Cancer Letters 454 (2019) 215–223

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Glycochenodeoxycholate promotes hepatocellular carcinoma invasion and migration by AMPK/mTOR dependent autophagy activation

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ARTICLE INFO

Keywords:
Hepatocellular carcinoma
Cholestasis
Metastasis
Autophagy

ABSTRACT

Metastasis and recurrence severely impact the treatment effect of hepatocellular carcinoma (HCC). HCC complicated with cholestasis is more prone to recurrence and metastasis. Previous studies have implicated pathogenesis of HCC by bile acid; however, the underlying mechanism is unknown yet. Glycochenodeoxycholate (GCDC) is one of most important component of bile acid (BA). In the present study, the role of GCDC in HCC cells invasion was detected by *in vitro* and *in vivo* assays. GCDC was found to significantly enhance the invasive potential of HCC cells; Further studies showed that GCDC could induce autophagy activation and higher invasive capability in HCC cells. Interestingly, inhibition of autophagy by chloroquine (CQ) reversed this phenomenon. Subsequently, the correlation between TBA expression level and clinicopathological characteristics was analyzed in HCC patients. Clinically, high TBA level in HCC tissue was found to be associated with more invasive and poor survival in HCC patients. Mechanistic study showed that bile acid induced autophagy by targeting the AMPK/mTOR pathway in HCC cells. Therefore, our results suggest that bile acid may promote HCC invasion via activation of autophagy and the level of bile acid may serve as a potential useful indicator for prognosis of HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is one of most common malignancies. Despite developments in medical and surgical treatments improved the overall survival of patients, long term outcomes remain poor because of high rates of tumor metastasis, which reduced the efficacy of therapeutic for a number of patients. Therefore, it is critical to identify the molecular mechanism of metastasis of liver cancer and find a proper management target for HCC.

Cholestasis often presents as a common complication in patients with HCC, especially in middle and advanced stage patients [1]. In cholestasis, impaired bile flow which caused by obstruction of bile ducts and genetic mutation of bile acid transporter genes or acquired dysregulation of bile transport, which can lead to accumulation of bile acid in the liver, can cause hepatocyte and biliary injury [2–4]. Glycochenodeoxycholate (GCDC) is one of most important component of bile acid [5,6]. It is a hydrophobic acid causing severe liver damage [2,7–9]. Wang et al. found that the hydrophobic bile acid induced hepatocyte apoptosis and necrosis, leading to cell death [10]. Therefore, GCDC directly simulates the pathophysiological change in the liver damage. Previous studies have shown that bile acid was a major endogenous promoter of liver cancer and the dysregulation of BA contributed to the development of HCC [11–14]. Knisely AS et al. found that a group of 10 children with genetic deficiency of the bile salt export pump (BSEP) or ABCB11 developed cholestasis and HCC [13]. The deregulation of bile acid homeostasis, which was also observed in liver cirrhosis and nonalcoholic steatohepatitis (NASH), was closely associated with HCC development [12,15]. Moreover, in wild type mice, a cholic acid (CA)-enriched diet strongly promoted N-nitrosodiethylamine-initiated hepatocarcinogenesis [16]. Although these studies have linked bile acid to hepatocellular carcinoma, the mechanisms by which bile acid promote HCC is still unknown.

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https://doi.org/10.1016/j.canlet.2019.04.009
Received 26 October 2018; Received in revised form 5 April 2019; Accepted 5 April 2019

Autophagy is a cellular self-degradation system that eliminates damaged or dysfunctional organelles to help maintain cellular metabolic needs. In response to stress, autophagy is also a catabolic process that generate nutrients and energy to facilitate the cells adaptation [17–19]. Our previous study also found the protective role of autophagy in cholestasis-induced liver injury [20]. Moreover, autophagy supports cancer survival and mediate resistance to anticancer therapies such as chemotherapy, radiation and some targeted therapies [21–23]. Various studies indicated that the defects of autophagy often restrained the proliferation, dissemination and metastatic potential of tumor cells [24–26]. Therefore, autophagy plays a significant role in the maintenance and promotion of tumor development.

In the present study, we provided evidence that bile acid enhanced the invasion and migration of HCC cells *in vitro* and *in vivo*, which was mediated by autophagy. Bile acid triggered autophagy as the protective mechanism, which enhanced cancer cells viability so that these cells became metastatic to the following damage. Inhibition of autophagy could reverse this phenomenon. Clinically, high total bile acid (TBA) level in HCC tissue was strongly associated with the poor survival of patients.

2. Materials and methods

2.1. Cell culture and reagents

Human HCC cell lines, SMMC7721 and Huh7 were cultured in Dulbecco's modified Eagle's Medium (DMEM), supplemented with10% fetal bovine serum (FBS) (Gibco, Invitrogen) in a humidified 5% CO_2 incubated at 37 °C. Glycochenodeoxycholate (GCDC), Chloroquine (CQ),AMPK inhibitor Compound C (Comp C) were purchased from Sigma-Aldrich (St Louis, MO,USA) and mTOR activator (MHY1485) was purchased from APExBIO (Boston, MA, USA).

2.2. Patients and specimens

From October 2000 to November 2003, specimens obtained from patients who underwent liver resection and pathology-proven HCC were examined in the study. The diagnosis of HCC and recurrence was confirmed by diagnostic criteria for HCC, as described previously [27]. The cohort consisted of 89 males and 15 females. The available characteristics of the patients were shown in Table 1. The study was approved by the Eastern Hepatobiliary Surgery Hospital Research Ethics Committee. Overall survival (OS) was defined as the interval between the dates of surgery or final follow-up, whereas the disease-free survival (DFS) was defined as the interval between the dates of surgery and recurrence or final follow-up.

