Suppressing ERK Pathway Impairs Glycochenodeoxycholate-Mediated Survival and Drug-Resistance in Hepatocellular Carcinoma Cells

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Abstract

Background This research aims to examine the mechanism of glycochenodeoxycholate (GCDA)-mediated survival and drug-resistance in hepatocellular carcinoma cells (HCC). Extracellular signal-regulated kinase 1/2 (ERK1/2) were extensively expressed in liver cancer cells.

Methods GCDA-induced survival of human liver carcinoma cells and chemoresistance was determined by CCK8 and flow cytometry, respectively. Functional of ERK1/2 and interaction of Bcl-2 family members was determined in western blot and immunofluorescence.

Results Silencing ERK1/2 by RNA interference suppressed GCDA-stimulated survival and promoted apoptosis. Furthermore, phosphorylation of endogenous ERK1/2 could be potently stimulated by GCDA in combination with enhanced chemoresistance in QGY-7703 hepatocellular carcinoma cells. And such GCDA-mediated proliferation and chemoresistance could be impaired by PD98059, who acts as an inhibitor to block phosphorylation of ERK1/2. Mechanistically, PD98059 was able to potently suppress GCDA-stimulated nuclear aggregation of ERK1/2 and p-ERK1/2, up-regulation of pro-survival protein Mcl-1 and decrease of pro-apoptotic protein Bim.

Conclusions Our work verified the function of ERK1/2 in GCDA-induced chemoresistance in hepatocellular carcinoma cells. Disruption of ERK1/2 by blocking phosphorylation or nuclear translocation may put forward new methods for treating of GCDA-related proliferation and drug-resistance in liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is the most common liver cancer nowadays, and more than 700,000 cases are diagnosed every year(1). The pathogenesis of HCC is extremely complex, but evolving information suggests that the major risk factors for HCC in contemporary clinical practice include alcoholic, nonalcoholic fatty liver disease (NFLD), hepatitis B virus (HBV) and hepatitis C virus (HCV)(2, 3). Most patients with advanced liver cancer will choose chemotherapy. However, patients with HCC usually develop resistance to 5-fluorouracil, doxorubicin or cisplatin, which are the traditional chemotherapeutics. Unfortunately, sorafenib, the new generation of drugs, did not achieve the desired results (4). Thus, it is very important to explore the resistance mechanism of HCC.

Bile salts are the major ingredients in bile, which are secreted by liver cells and involved in fat digestion and absorption. Glycochenodeoxycholate (glycine conjugate of chenodeoxycholate, GCDA), a toxic component in bile salts, is involved in carcinogenesis of gastrointestinal tumours(5). Previous researches have indicated that GCDA could stimulate the growth of Barrett's adenocarcinoma cells and non-neoplastic Barrett cell lines through PI3 kinase/Akt pathway and p38/ERK/MAPK pathway respectively (6, 7). Satoshi et al. (8) found that glycochenodeoxycholate acid could promote the proliferation of intestinal epithelia via decreasing cyclic AMP and increasing histone H2AX phosphorylation after exposure to γ-rays. Another study demonstrated that the biliary tract cancer could be induced by GCDA via aggregation

of 8-OHdG and oxidative DNA damage (9). The metabolic disorder of bile salt could lead to abnormal bile salt accumulation and be a direct factor in the development of HCC. A study by Wang et al. (10) found that GCDA might up-regulate pro-survival proteins (Mcl-1, Survivin and Bcl-2) and eventually result in chemoresistance of HCC cells. However, the specific intracellular mechanism of GCDA-mediated hepatocellular carcinoma development remains to be further studied.

As a member of the mitogen activated protein kinase family, the extracellular signal-regulated kinase (ERK) takes a key part in transmission signals from receptors on the cell surface into the nucleus (11). Signals transmitted from MEK1/2 can phosphorylate ERK1/2 at Thr and Tyr residues (12). Then the activated ERK1/2 will phosphorylate downstream substrates and eventually cause cell proliferation, differentiation and canceration(13). Usually, ERK1/2 are mainly distributed in the cytoplasm of normal cells. Upon stimulation, many ERK1/2 molecules shift to the nucleus, Golgi, mitochondria, endosomes/lysosomes and endoplasmic reticulum(14). The main translocation seems to be the entry into the nucleus, which is an important place for signal transmission downstream of ERK(13). Because the nuclear translocation of ERK is mainly important for cell proliferation, prevention of such translocation can be used as a novel strategy to combat cancer (15). Furthermore, ERK1/2 signalling is an important regulator of cell-intrinsic Bcl-2-regulated apoptotic signalling (16). In most situations, ERK1/2 signalling accelerates cell growth via stimulating anti-apoptosis proteins (Bcl-2, Mcl-1 and Bcl-xL) and inhibiting pro-apoptotic proteins (Bim, Bad, Bmf and Puma) (14). Thus, suppression of ERK1/2 pathway in tumor cells might serve as an effective way to prevent cancer development.

The chemoresistance of ERK1/2 has been extensively studied in other cancers. In radioresistant glioblastoma multiforme cells, cell survival could be promoted through ERK1/2 signalling when pSTAT3(Y705) was inhibited (17). ERK1/2 and p38 MAPK signalling pathway were significantly involved in neoplastic transformation and cisplatin-resistance in nasopharyngeal carcinoma cell lines (18). However, there was few in-depth research for the chemoresistance of ERK1/2 in HCC. A published study has shown that the activation of ERK1/2 could decrease the sensitivity to sorafenib in the HCC cells (Bel-7402 and SMMC-7721) (19). Our previous researches have confirmed the association of GCDA with drug resistance in HCC cells (10, 20). But the exact function of ERK1/2 in such process has not been clarified. In this research, we have proved that GCDA mediates activation and nuclear accumulation of ERK1/2, which finally results in promoting anti-apoptotic function in human liver cancer cells.

Materials And Methods

Cell culture. HepG2, Bel-7402, Bel-7404, QGY-7703 and SMMC-7721 HCC cell lines were originally from the Institute of Biochemistry and Cell Biology (CAS, Shanghai, China). Bel-7402 cell line was maintained in RPMI-1640 medium (Thermo Fisher Scientific, USA) with 10% fetal bovine serum (ExCell Bio, USA). HepG2, Bel-7404, QGY-7703 andSMMC-7721 cell lines were cultivated in Dulbecco's modified Eagle's medium (Hyclone, USA) supplemented 10% FBS. Cell lines were incubated at 37℃ with 5% CO_2.

