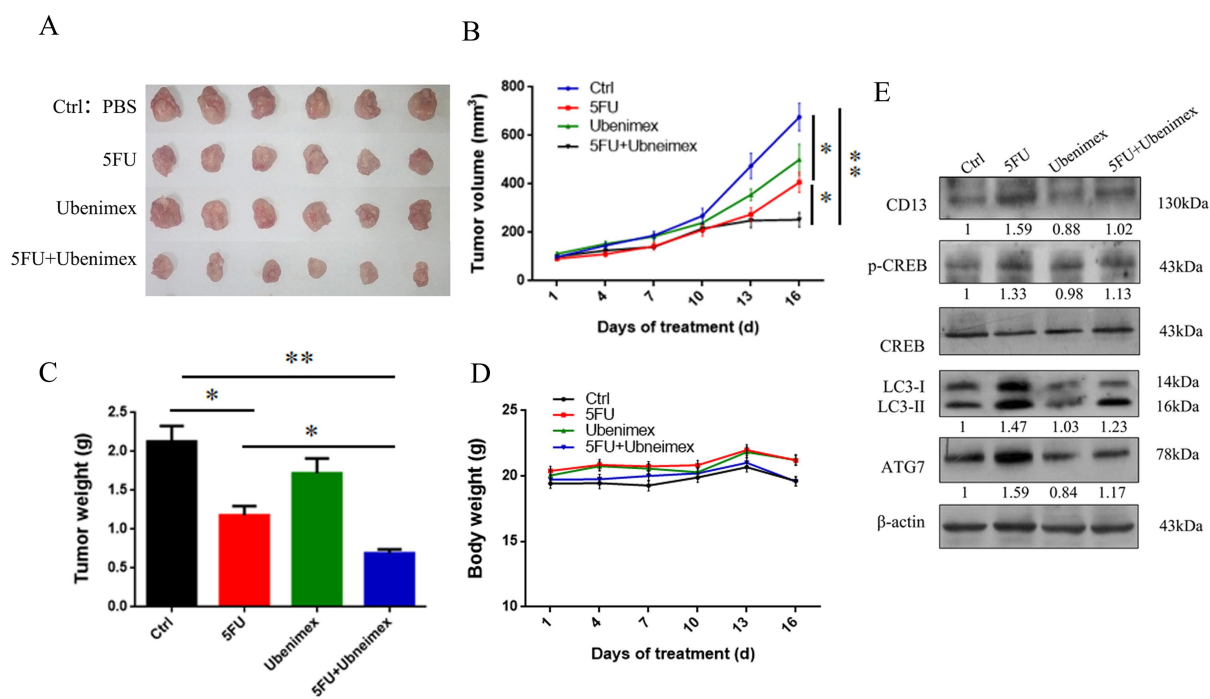


Figure 5

CD13 induces autophagy to promote hepatocellular carcinoma cell chemoresistance through the P38/Hsp27/CREB/ATG7 pathway

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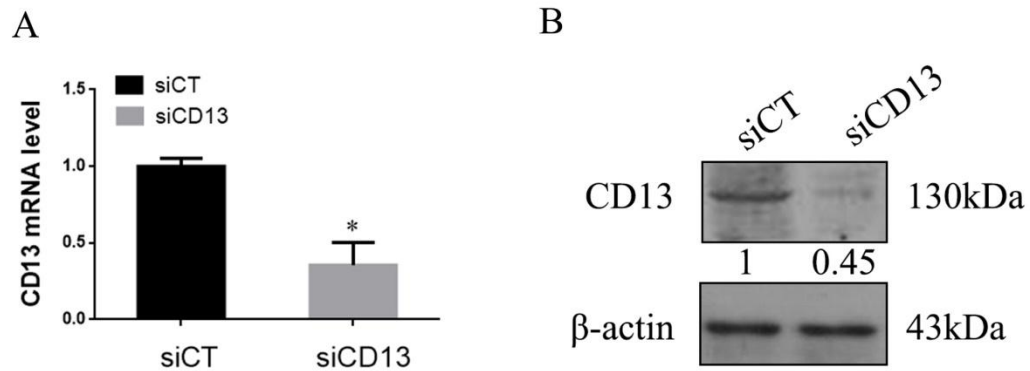
Journal title: Journal of Pharmacology and Experimental Therapeutics

Supplemental Methods:

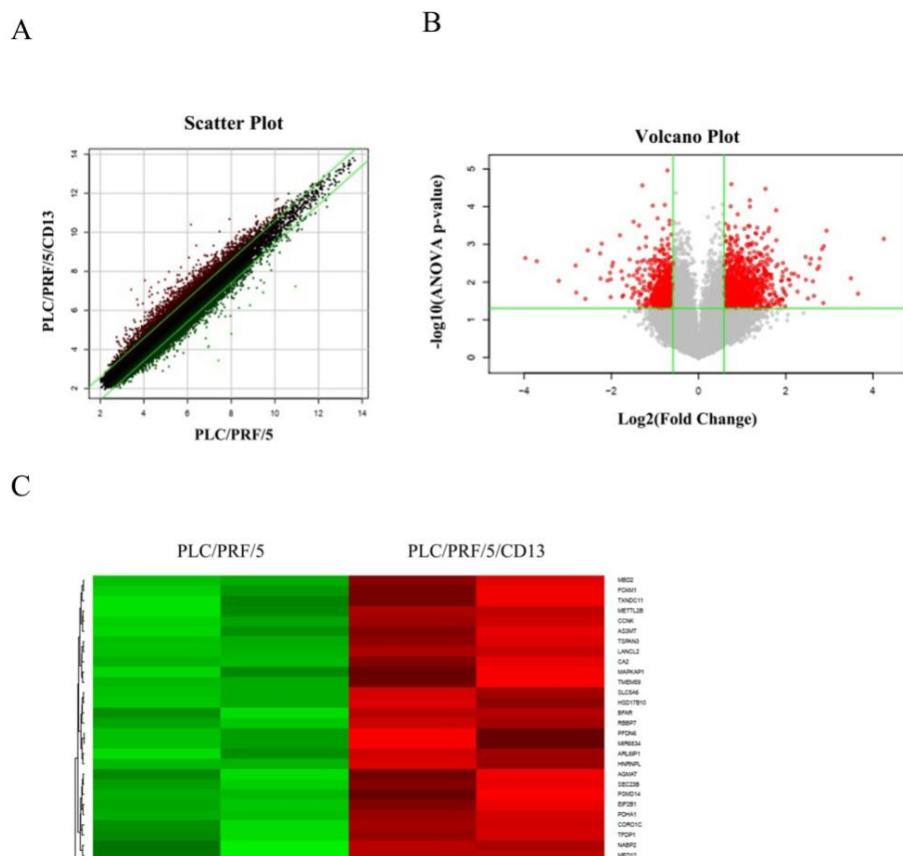
Transfection Assay

Cells were seeded into a 6-well plate and transfected with siRNA targeting the sequence CCGAAATGCCACACTGGTCAA of the human CD13 sequence (NM_001150) (Zhang et al., 2018, PMID: 30258365). Non-specific scrambled siRNA duplex was purchased from GenePharma (Shanghai, China). Transfection was conducted according to the Lipofection 2000 kit instructions.

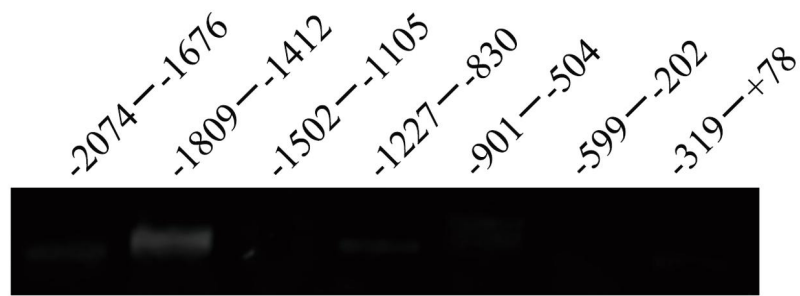
Supplemental Figures:



Supplemental Figure 1: CD13-targeting siRNA down-regulated the expression level of CD13 mRNA and protein. PLC/PRF/5 cells were transfected with CD13-targeting siRNA (siCD13) or control (siCT). qPCR (A) and western blot (B) were used to detect CD13 expression in PLC/PRF/5 cells. Data are represented as mean \pm SD (n=3). *P \leq 0.05. Statistical analysis was carried out using Student's *t*-test.



Supplemental Figure 2: RNA transcriptome chip technology was employed to detect mRNA levels in Ctrl and CD13 overexpression PLC/PRF/5 cells. (A) Scatter plot showing the distribution of signal intensity in a rectangular coordinate plane. Red and green dots outside intervals represent up-regulated probes in the CD13 over-expression and control cells, respectively. (B) Volcano plot image for all genes. Gray and red puncta represent equally (fold change < 1.5) and differentially (fold change ≥ 1.5) expressed mRNAs. (C) Heat map generated using the R package to depict 1891 transcripts that are significantly differentially expressed in PLC/PRF/5/CD13 cells (fold change ≥ 1.5 , $*P < 0.05$). Red and green respectively colors indicate up-regulated and down-regulated transcripts.



Supplemental Figure 3: A series of primers flanking the *ATG7* promoter from - 2074 to +100 were designed for ChIP assay followed by PCR analysis. The region of the *ATG7* promoter from -1809 to -1412 was detected to evaluate CREB binding.