2.3. Wound-healing assay

The wound healing assay followed by method that had been described previously [28]. For the wound-healing assay, cells were seeded in 6-well plates at 1×10^5 cells/well and incubated for 48 h. Monolayer were scratched with a 200 μL pipette tip. The cell migration was recorded by a phase-contrast microscope (Olympus, Japan) at 48 h after wound scratch. Experiments were performed three times, and four fields were recorded.

2.4. Cell migration and invasion assay

For the transwell assay, matrigel invasion chambers were purchased from Corning (NY, USA). The chamber has been coated with matrigel. Approximately 5×10^4 cells from different groups were resuspended in 200 μL serum-free medium and placed in the upper chamber, and 600 μL medium containing 5% FBS was added to the lower chamber. After 48 h, cells on the lower surface of the membrane were fixed with 4% paraformaldehyde in PBS buffer and stained with 0.1% crystal violet. The migrated cells were counted and imaged at 200 × magnification. These experiments were performed three times.

Table 1
Correlations between TBA level and clinicopathological parameters of HCC.

Clinicopathologic Parameters	TBA			*P*-Value
	Number	Negative	Positive	
Frequency		60	44	
Gender				
Male	89	53	36	0.404
Female	15	7	8	
Age(years)				
≤60	85	47	38	0.320
> 60	19	13	6	
Hbsag				
Negative	82	51	31	0.091
Positive	22	9	13	
Cirrhosis				
Absence	27	21	6	**0.023***
Presence	77	39	38	
AFP				
≤20	52	35	17	0.074
> 20	52	25	27	
TNM				
T1/T2	43	31	12	**0.017***
T3	61	29	32	
Tumor size(cm)				
≤5	48	30	18	0.427
> 5	56	30	26	
Tumor number				
Single	93	56	37	0.196
Multiple	11	4	7	
Microvacular invasion				
Absence	43	30	13	**0.045***
Presence	61	30	31	
Macrovacular invasion				
Absence	6	6	0	**0.037***
Presence	98	54	44	
Encapsulation				
Absence	30	21	9	0.128
Presence	74	39	35	
LC3				
Low	43	31	12	**0.016***
High	61	29	32	
p62				
Low	46	21	25	**0.030***
High	58	39	19	

*$p < 0.05$ was considered statistically significant.

2.5. Western blot analysis

The cells were harvested, washed, and lysed in RIPA buffer (Beyotime). An equivalenta mount of proteins was separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 5% non-fat-milk in TBS for 1 h at roomtemperature and incubated overnight at 4 °C with rabbit primary antibodies against LC3(1:1000, Cell Signaling Technology), p62 (1:500, Cell Signaling Technology), AMPK (1:1000, Cell Signaling Technology), P-AMPK (1:1000,Cell Signaling Technology), mTOR (1:1000, Cell Signaling Technology), p-mTOR (1:1000,Cell Signaling Technology), and GAPDH (1:5000, Bioworld Technology, Inc.) as a control. The membranes were washed threetimes in TBS-Tween20 and incubated with goat polyclonal secondary antibody to rabbit IgG (1:10000, Bioworld Technology, Inc.) for 1 h at room temperature. Finally, the proteins were detected using an enhanced chemiluminescence (ECL) system (Beyotime).

2.6. Animal experiments

Eight-week-old male BALB/c nude mice weighing 20–25 g were purchased from Shanghai experimental animal center of Chinese