Reagents and antibodies. The antibodies of ERK1 + ERK2 and ERK1 (pT202/pY204) + ERK2 (pT185/pT187) were obtained from Abcam (Cambridge, UK). Goat-anti Rabbit HRP antibody and anti-GAPDH antibody were from Cell Signalling Technology (Danvers, MA, USA). PD98059, a specific inhibitor of ERK kinase, was from Calbiochem (San Diego, CA, USA). Glycochenodeoxycholate (GCDA) was obtained from Sigma-Aldrich (St. Louis, USA). 5-Fluorouracil (5-FU) and Irinotecan were purchased from Xudong Haipu Pharmaceutical (Shanghai, China). The Annexin V-FITC apoptosis detection kit was purchased from Becton, Dickinson and Company (BD, Franklin Lake, NJ).

siRNA and transfections. For RNA interference, siRNA 225 (ACACGCAGUUGCAGUACAU), 888 (GACCGGAUGUUAACCUUUA) and 933 (GAAACUACCUACAGUCUCU) targeting human ERK1, siRNA 355 (GUGCUCUGCUUAUGAUAAU), 513 (CACCAACCAUCGAGCAAAU) and 714 (CCACCUGUGAUCUCAAGAU) targeting human ERK2 and Negative control siRNA (UUCUCCGAACGUGUCACGU) were from Shanghai Gene Pharma, Co., Ltd (Shanghai, China). QGY-7703 cells were transfected with siRNAs for 24h using Lipofectamine RNAi max (Invitrogen, NY, USA).

CCK8 assay. QGY-7703 cells were seeded in 96 well plates. Then GCDA, drugs or inhibitors were used to treat cells. After various treatments, each well was supplemented with 10μl of CCK8 solution and incubated for 1.5 h. After that, the absorbance was determined by microplate microscopy at 450 nm (BioTek, Winooski, VT).

Western blot analysis. The samples of QGY-7703 cells were lysed with detergent buffer for 30 minutes on ice. Then cell products were scraped from the wells and centrifuged for 15 min at 12,000 rpm. 30μg protein was loaded onto 10% SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with blocking solution for 2 h at room temperature and then incubated at 4°C overnight with primary antibodies. Following washing with 1×TBST andincubating with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5000) with shaking for 1h. Results were detected using WesternBright™ ECL (Advansta,USA) and the bands were scanned and quantified using the FluorChem FC3 system.

Flow cytometry. QGY-7703 cells were transfected with siRNA888 and siRNA513 together for 24 hours. Following treating with 100μM GCDA, cells were collected and washed with cold PBS. After resuspending with 1×binding buffer.3μL Annexin V-FITC and propidium iodide (PI) (Becton, Dickinson and Company, NJ) were used to treat for 15min. The apoptotic rate was detected by flow cytometry.

Immunofluorescence. In 24-well plates, QGY-7703 cells were cultured with a glass coverslip overnight. After cells were exposed in GCDA or GCDA + PD98059 for 8h, 4% paraformaldehyde was used to fix cells for 15 minutes. Then cells were washed with TBST and performed using ERK1/2 or p-ERK1/2 antibody at 4°C overnight. After incubating with Alexa Fluor®594 goat antibody at 37°C for 1 h. Cell nuclei were stained with DAPI for 2min. At last, the resulting was photographed by fluorescence microscopy.

Statistical analysis. SPSS software V17.0 was used to perform the statistical analysis. All data were displayed as the means ± SD deviation. Inter-group differences were assessed by Student's t-test. $P < 0.05$

was the considered level of statistical significance.

Results

ERK1/2 act a part in GCDA-induced survival of human liver carcinoma cells.

The ERK1/2 cascade is best known for its role in proliferation, differentiation and tumorigenesis (13). Firstly, we measured the endogenous protein levels of ERK1/2 in five HCC cell lines (HepG2, Bel-7402, Bel-7404, QGY-7703 and SMMC-7721). The result of western blot showed that ERK1/2 were extensively expressed in all the liver cancer cells we detected (**Figure.1A**). Next, to test whether GCDA promoted HCC cell proliferation, we treated QGY-7703 cell line with 100μM GCDA for 0h, 24h, 48h, and 72h, and then checked the viable cells by CCK8. Results indicated that viable cells significantly increased after treatment with GCDA until 72h (Fig. 1B).

To determine whether ERK1/2 affected the GCDA-induced survival of HCC cells, we designed siRNAs targeting ERK1 (225, 888 and 933) and ERK2 (355, 513 and 714). All the siRNAs were transfected into QGY-7703 cells. Then immunoblot was done to determine the interference efficiency. As shown in Fig. 1C, ERK1 and ERK2 proteins expression were inhibited by siRNA888 and siRNA513 mostly, respectively. After siRNA888 targeting ERK1 and siRNA513 targeting ERK2 were transfected into QGY-7703 cell line together, GCDA was used to treat cells for 24h. Apoptotic cells were analyzed using annexin V binding on FASC. Flow cytometry results demonstrated that GCDA could repress apoptosis. But after ERK1/2 were silenced, the apoptotic cells were increased (Fig. 1D**&E**). In other words, specific depletion of ERK1/2 blocked GCDA-stimulated cell survival. These results indicated that ERK1 and ERK2 molecules might have functions in survival of hepatoma cells mediated by GCDA.

GCDA induces ERK1/2 phosphorylation, which may be involved in prolonged survival of human liver cancer cells.

Next, we investigated potential mechanisms involved in the GCDA-induced HCC cell survival. QGY-7703 cells were treated with 100μM GCDA for 0h, 0.5h, 1h, 2h, 4h, 8h, 12h and 24h. Results demonstrated that the activated ERK1/2 increased obviously after GCDA treatment in QGY-7703 cells, while the expression of endogenous ERK1/2 changed little (Fig. 2A).

Irinotecan has been known as a topoisomerase I inhibitor, which was effective in a variety of cancers (21). The antimetabolite 5-fluorouracil (5-FU), which could inhibit thymidylate synthase, was a widely used antitumor agent (22). In order to check the effect of GCDA-induced ERK1/2 activation on cell survival, QGY-7703 cells were treated with antitumor drug (10μM Irinotecan or 5μg/mL 5-FU) or GCDA (100μM) + antitumor drug (10μM Irinotecan or 5μg/mL 5-FU) for 72h. We observed that Irinotecan or 5-FU induced > 80% of cells undergoing death and GCDA significantly prolonged cell survival following treatment with Irinotecan or 5-FU (Fig. 2B). Therefore, we speculated that phosphorylation of ERK1/2 might participate in chemoresistance induced by GCDA.

The MAPK/ERK1/2 inhibitor PD98059 decreases GCDA-stimulated cell proliferation.