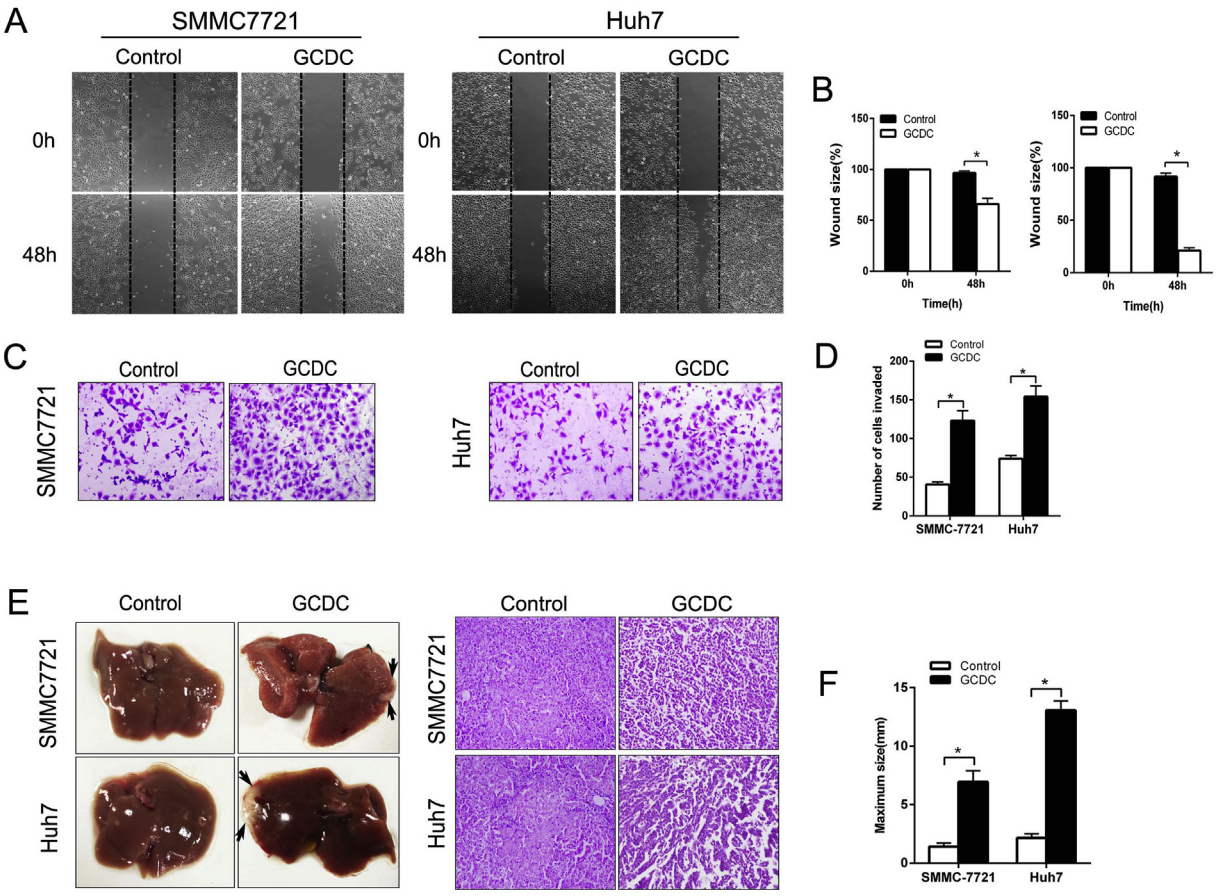

Fig. 1. Migratory capacity of HCC cells was up regulated in response to GCDC. (A) The migration of SMMC7721 and Huh7 cells was measured by wound healing assay. Cells were stimulated with GCDC (200 μM) for 48 h to evaluate the rate of migration into the scratched area (× 200). (B) Quantitative analysis in invasion experiment was performed and the results were showed as mean ± SD (*p < 0.05). (C) Invasion of GCDC treated HCC cells were measured by transwell assay in comparison with the control groups. SMMC7721 and Huh7 cells were monitored for 48 h, and the invasion ability was determined 48 h later. Cells were plated in the upper chamber of the transwell and allowed to grow for 48 h in serum-free medium. 5% FBS was simultaneously plated in the lower chamber. (D) The number of cells was counted under × 200 and represented as mean ± SD (*p < 0.05). (E) Representative macroscopic images from liver nodules in nude mice injected with GCDC-treated SMMC7721 and Huh7 cells through the splenic vein. HCC cells were treated with GCDC for 72 h before injecting HCC cells into mice. The mice were sacrificed 8 weeks later and the maximum size of surface liver metastases was counted. The arrow indicates the metastatic tumor on the surface of the liver. H&E staining was performed on the sections of liver samples (× 200). (F) The maximum size of liver surface nodules were quantified on nude mice livers. Values for individual mice are shown above the bars (*$p < 0.05$).

Academy of Science. All the animals were maintained under specific pathogen-free environment before the experiment. HCC cells were injected into the splenic vein of 8-week-old nude mice with 1×10^6 cells/injection site. The animals were sacrificed at the 8th weeks and the maximum size of surface liver metastases was counted. All the animal experiments were performed in accordance with the Institutional Animal Welfare Guidelines of the Eastern Hepatobiliary Surgery Hospital of the Second Military Medical University, Shanghai, China.

2.7. Transient transfection and identification of autophagy

GFP-LC3 expression vector was utilized to demonstrate the occurrence of autophagy. HCC cells were seeded (1×10^4 cells/well) in 96-well plates and cultured overnight. Then, GFP-LC3 expression plasmids were transiently transfected into the cells using Fugene HD transfection reagent (Roche, Basle, Switzerland) according to the manufacturer's instructions. The cells were subjected to the indicated treatments 48 h after transfection. At the end of the treatments, the GFP-LC3-positive expression was detected by assessing the percentage under a fluorescent microscope (OlympusIX71, Olympus Optical Co. Ltd, Tokyo, Japan). A minimum of 200 cells/sample was counted in triplicate for each experiment.

2.8. Electron microscopic analysis

After treatment, the cells were collected and fixed in 2.5% glutaraldehyde in phosphate buffer for 2 h at 4 °C, followed by postfixation with 1% osmium tetroxide for 1 h at room temperature. The samples were dehydrated in gradient ethanol and then embedded in araldite. Ultrathin sections of 50–60 nm were cut on a Leica microtome and placed on copper grids and stained with uranylacetate and lead citrate. Images were obtained with a CM-120 electron microscope (Philips).

2.9. CCK8 assay

Cell counting kit-8 (CCK-8) assay was used to determine cell survival. Cell count was adjusted to 1×10^4 cells/well seeded to 96-well plates. After overnight incubation, cells were exposed to expriments for short-term (0–48 h). After treatment, 10 μl/well of CCK-8 solution was added. Cells were incubated at 37 °C for 1 h and the absorbance of the samples were detected at 450 nm using a microplate reader (Synergy HT).

2.10. Immunohistochemistry staining

The sample staining procedures were performed as described

previously [29]. The slides were deparaffinized in xylene and rehydrated through gradient alcohol. Endogenous peroxidase was then inactivated with 3% hydrogen peroxide at room temperature for 20min. Next, the antigen retrieval was enhanced by autoclaving the slides in 0.1 mol/L citrate buffer (pH 6.0) for 2min. After washing with PBS (pH7.4), the sections were blocked with 1% BSA at 37 °C for 30min.The slides were then incubated overnight at 4 °C with primary rabbit polyclonal anti-LC3 and anti-p62. Subsequently, the HRP-conjugated goat anti-rabbit antibody and DAB (Dako, Carpinteria, CA, USA) were used. Images were captured with the microscope. The positive areas of LC3 and p62 in the images were measured by Image Pro Plus (IPP).

2.11. Statistical analysis

All experiments were performed at least three times. The data were expressed as the mean ± SD. Statistical analysis was performed using the SPSS 21.0 for Windows (SPSS Inc., Chicago, IL) for Student's t-test for clinical analysis. The differences between categorical variables were assessed by chi-square test or Fisher's exact test. Pearson's correlation coefficient was used to determine the correlations between continuous normally distributed variables. OS and DFS were calculated by Kaplan-Meier analyses, and differences in survival rate were compared using the log-rank test. $p < 0.05$ was considered significant.

3. Results

3.1. GCDC induced metastasis and invasion of HCC cells in vitro and in vivo

Effect of bile acid on the migratory and invasive potential of HCC cell lines was evaluated *in vitro* and *in vivo*. Firstly, wound healing and transwell assay were employed to examine the role of GCDC in migration and invasion of two human HCC cell lines (SMMC-7721 and Huh7). Compared with the control group, shorter distances were observed in the presence of GCDC stimulation (200 μM) for 48 h in SMMC-7721 and Huh7 cells by wound healing assay (Fig. 1A and B). And the data of transwell assays showed significant higher invasive ability of SMMC-7721 cell lines treated with GCDC than untreated one. Consistent results were obtained from the Huh7 cell line (Fig. 1C and D). Then, nude mice were used to validate the *in vivo* findings through tumorigenic and splenic vein metastasis assays. HCC cells were treated with or without GCDC for 72 h, and then intrasplenically injected into the nude mice. After 8 weeks, we collected liver tissues. As shown in Fig. 1E, the injections resulted in hepatic metastasis, especially in the Huh7 group. Cells treated with GCDC in SMMC7721 and Huh7 group exhibited a higher maximum size of liver tumor nodules as compared with those of control group (Fig. 1F). These results indicate that GCDC treatment may enhance the invasion and migration potential of HCC cells.

3.2. GCDC promoted invasive capability of HCC cells through autophagy activation

In our previous study, GCDC could activate autophagy which protected the liver from GCDC-induced hepatic injury [20]. Thus, GCDC can stimulate autophagy to promote the tumor development in HCC. Herein, we attempted to determine whether autophagy was involved in the effect of GCDC on the migration and invasion of HCC cells. Firstly, SMMC-7721 and Huh7 cells were transfected with GFP-LC3 plasmid and analyzed by microscopy. Our results showed that GCDC treatment exhibited a significantly higher percentage of punctate GFP (green dots), while untreated cells showed primary diffusion (Fig. 2A). Secondly, electron microscopic analysis also showed an increase in autophagosomes in GCDC treated tumor cells (Fig. 2B). In addition, we tested the effect of GCDC on isoform conversion of LC3, an indicator of autophagosome formation and an autophagy-related marker on the progressive reduction of p62, which was a selective substrate of

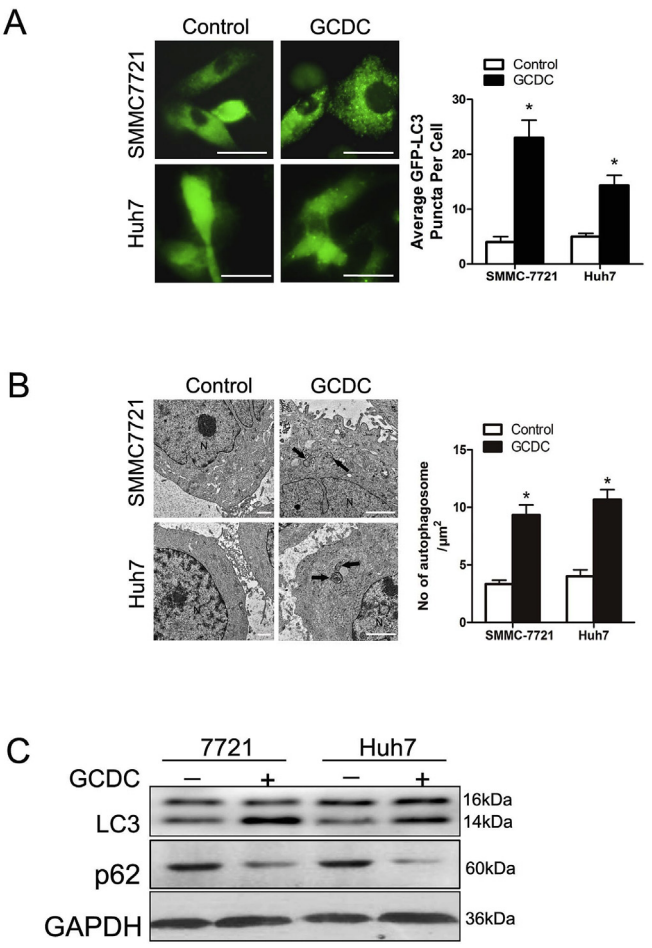

Fig. 2. GCDC activates autophagy in HCC cells. (A) SMMC7721 and Huh7 cells were transfected with GFP-LC3 and treated with GCDC (200 μM) for 48 h. Images were captured under a fluorescence microscope. GFP-LC3 puncta (mean ± SD) were quantified for each experiment. A minimum of 20 cells were counted in each experiment (scale bar, 50 μm). (B) Electron micrograph showed autophagosome of HCC cells under GCDC exposure. Black arrows indicated autophagosome formation. (scale bar, 1 μm). (C) Expression of LC3 and p62 were analyzed by western blotting using the indicated antibodies.

autophagy. Western Blotting analysis showed that LC3-II expression was increased upon GCDC treatment, while the p62 level was decreased (Fig. 2C). These results reveal that GCDC can activate autophagy in HCC cells.