To further verify the role of activated ERK1/2 in HCC cells, the MAPK/ERK1/2 inhibitor PD98059, which could inhibit phosphorylation of ERK1/2, was used (23). We treated QGY-7703 cells with GCDA (100μM) or GCDA (100μM) + PD98059 (10μM) for 24h, 48h and 72h. Then, CCK8 was done to test the viability of QGY-7703 cells. CCK8 experiments showed that suppression of ERK1/2 activation by PD98059 would decrease proliferation of liver cancer cells (Fig. 3A). Next, QGY-7703 cells were treated with or without PD98059 (10μM) for 0.5 h, followed by treatment with GCDA (100μM) or GCDA (100μM) + antitumor drug (10μM Irinotecan or 5μg/mL 5-FU) for 72h. Results of CCK8 showed that PD98059 significantly attenuated the chemoresistance induced by GCDA, which could prolong cell survival following treatment with Irinotecan or 5-FU (Fig. 3B). In conclusion, these findings implied that phosphorylation (or activation) of ERK1/2, which could be attenuated by PD98059, might be important for GCDA-mediated HCC cells' survival and chemoresistance.

PD98059 suppresses GCDA-induced nuclear aggregation of ERK1/2 and p-ERK1/2.

In unstimulated cells, ERK1/2 molecules are usually located in the cytoplasm (15). Under stimulation, lots of ERK1/2 molecules were translocated to the nucleus (15). ERK1/2 localization plays a significant role in determining the strength of this pathway. Therefore, we examined the localization of ERK1/2 and p-ERK1/2 following GCDA (100μM) or GCDA (100μM) + PD98059 (10μM) treatment. The results of immunofluorescence staining showed that ERK1/2 proteins were distributed in both cytoplasm and nucleus and more p-ERK1/2 proteins accumulated in the nuclear as small spots in resting HCC cells (Fig. 4A**&B**). Following GCDA treatment, most ERK1/2 proteins gathered in the nucleus, while more p-ERK1/2 proteins accumulated in the nucleus as bigger speckles. However, after PD98059 treatment, the aggregation of ERK1/2 and p-ERK1/2 proteins in nucleus significantly decreased (Fig. 4A**&B**). Collectively, the above data suggested that nuclear accumulation of ERK1/2 and p-ERK1/2 induced by GCDA could be impaired by PD98059.

PD98059 restrains GCDA-induced increase of Mcl-1 and decrease of Bim.

ERK1/2 signalling has been verified to regulate some members of Bcl-2 family, which can contribute to tumour cell survival via increasing anti-apoptotic factors and decreasing pro-apoptotic members of Bcl-2 family(16). Hence, we inspected the level of some Bcl-2 family members following GCDA (which can activate ERK1/2 pathway) or PD98059 (which can repress ERK1/2 pathway) treatment. Firstly, 100μM GCDA was used to treat QGY-7703 cells for 0h, 0.5h, 1h, 2h, 4h and 8h. Immunoblot had been done to check the levels of Bcl-2, Mcl-1, Bim and Bak. We observed that GCDA could promote expression of Bcl-2 and Mcl-1, both of which are anti-apoptotic Bcl-2 family members, and decrease expression of Bim and Bak, both of which are pro-apoptotic Bcl-2 family members (Fig. 5A). Next, in order to determine if suppression of ERK1/2 signalling regulated Bcl-2 family members' expression, GCDA (100μM) or GCDA (100μM) + PD98059 (10μM) were used to treat QGY-7703 cells for 8h. Results showed that inhibition of ERK1/2 by PD98059 could block GCDA-induced increase of Mcl-1 and decrease of Bim. However, Bcl-2 and Bak did not change significantly (Fig. 5B). Our data supported the notion that GCDA might facilitate

cell survival via regulation proteins of Bcl-2 family, some of which could be inhibited by PD98059. Such results indicated that activation of ERK1/2 pathway induced by GCDA could mediate certain members of Bcl-2 family.

Discussion

Glycochenodeoxycholate is the toxic bile salts, and may promote HCC invasion via activation of autophagy (24, 25). In the current study, survival and chemoresistance to Irinotecan and 5-FU induced by GCDA had been verified in QGY-7703 cell line (Fig. 1B and 3A).

The ERK1/2 signalling pathway was considered to have great effects on proliferation, invasion and migration in cancer cells. Numerous researches have confirmed that ERK1/2 signalling might be a main regulator to promote the progression of human hepatocellular carcinoma (26–30). The ERK1/2 participated in liver injury in human liver stem cells (31, 32). Also, the aggressive behavior of HCC cells have a positive relationship with the level of phosphorylated ERK and activated level of hepatic stellate cells (aHSCs) (33). Thus, we speculated whether GCDA mediated survival and chemoresistance via ERK1/2 pathway in liver cancer cells. Our results showed that activation levels of ERK1/2 increased significantly following GCDA treatment in hepatocellular carcinoma cells (Fig. 2A). After ERK1/2 were silenced by siRNA or phosphorylation of ERK1/2 was blocked by PD98059, the cell proliferation was significantly decreased (Fig. 1D**&E and 3A**). In the light of those results, it is reasonable to believe that ERK1/2 pathway is involved with GCDA-induced survival in HCC cells.

Because of binding to many scaffold proteins or cytoplasmic anchors in resting cells, ERK1/2 are usually localized in the cytoplasm (15). Upon stimulation, lots of the ERK1/2 molecules are translocated to the nucleus (14). In QGY-7703 cells, ERK1/2 and p-ERK1/2 could aggregate in the nucleus after treatment with GCDA (Fig. 4A**&B**). Therefore, nuclear aggregation of ERK1/2 molecules must be relevant to HCC cells proliferation signal transduction following GCDA treatment. However, such nuclear accumulation could be decreased by inhibitor PD98059 (Fig. 4A**&B**), which meant that the GCDA-induced survival signal might be impaired by PD98059. Based on the evidence in this study, preventing ERK1/2 from entering the nucleus may be considered as a novel strategy to arrest liver cancer growth.

Activated ERK1/2 are also translocated to mitochondria, Golgi, the endoplasmic reticulum or endosomes/lysosomes, resulting in influence on cell physiology(34). Among them, the mitochondrial anchored ERK1/2 molecules are involved with the mitochondrial apoptosis pathway via affecting Bcl-2 family members (16). Usually, ERK1/2 signalling facilitates cell survival via activating pro-survival proteins (Bcl-2, Mcl-1 and Bcl-xL) and inhibiting pro-apoptotic proteins (Bim, Bad, Bmf and Puma) (16). Among them, the transcription of pro-survival protein Bcl-2 can be promoted by ERK1/2 signalling through cAMP-responsive element-binding protein (CREB) (35). Besides, Bcl-2 itself can also be phosphorylated at Ser87 by ERK1/2, which is proposed to inhibit its pro-survival function (14). The mRNA level of Mcl-1 is verified to be promoted in response to ERK1/2 pathway via CREB or transcription factor ELK1 (36). Also, the short half-life of Mcl-1 protein can be prolonged via direct phosphorylation by

ERK1/2 (37). Bim, is a prominent target of ERK1/2 signalling(38). ERK1/2-induced activation of Bim leads it to ubiquitylation and degradation (39). Bak is the apoptotic effector protein of Bcl-2 family. Bak can be directly activated by Bim and cause the release of cytochrome c (40). In the present research, we observed that inhibiting ERK1/2 phosphorylation by PD98059 blocked GCDA-induced increase of Mcl-1 and decrease of Bim. However, Bcl-2 and Bak did not change significantly (Fig. 5A**&B**). These results showed that the GCDA-induced change of Mcl-1 and Bim might be regulated by ERK1/2 pathway, while the variation of Bcl-2 and Bak induced by GCDA maybe in an ERK-independent manner.

Conclusion

The present results found that GCDA-stimulated cell proliferation and chemoresistance could be attenuated via targeting ERK pathway. GCDA was able to potently promote phosphorylation and nuclear aggregation of ERK1/2 molecules, which eventually led to increased level of anti-apoptotic Bcl-2 family members proteins (Bcl-2 and Mcl-1) and decreased expression of pro-apoptotic Bcl-2 family members (Bim and Bak). The inhibitor PD98059 could not only suppress the phosphorylation of ERK1/2, but also block ERK1/2 molecules' nuclear accumulation and attenuate GCDA-stimulated increase of Mcl-1 and decrease of Bim. Therefore, disruption of the pro-survival function of GCDA by blocking phosphorylation and nuclear accumulation of ERK1/2 molecules might represent tactics for treating GCDA-related liver cancer and chemoresistance.

Abbreviations

HCC: hepatocellular carcinoma cells; ERK1/2: Extracellular signal-regulated kinase 1/2; NFLD: nonalcoholic fatty liver disease; HBV: hepatitis B virus; HCV: hepatitis C virus; GCDA: glycochenodeoxycholate; PVDF: polyvinylidene difluoride; PI: propidium iodide.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors have read and approved this version of the article. Neither the entire paper nor any part of its content has been published or has been accepted elsewhere. It is not being submitted to any other journal.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Manyi Yang contributed to conceive and designed the experiments; Bingxin Li performed the data analyses and wrote the manuscript; Maojun Zhou contributed significantly to analysis and manuscript preparation; Jue Wang and Hongjuan Xu helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

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References

1. Bruix J, Gores GJ, Mazzaferro V. Hepatocellular carcinoma: clinical frontiers and perspectives. Gut. 2014;63(5):844–55.
2. Ioannou GN, Feld JJ. What Are the Benefits of a Sustained Virologic Response to Direct-Acting Antiviral Therapy for Hepatitis C Virus Infection? Gastroenterology. 2019;156(2):446–60. e2.
3. Kulik L, El-Serag HB. Epidemiology and Management of Hepatocellular Carcinoma. Gastroenterology. 2019;156(2):477. :– 91.e1..
4. Dhanasekaran R, Nault JC, Roberts LR, Zucman-Rossi J. Genomic Medicine and Implications for Hepatocellular Carcinoma Prevention and Therapy. Gastroenterology. 2019;156(2):492–509.
5. Zhou M, Zhang Q, Zhao J, Liao M, Wen S, Yang M. Phosphorylation of Bcl-2 plays an important role in glycochenodeoxycholate-induced survival and chemoresistance in HCC. Oncol Rep. 2017;38(3):1742–50.
6. Jaiswal K, Lopez-Guzman C, Souza RF, Spechler SJ, Sarosi GA. Jr. Bile salt exposure increases proliferation through p38 and ERK MAPK pathways in a non-neoplastic Barrett's cell line. Am J Physiol Gastrointest Liver Physiol. 2006;290(2):G335-42.
7. Jaiswal K, Tello V, Lopez-Guzman C, Nwariaku F, Anthony T, Sarosi GA. Jr. Bile salt exposure causes phosphatidyl-inositol-3-kinase-mediated proliferation in a Barrett's adenocarcinoma cell line. Surgery. 2004;136(2):160–8.

8. S I MS. M H, T S, T H, H H. - Glycochenodeoxycholic acid promotes proliferation of intestinal epithelia via. D – 8100317. 2012(– 1880-313X (Electronic)):- 159 – 65.
9. Komichi D, Tazuma S, Nishioka T, Hyogo H, Chayama K. Glycochenodeoxycholate plays a carcinogenic role in immortalized mouse cholangiocytes via oxidative DNA damage. Free Radic Biol Med. 2005;39(11):1418–27.
10. Wang C, Yang M, Zhao J, Li X, Xiao X, Zhang Y, et al. Bile salt (glycochenodeoxycholate acid) induces cell survival and chemoresistance in hepatocellular carcinoma. J Cell Physiol. 2019;234(7):10899–906.
11. Sun MH, Chen XC, Han M, Yang YN, Gao XM, Ma X, et al. Cardioprotective effects of constitutively active MEK1 against H2O2-induced apoptosis and autophagy in cardiomyocytes via the ERK1/2 signaling pathway. Biochem Biophys Res Commun. 2019;512(1):125–30.
12. BJ C, MH AK. C, EJ G. - Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. D – 0413066. (– 0092-8674 (Print)):- 859 – 69.
13. Yao Z, Seger R. The ERK signaling cascade–views from different subcellular compartments. Biofactors. 2009;35(5):407–16.
14. Cook SJ, Stuart K, Gilley R, Sale MJ. Control of cell death and mitochondrial fission by ERK1/2 MAP kinase signalling. FEBS J. 2017;284(24):4177–95.
15. E W, R S. - The dynamic subcellular localization of ERK: mechanisms of translocation and role. D – 8913428. (– 1879 – 0410 (Electronic)):- 15–20.
16. MJ S, SJ C. - That which does not kill me makes me stronger; combining ERK1/2 pathway. D – 7502536. (– 1476–5381 (Electronic)):- 1708-22.
17. Xie B, Zhang L, Hu W, Fan M, Jiang N, Duan Y, et al. Dual blockage of STAT3 and ERK1/2 eliminates radioresistant GBM cells. Redox Biol. 2019;24:101189.
18. Hsieh MJ, Wang CW, Lin JT, Chuang YC, Hsi YT, Lo YS, et al. Celastrol, a plant-derived triterpene, induces cisplatin-resistance nasopharyngeal carcinoma cancer cell apoptosis though ERK1/2 and p38 MAPK signaling pathway. Phytomedicine. 2019;58:152805.
19. Zhong J, Yu X, Dong X, Lu H, Zhou W, Li L, et al. Downregulation of secreted clusterin potentiates the lethality of sorafenib in hepatocellular carcinoma in association with the inhibition of ERK1/2 signals. Int J Mol Med. 2018;41(5):2893–900.
20. Liao M, Zhao J, Wang T, Duan J, Zhang Y, Deng X. Role of bile salt in regulating Mcl-1 phosphorylation and chemoresistance in hepatocellular carcinoma cells. Mol Cancer. 2011;10:44.
21. K AM, C AGH, Y M. M, T S, et al. - Irinotecan monotherapy as third-line or later treatment in advanced gastric. D – 100886238. 2018(– 1436–3305 (Electronic)):- 464 – 72.
22. Marin JJG, Cives-Losada C, Asensio M, Lozano E, Briz O, Macias RIR. Mechanisms of Anticancer Drug Resistance in Hepatoblastoma. Cancers (Basel). 2019;11(3).
23. Chen LC, Shibu MA, Liu CJ, Han CK, Ju DT, Chen PY, et al. ERK1/2 mediates the lipopolysaccharide-induced upregulation of FGF-2, uPA, MMP-2, MMP-9 and cellular migration in cardiac fibroblasts.