Next, to detect whether autophagy induced by GCDC could affect tumor invasion, we used autophagy inhibitor-chloroquine (CQ). The scratch wound-healing results showed that CQ reversed the changes in cell motility induced by GCDC in both the HCC cells (Fig. 3A and B). Similar effects were further confirmed by the invasive assay, and the results displayed that CQ treatment markedly reduced the HCC cells invasion induced by GCDC (Fig. 3C and D). To test the effect of CQ on cell migration and invasion was not dependent the proliferative ability, CCK8 assay was performed. The results indicated that CQ was no effect on the proliferation of HCC cells (Supplementary Fig. 1). To further investigate the efficacy of the combined treatment *in vivo*, SMMC7721 and Huh7 cells stimulated with GCDC in the presence of CQ were intrasplenically injected into nude mice, respectively. As shown in Fig. 3E and F, GCDC + CQ group displayed a lower maximum size of liver tumor nodules on the liver surface as compared with the GCDC-treated group. The histological analysis suggested that GCDC caused prominent necrosis in the tumor. On the contrary, necrosis in GCDC + CQ group was significantly lower than that of the GCDC group (Fig. 3G). These findings exhibited that inhibition of autophagy resulted in fewer

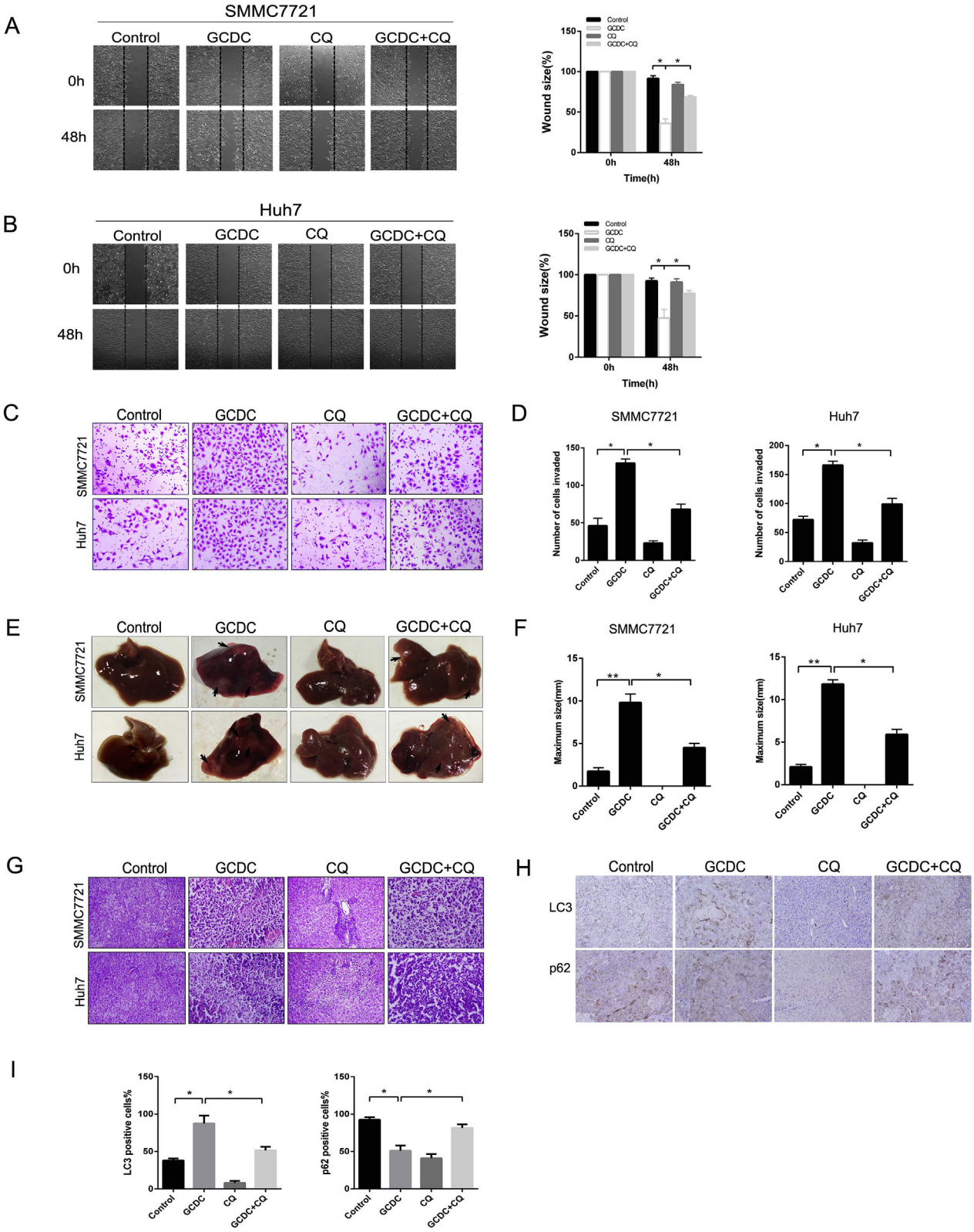

Fig. 3. CQ inhibits the migration of HCC cells caused by GCDC *in vitro* and *in vivo*. (A–B) The migration of HCC cells was determined by wound healing assay (× 200). Quantitative analysis in each group was performed and the result was shown as mean ± SD (*$p < 0.05$). (C) The invasiveness of HCC cells was assessed by transwell assay (× 200). (D) Quantitative analysis in each group was performed and the result was shown as mean ± SD (*$p < 0.05$). (E–F) CQ was used as autophagy inhibitor. HCC cells were treated with GCDC (200 μM) and CQ (50 mM) or both for 72 h before injecting HCC cells into mice. A representative image of harvested livers in different groups was shown. The arrow indicated the metastatic tumor on the liver surface (n = 10/group). Quantification of animal experiments was shown as means ± SD (*$p < 0.05$, **$p < 0.01$). (G) Corresponding H&E staining of metastatic liver tumor tissue was shown (× 200). (H) Representative IHC image of LC3 and p62 in tumor tissues (× 200). (I) Quantification of LC3 and p62-positive cells was shown as means ± SD (*$p < 0.05$).