Chem Biol Interact. 2019;306:62–9.

24. Wang K, Brems JJ, Gamelli RL, Ding J. Reversibility of caspase activation and its role during glycochenodeoxycholate-induced hepatocyte apoptosis. J Biol Chem. 2005;280(25):23490–5.
25. Gao L, Lv G, Li R, Liu WT, Zong C, Ye F, et al. Glycochenodeoxycholate promotes hepatocellular carcinoma invasion and migration by AMPK/mTOR dependent autophagy activation. Cancer Lett. 2019;454:215–23.
26. Odagiri N, Matsubara T, Higuchi M, Takada S, Urushima H, Sato-Matsubara M, et al. Involvement of ERK1/2 activation in the gene expression of senescence-associated secretory factors in human hepatic stellate cells. Mol Cell Biochem. 2019;455(1–2):7–19.
27. Li H, Zhang M, Linghu E, Zhou F, Herman JG, Hu L, et al. Epigenetic silencing of TMEM176A activates ERK signaling in human hepatocellular carcinoma. Clin Epigenetics. 2018;10(1):137.
28. Hou H, Ge C, Sun H, Li H, Li J, Tian H. Tunicamycin inhibits cell proliferation and migration in hepatocellular carcinoma through suppression of CD44s and the ERK1/2 pathway. Cancer Sci. 2018;109(4):1088–100.
29. Chen P, Lei L, Wang J, Zou X, Zhang D, Deng L, et al. Downregulation of Talin1 promotes hepatocellular carcinoma progression through activation of the ERK1/2 pathway. Cancer Sci. 2017;108(6):1157–68.
30. Cong N, Li Z, Shao W, Li J, Yu S. Activation of ETA Receptor by Endothelin-1 Induces Hepatocellular Carcinoma Cell Migration and Invasion via ERK1/2 and AKT Signaling Pathways. J Membr Biol. 2016;249(1–2):119–28.
31. Wei Y, Zhang X, Wen S, Huang S, Huang Q, Lu S, et al. Methyl helicterate inhibits hepatic stellate cell activation through downregulating the ERK1/2 signaling pathway. J Cell Biochem. 2019.
32. Raska J, Ctverackova L, Dydowiczova A, Sovadinova I, Blaha L, Babica P. Cylindrospermopsin induces cellular stress and activation of ERK1/2 and p38 MAPK pathways in adult human liver stem cells. Chemosphere. 2019;227:43–52.
33. Xie YX, Liao R, Pan L, Du CY. ERK pathway activation contributes to the tumor-promoting effects of hepatic stellate cells in hepatocellular carcinoma. Immunol Lett. 2017;188:116–23.
34. - The IWRS, Cascade ERK. Distinct Functions within Various Subcellular Organelles. D – 101516546. (– 1947–6027 (Electronic)):- 195–209.
35. YL L, JS FL, XG W, XY Y. L, Q L, et al. - Noxa upregulation by oncogenic activation of MEK/ERK through CREB promotes. D – 101532965. (– 1949–2553 (Electronic)):- 11237–51.
36. AM D, MA JAV, SR G. H, RW C. - MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in. - Oncogene 2004 Jul 8;23(31):5301-15 doi: 101038/sjonc1207692. (– 0950–9232 (Print)):- 5301-15.
37. U M, C C, AS W. E D, DR G. - Glycogen synthase kinase-3 regulates mitochondrial outer membrane. D – 9802571. (– 1097–2765 (Print)):- 749 – 60.

38. JY Y, CS Z. W X, H Y, Q D, X X, et al. - ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation. D – 100890575. (– 1476–4679 (Electronic)):- 138 – 48.
39. R L, K B, K H, C W, SJ C. - Activation of the ERK1/2 signaling pathway promotes phosphorylation and. D – 2985121r. (– 0021-9258 (Print)):- 18811–6.
40. L B-H TK, C JEC, BA B, DR S. G, et al. - BH3 domains of BH3-only proteins differentially regulate Bax-mediated. - Mol Cell 2005 Feb 18;17(4):525 – 35 doi: 101016/jmolcel200502003. (– 1097–2765 (Print)):- 525 – 35.