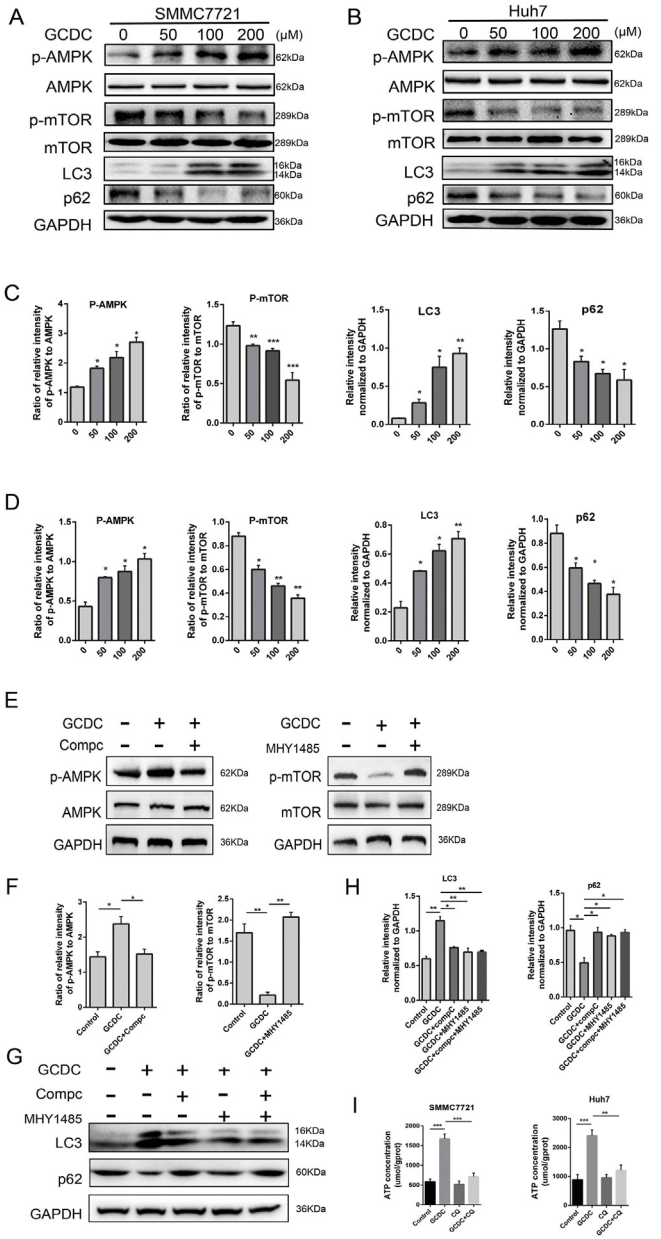

Fig. 4. GCDC target the AMPK/mTOR pathway in HCC cells. (A–B) Immunoblotting analyses of p-AMPK, AMPK, p-mTOR, mTOR, LC3, p62 in SMMC7721 and Huh7 cells with different concentrations of GCDC 48 h. (C–D) Quantification of image was shown as means ± SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (E) p-AMPK, AMPK, p-mTOR and mTOR in HCC cells treated with GCDC, Comp C and MHY1485 by western blotting analyses. (F) Quantification of image was shown as means ± SD (*$p < 0.05$, **$p < 0.01$). (G) LC3 and p62 expression was examined by western blotting in HCC cells after treatment of GCDC with Comp C (20 μM) and MHY1485 (10 μM). (H) Quantification of above image was shown as means ± SD (*p < 0.05, **p < 0.01). (I) ATP colorimetric assay was used to observe the effect of GCDC on the ATP concentration with or without the presence of CQ. ATP concentrations were quantitated and image was shown as means ± SD (**$p < 0.01$, ***$p < 0.001$).

nodules in HCC. Additionally, the autophagy level was detected in tumor tissue, and the results showed that GCDC treatment upregulated the expression of LC3 and downregulated p62 expression, however, the effect of GCDC on autophagy weakened when HCC cells were treated with CQ (Fig. 3H and I). Thus, autophagy contribute to HCC metastasis which induced by GCDC.

3.3. GCDC induced autophagy via the AMPK/mTOR signaling pathway

Previous study has shown that the AMPK/mTOR signaling pathway is involved in cell autophagy and AMPK negatively regulates mTOR and triggers autophagy flux. Therefore, the expression levels of p-mTOR, mTOR, p-AMPK and AMPK after treated with different concentrations of GCDC (0, 50, 100, 200 μM) was analyzed in the current experiment (Fig. 4A and D). Compared with untreated cells, the expression of p-AMPK was upregulated, while the p-mTOR protein level was downregulated in a dose dependent manner with GCDC for 48 h. This was followed by increased levels of LC3 and decreased levels of p62. Activated AMPK negatively regulated mTOR and thereby enhanced autophagy flux. Furthermore, to determine the role of the AMPK pathway in GCDC-induced autophagy, HCC cells were treatment with AMPK inhibitor Compound C and mTOR activator MHY1485. As shown in Fig. 4E and F, p-AMPK and p-mTOR expression was downregulated and upregulated respectively when HCC cell treated with Compound C or MHY1485. Then LC3 and p62 expression was also detected in HCC cells treated with GCDC and compound C, MHY1485 or both. The results showed that GCDC-induced autophagic activity was inhibted by Compound C and MHY1485 (Fig. 4G and H). These findings indicated that AMPK/mTOR pathway was involved in GCDC-induced autophagy in HCC cells.

AMPK is known as essential energy sensor. In the present study, we wondered whether GCDC affects the energy at the ATP levels. As shown in Fig. 4I, ATP concentration increased significantly when HCC cells treated by GCDC. Furthermore, CQ combined with GCDC could inhibit ATP elevation induced by GCDC, suggesting that autophagy provided energy for invasion of liver cancer.