Figures

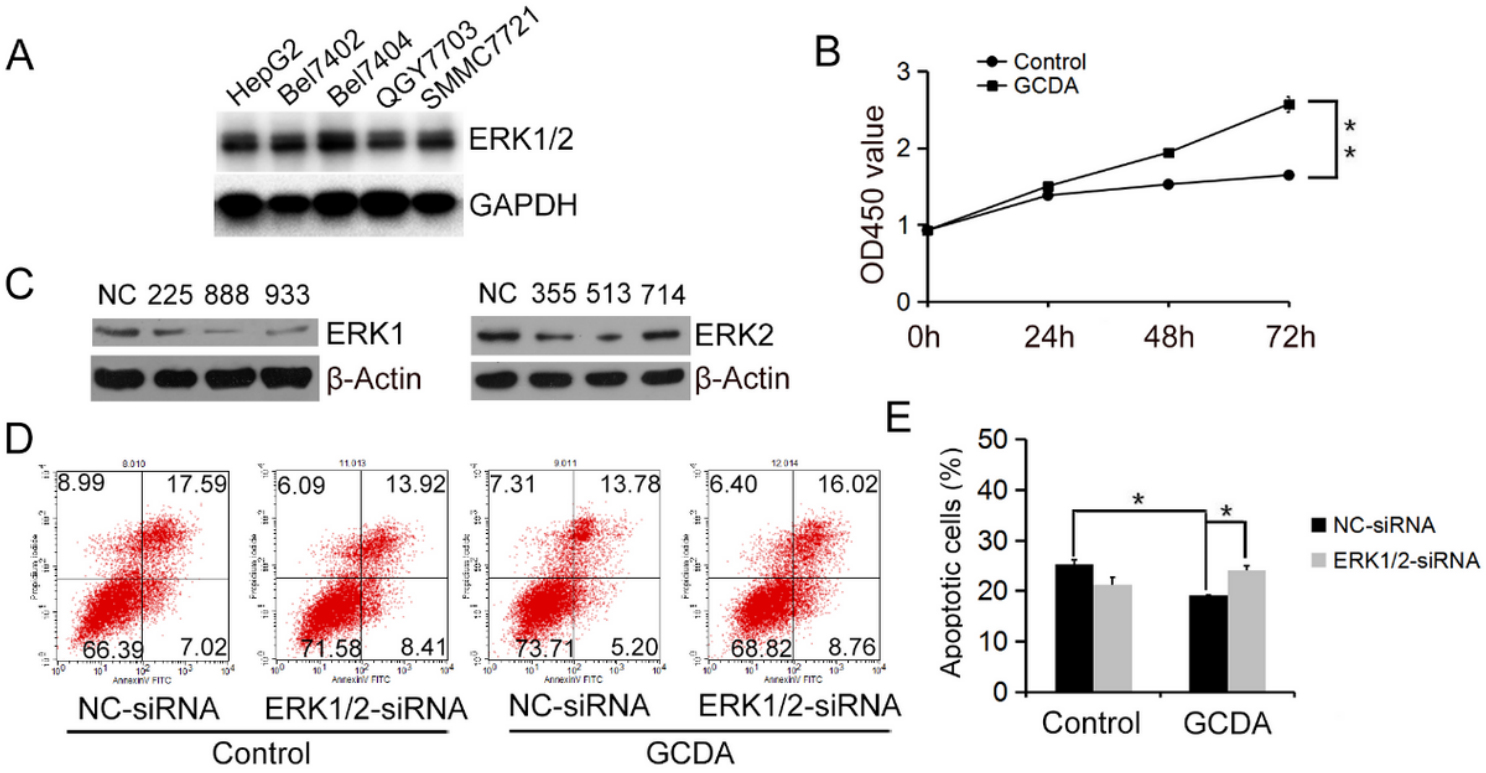

Figure 1

ERK1/2 act a part in GCDA-mediated survival of human liver carcinoma cells. (A) Expression of ERK1/2 in five liver carcinomas cell lines (HepG2, Bel7402, Bel7404, QGY7703 and SMMC7721) was detected by western blot. (B) GCDA (100μM) was used to treat QGY7703 cells for 0h, 24h, 48h and 72h. CCK8 was performed to determine the viable cells. (C) QGY7703 cells were transfected with siRNA targeting ERK1 (225, 888 and 933) and ERK2 (355, 513 and 714). After 24h, whole cell extracts were analyzed by western blot using ERK1 and ERK2 antibodies. NC, negative; control siRNA. (D&E) siRNA888 targeting ERK1 and siRNA513 targeting ERK2 were transfected into QGY7703 cells together. 24 hours later, 100μM GCDA was

used to treat cells for 24h. Apoptosis were determined using flow cytometry. All data represent the mean±SD and were obtained from at least three independent experiments. *P<0.05, **P<0.01 (Student's t-test)