3.4. TBA levels were correlated with autophagy and poor prognosis in HCC patients

Clinically, TBA abnormalities are commonly used to reflect the occurrence of cholestasis. Changes in the TBA values can track hepatic disorders [30]. Earlier studies have confirmed the clinical applicability of measuring serum TBA concentrations as a diagnostic for hepatobiliary diseases [31–33]. In order to evaluate the prognostic value of TBA in HCC, 104 HCC patients were recruited and tumor tissues were collected. According to the standard clinical value, the patients were divided into two groups: the high TBA level group (n = 44) and the low TBA level (n = 60) group. The correlation between the TBA level and clinicopathological factors in the 104 patients was shown in Table 1. High TBA level was significantly associated with severe cirrhosis ($p = 0.023$), TNM stage ($p = 0.017$), microvascular invasion ($p = 0.045$), and macrovascular invasion ($p = 0.037$). Then, we identified the correlation between the TBA level and autophagy-related markers' expression. The TBA level was significantly associated with LC3 ($p = 0.016$) and p62 ($p = 0.030$) in HCC tissues. Furthermore, IOD of LC3 and p62 expression was calculated by IPP analysis following immunohistochemistry (Fig. 5A). By Spearman's correlation analysis, we found that TBA level in HCC patients was positively correlated with LC3($r = 0.573$, $p < 0.01$) and negatively correlated with p62($r = -0.265$, $p < 0.01$) (Fig. 5B and C). These results showed that TBA level in HCC patients was associated with the autophagy activation in tumor tissues. High TBA level may confer to upregulated autophagy and increase the potential of invasion in HCC patients. Next, the survival rate of the recruited HCC patients was evaluated using the Kaplan-Meier survival analysis. Patients with high TBA level were likely to have significantly poor OS and DFS. The median (95% CI) OS was 33.13 (27.20–39.06) and 53.06 (39.97–66.16) months for patients with high TBA level group and low TBA level group, respectively ($p < 0.05$, Fig. 5D). The median (95% CI) DFS was 24.43 (18.93–29.93) and 33.66(24.67–42.66) months for patients with high TBA level group and low TBA level group, respectively ($p < 0.01$, Fig. 5E). The results demonstrate that high TBA level is a risk factor in prognosis of HCC

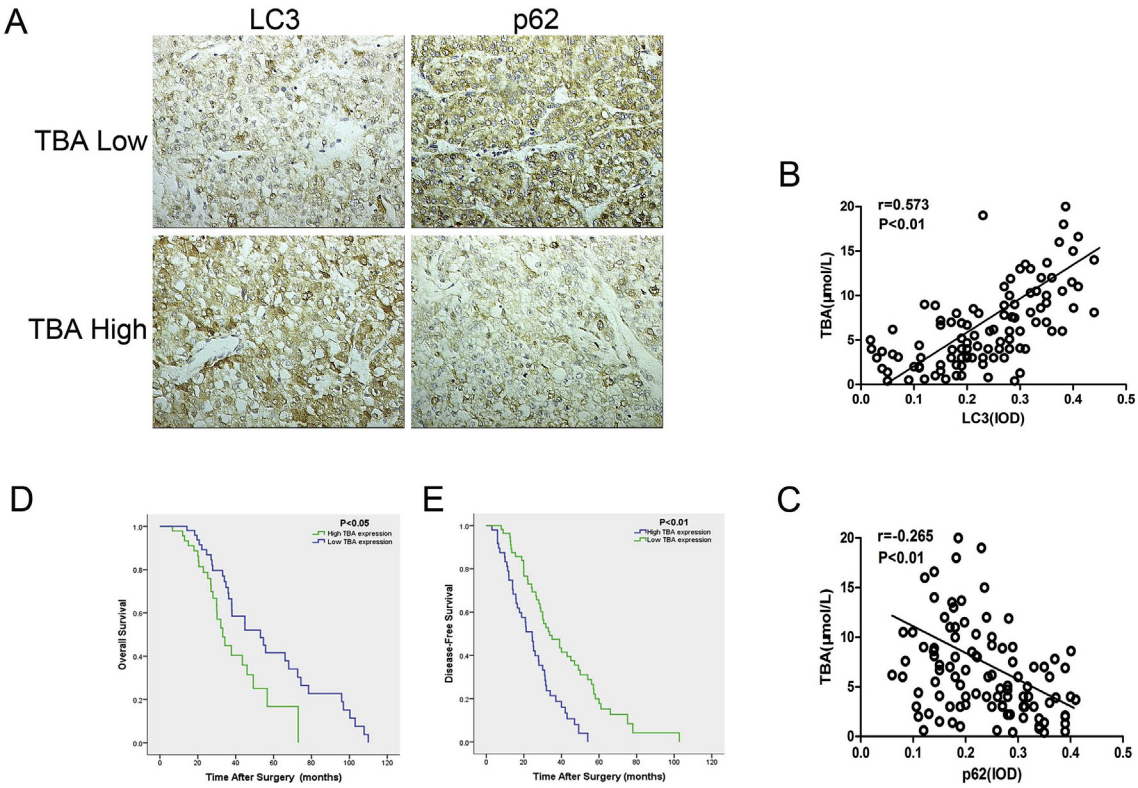

Fig. 5. TBA level in HCC patients is associated with autophagy marker and survival. (A) Representative IHC image of LC3 and p62 in HCC patients (× 200). (B) Pearson's correlation analysis provides a correlation coefficient (r) and *p*-value between TBA level and autophagy marker. TBA level is positively correlated with LC3 expression (r = 0.573, $p < 0.01$). (C) TBA level is negatively correlated with p62 expression (r = 0.265, $p < 0.01$). (D–E) Kaplan-Meier survival analysis of HCC patients with high or low TBA. Low TBA in HCC patients was associated with prolonged overall survival ($p < 0.05$). High TBA in HCC patients was associated with poor disease-free survival ($p < 0.01$). The *p*-value was calculated by the log-rank test.

patients.