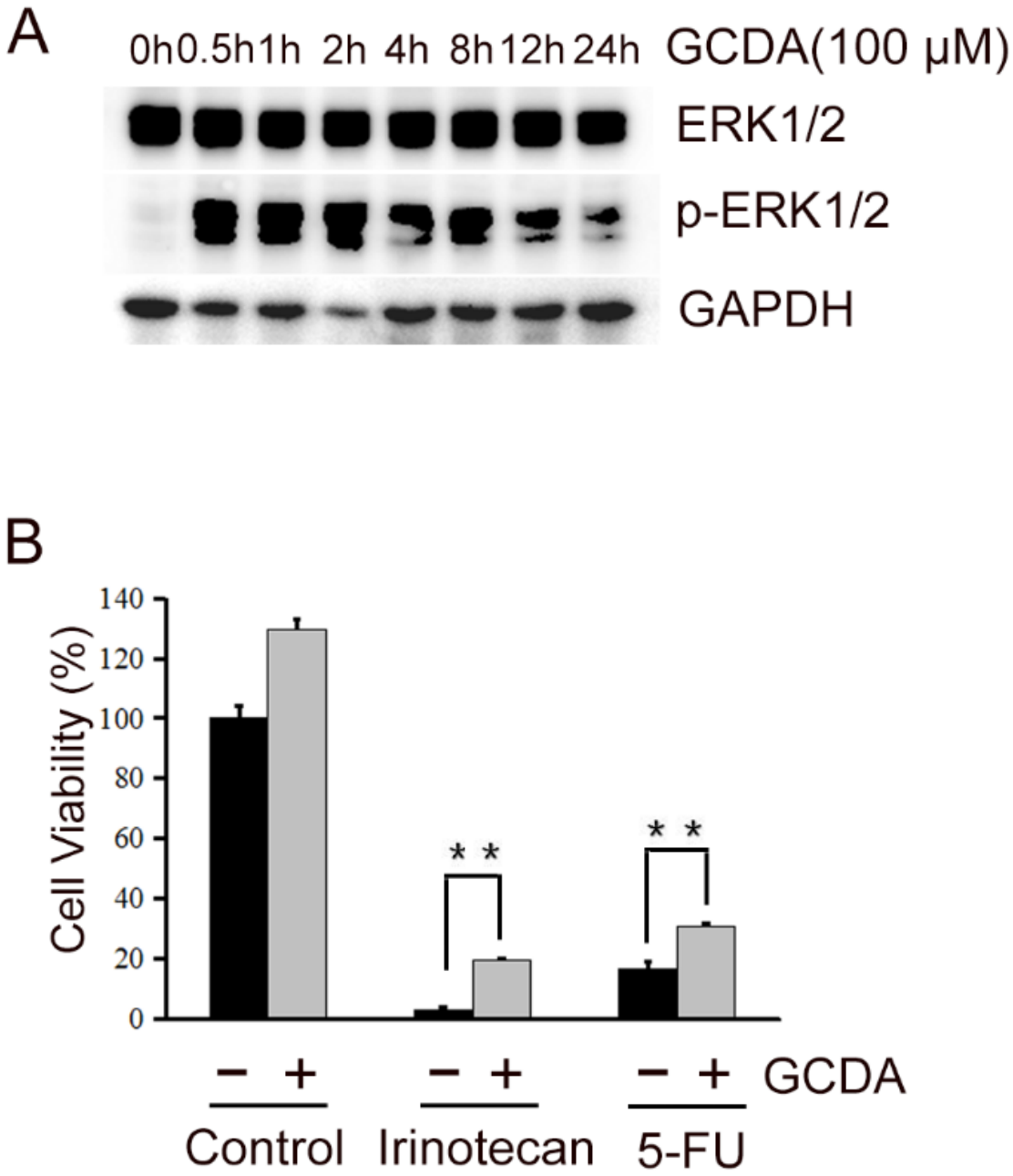

Figure 2

GCDA induces ERK1/2 phosphorylation, which may be involved in prolonged survival of human liver cancer cells. (A) 100μM GCDA was used to treat QGY7703 cells for 0h, 0.5h, 1h, 2h, 4h, 8h, 12h and 24h.

The expression level of ERK1/2 and p-ERK1/2 were tested by western blot. (B) Antitumor drug (10μM Irinotecan or 5μg/mL 5-FU) or GCDA (100μM) + antitumor drug (10μM Irinotecan or 5μg/mL 5-FU) were used to treat QGY-7703 cells for 72h. CCK8 was performed to determine the viable cells. All data represent the mean±SD and were obtained from at least three independent experiments. **P<0.01 (Student's t-test)

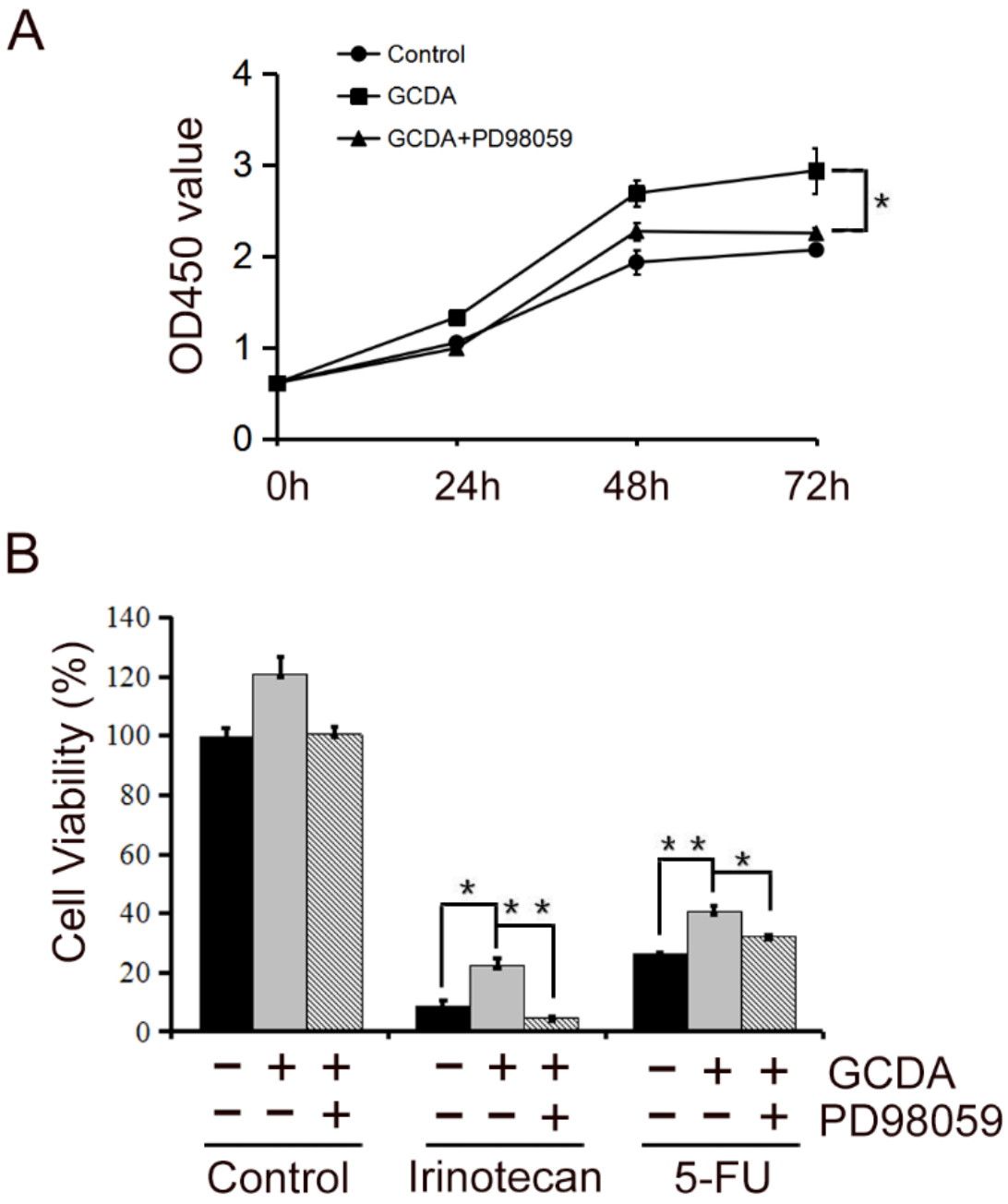

Figure 3

PD98059, the ERK1/2 inhibitor, attenuates GCDA-mediated survival and drug-resistance in HCC cells. (A) PD98059 could inhibit phosphorylation of ERK1/2. QGY-7703 cells were preincubated with PD98059 (10μM) for 0.5 h, followed by treatment with 100μM GCDA for 24h, 48h and 72h. CCK8 was performed to determine the viable cells. (B) QGY7703 cells were treated with or without PD98059 (10μM) for 0.5 h, followed by treatment with GCDA (100μM) or GCDA (100μM) + antitumor drug (10μM Irinotecan or 5μg/mL 5-FU) for 72h. Then CCK8 was performed to determine the viable cells. Data in graphs are as mean±SD. All experiments data were repeated at least three independent experiments. *P<0.05, **P<0.01 (Student's t-test)

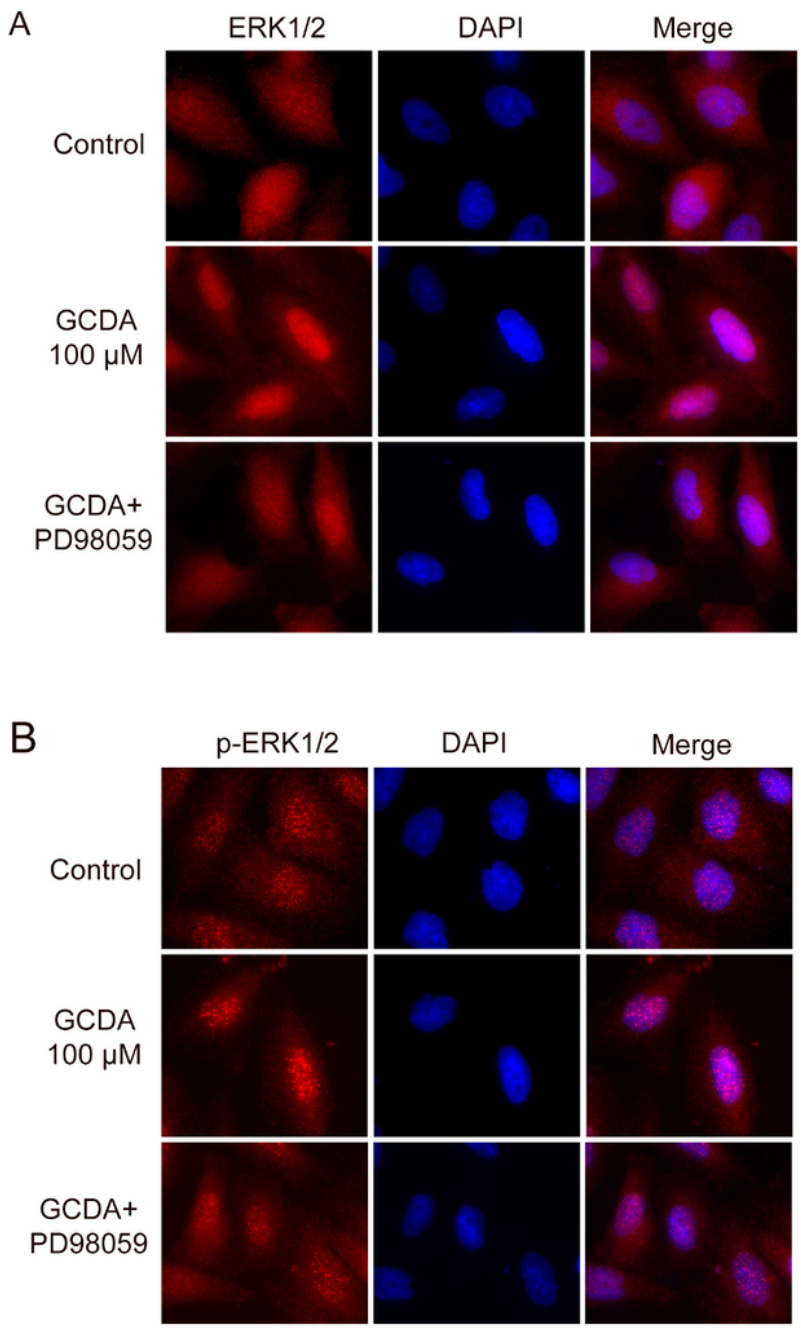

Figure 4

PD98059 suppresses GCDA-induced nuclear aggregation of ERK1/2 and p-ERK1/2. (A&B) QGY-7703 cells were preincubated with PD98059 (10μM) for 0.5 h, followed by treatment with 100μM GCDA for 8h. Immunofluorescence staining was done using ERK1/2 or p-ERK1/2 antibody. Cell nuclei were stained with DAPI for 2 min. The experiments were repeated three times.

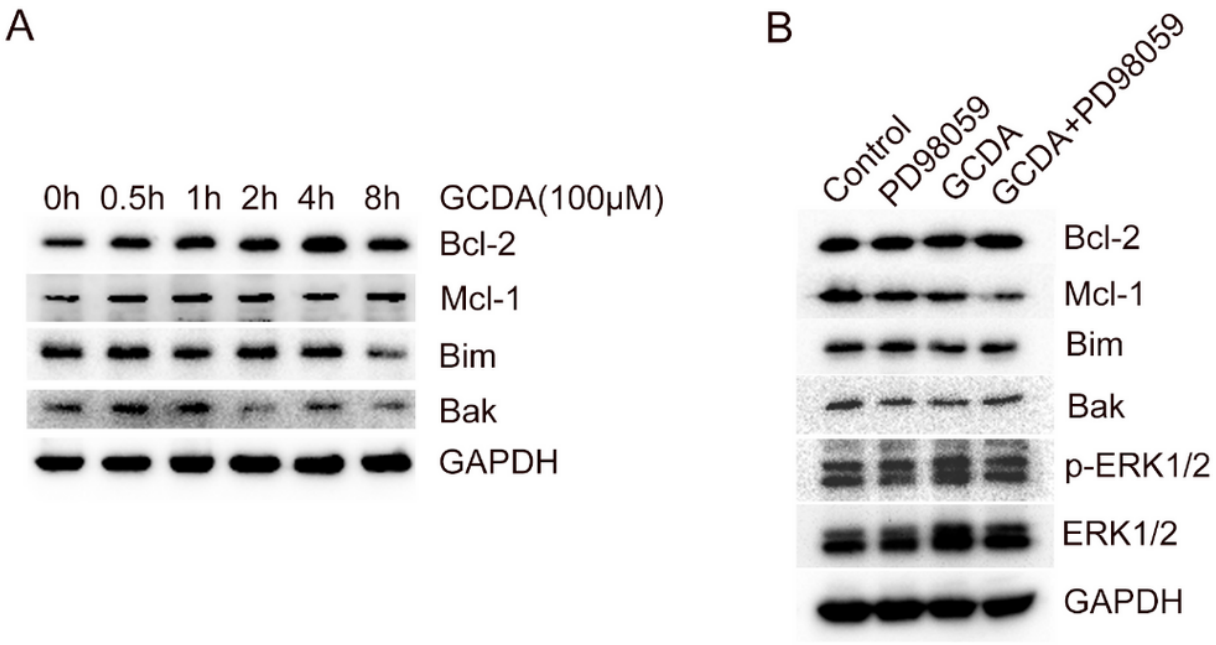

Figure 5

PD98059 suppresses GCDA-stimulated increase of Mcl-1 and decrease of Bim. (A) 100μM GCDA was used to treat QGY-7703 cells for 0h, 0.5h, 1h, 2h, 4h and 8h. Expression of Bcl-2, Mcl-1, Bim and Bak was tested by western blot. (B) QGY-7703 cells were preincubated with 10μM PD98059 for 0.5 h, followed by treatment with 100μM GCDA for 8h. All cell extracts were analyzed using Bcl-2, Mcl-1, Bim, Bak, p-ERK1/2 and ERK1/2 antibodies by Western blotting.