4. Discussion

The present study demonstrateed autophagy as the mechanism of bile acid-mediated HCC invasion and metastasis. We found that HCC cell lines presented higher invasive capacity induced by GCDC. Additionally, we reported that GCDC could induce autophagy and inhibition of autophagy reversed the alterations in cell motility, decreased the invasion and metastasis of HCC *in vitro* and *in vivo*. Finally, we revealed that high levels of TBA in HCC patients were strongly correlated with autophagy level and poor survival. TBA levels were higher in some HCC patients who had a worse prognosis. Our data suggest that bile acid contribute to invasion and metastasis in HCC development and show that autophagy may be as the possible mechanism of bile acid-related metastasis in HCC.

In this study, we provided the evidence that bile acid promoted migration and invasion of HCC cells. Similar results were further confirmed in nude mice. Metabolic imbalance of BA may affect the liver regeneration and tumorigenesis [34–36]. Aged male and female FXR-null mice spontaneously developed liver cancer owing to increased total bile acid [16]. Based on the studies that bile acid are a risk factor on hepatocarcinogenesis, we hypothesize the auxo-action of bile acid on HCC progression. In our study, HCC cell lines were tested by the scratch wound-healing and matrigel invasion assay. After GCDC induction, the two HCC cell lines achieved significantly higher potential for both migration and invasion. Results of splenic vein metastasis assay *in vivo* were consistent with this tendency. Besides, reports showed that TBA are highly specific and moderately sensitive indicators of hepatobiliary diseases in human and animal models, particularly in patients with cholestatic liver diseases [31,37–39]. Therefore, we hypothesize that the bile acid in serum may affect the prognosis of HCC patients. In order to test this hypothesis, our clinical study enrolled 104 HCC patients. Statistical analysis found invasive characteristics of HCC tissues, including severe cirrhosis, TNM stage, microvascular invasion, macrovascular invasion, correlated with the TBA level. Additionally, TBA level had a correlation with the autophagy expression and was significantly associated with HCC vascular invasion and poor survival. Kaplan-Meier analysis showed that the HCC patients with high TBA level had a worse OS than those with low TBA level. In our study, we observed TBA level was 97.43 ± 22.0 μM in HCC patients. As reported, the level of TBA was 3.3 ± 2.1 μM in health person [30]. GCDC is one of most important component of bile acid which present in serum of cholestatic patients [40]. Therefore, GCDC can directly reflect the level of bile acid. As reported, the concentration of GCDC was 564.5 ± 69.8 ng/mL and 7785.7 ± 771.5 ng/mL in healthy person and HCC patients, respectively [30]; Another paper pointed out that the mean value of the most abundant bile acid in human serum, GCDC, was 745 ± 54.2 nM [41]. In general, the mean concentrations of GCDC in HCC people were higher than those reported from healthy people. These data are consistent with previous observation that HCC patients have higher total serum BA than patients with hepatitis, cirrhosis and healthy people [42], suggesting that increased bile acid could be a promoter of HCC development and a useful biomarker of HCC prognosis.

Autophagy has been extensively studied in recent years. Substantial clinical evidence indicates that autophagy can be upregulated in response to a multitude of stresses including starvation, hypoxia and pathogenic et al. [43]. Invasion and metastasis are common characteristics of cancer cells, autophagy accelerates invasion of cancer cells during starvation or hypoxia [44–46]. Hence, autophagy plays a pro-survival role in tumor development. In this study, we found that bile

acid induced autophagy, which was confirmed by evaluating the expression of autophagy markers and by electron microscopy. Therefore, we demonstrated that bile acid induced autophagy to promote migration and invasion of HCC cells. Meantime, we used autophagy inhibitor-chloroquine (CQ) to investigate the function of autophagy in bile acid treatment. Inhibition of autophagy displayed reduced the HCC cells invasion *in vitro* and showed fewer nodules in HCC *in vivo*. Thus, it is possible that autophagy is responsible for supporting the bile acid growth and progression and suppression of autophagy may be a promising strategy for HCC therapy with cholestasis. Autophagy is regulated by a complex network and most of which was regulated by AMPK/mTOR pathway [47]. The mTOR is the downstream target of AMPK. Activation of mTOR inhibit autophagy and pharmacological mTOR inhibitors induce autophagy in most models [48]. Our study showed that the GCDC increased the level of AMPK and suppressed mTOR level in HCC cells. Meanwhile, the treatment of AMPK inhibitor reversed this phenomenon. These results illustrates that the AMPK/mTOR plays an important role in activation of autophagy in HCC cells.

In summary, our findings show that GCDC leads to enhance invasive ability in HCC cells through activation of autophagy, which is mediated by AMPK/mTOR pathway. Autophagy plays a crucial role in the invasion of HCC cells while exposure to bile acid. We also confirm the role of high TBA level in HCC patients' prognosis. These results expand our understanding of cholestasis in metastasis of HCC and provide a new target for prevention of HCC metastasis.

Declaration of interests

The authors declare that they have no competing interests.

Acknowledgments

This project was supported by National Key R&D Program of China (Grant No. 2017YFA0504503). National Natural Science Foundation of China (Grant No. 81502543, 81502417, 81630070, 81572444, 31700788, 81702320, 81772940, 81673641). Special Funds for National Key Sci-Tech Sepcial Project of China (Grant No. 2018ZX10723204-005-004). Shanghai Science and Technology Committee (Grant No. 16YF1415000, 16ZR1400200, 16JC1405200); Science Fund for Creative Research Groups, NSFC, China (Grant NO. 81521091).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.04.009.

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