GSK-3β inhibition promotes doxorubicin-induced apoptosis in human cholangiocarcinoma cells via FAK/AKT inhibition

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Abstract. Cholangiocarcinoma (CCA) is the most common type of malignant tumor of the bile duct and is characterized by high morbidity and mortality; it is difficult to diagnose in the early stages and responds poorly to current conventional radiotherapy and chemotherapy. The present study investigated the role of GSK-3β signaling on the anticancer effects of doxorubicin in human CCA cells. Blocking GSK-3β enhanced the sensitivity of human CCA cells to doxorubicin (Dox)-induced apoptosis, which was accompanied by decreased AKT and focal adhesion kinase (FAK) activity. Moreover, inhibiting GSK-3β using 6-bromoindirubin-3'-oxime, CHIR99021 or small interfering RNA decreased phosphorylation of FAK and AKT, and promoted apoptosis of Dox-induced human CCA cells. Moreover, FAK inhibition suppressed AKT activity independently of phosphoinositide 3-kinase activity. These results indicated that GSK-3β protects human CCA cells against Dox-induced apoptosis via sustaining FAK/AKT activity.

Introduction

Cholangiocarcinoma (CCA) is a fatal tumor that arises from the biliary epithelium, which is characterized by late diagnosis and poor outcome (1-4). Over the past three decades the global incidence of CCA (1/100,000) has steadily increased, whereas the 5-year survival rate of diagnosed patients has remained ~10% (5-8). Resection is the best option for the curative treatment of patients with CCA. However, high rates of recurrence and short survival times are associated with resection in patients with CCA (9-13).

Chemotherapy is used to improve the outcomes of patients with CCA. Doxorubicin (Dox), which blocks DNA replication (14,15), is a first-line drug widely used to treat numerous types of tumors, including CCA. Nevertheless, objective response rates of Dox treatment in clinical trials are modest and the results vary (16,17). Thus, it is crucial to investigate whether combination approaches can enhance the antitumor activity of Dox.

GSK-3β is a serine/threonine kinase involved in numerous disease processes, including tumorigenesis (18). Previous studies have indicated that GSK-3β inhibition may be a potential therapeutic strategy for cancer treatment (18,19). However, whether GSK-3β inhibition affects the antitumor activity of Dox in patients with CCA remains unknown. The present study investigated the effect of GSK-3β inhibition on the antitumor activity of Dox in human CCA cells, and suggested that GSK-3β inhibition promotes Dox-induced human CCA cells apoptosis, in part by decreasing focal adhesion kinase (FAK)/protein kinase B (AKT) activity. The present study demonstrated that a combination of GSK-3β inhibitors and Dox may be an effective therapeutic strategy for patients with CCA.

Materials and methods

Chemicals and antibodies. Dox and inhibitors of GSK-3β [6-bromoindirubin-3'-oxime (BIO) and CHIR99021], mTOR (rapamycin and AZD8055), PI3K (LY294002, PI828 and Wortmannin), phosphoinositide-dependent protein kinase1 (PDK1; OSU-03012) and focal adhesion kinase 1 (FAK; PF-573228) were purchased from Selleck Chemicals. Human PDK1 small interfering (si)RNA, rapamycin-insensitive companion of mTOR (RICTOR) siRNA, antibodies against poly [ADP-ribose] polymerase, phosphorylated (p)-AKT (S473; cat. no. 9271), p-AKT (T308; cat. no. 13038), AKT (cat. no. 9272), p-ribosomal protein S6 (S6; cat. no. 4856), S6 (cat. no. 2217), GSK-3β (cat. no. 12456), p-PDK1 (cat. no. 3438), PDK1 (cat. no. 5662), p-70 kDa ribosomal protein S6 kinase (P70S6K; cat. no. 9234), P70S6K (cat. no. 2708), RICTOR (cat. no. 2114), p-FAK (T397; cat. no. 8556), p-FAK (T576/577;

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cat. no. 3281), FAK (cat. no. 3285), hemagglutinin (cat. no. 3724), cleaved caspase-3 (cat. no. 9661), caspase-3 (cat. no. 9662) and GAPDH (cat. no. 5174) were purchased from Cell Signaling Technology, Inc., p-protein kinase C (PKC; cat. no. ab180848), PKC (cat. no. ab32376) were purchased from Abcam, and p-GSK-3a/β (cat. no. sc81496) was purchased from Santa Cruz Biotechnology, Inc.

Cell culture and treatment. Human CCA cell lines QBC939 and RBE (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were grown in a humidified incubator at 37˚C and 5% CO_2 in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (cat. no. 04-001-1ACS; Biological Industries), 1% penicillin and 1% streptomycin. The human CCA cells were treated with the following drugs: Dox (2 μM), LY294002 (30 mM), PI828 (20 nM), BIO (5 μM), CHIR99021 (10 μM), PF-573228 (10 μM), OSU-03012 (10 μM), rapamycin (100 nM), AZD8055 (1 μM) and Wortmannin (2 μM), for the indicated time periods at 37˚C.

RNA interference. Cells were seeded into 6-well plates at a density of $1x10^6$ cells per well and transiently transfected with 100 nM siRNA duplexes using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Following addition of siRNA duplexes at 37˚C for 6 h, the cells were washed and recovered for 30 h. GSK-3β siRNA sequences were synthesized by ViewSolid Biotech, Inc., as follows: siGSK-3β #1, 5'-CUCAAG AACUGUCAAGUAATT-3'; siGSK-3β #2, 5'-CGAGAGCUC CAGAUCAUGATT-3'; siGSK-3β #3, 5'-GCUAGAUCACUG UAACAUATT-3'. A scrambled siRNA sequence (5'-UUCUCC GAACGUGUCACGTT-3') was used as an internal negative control.

Plasmid cell transfection. The constitutively activated AKT plasmid (myr-HA-AKT) was provided by Professor JinQuan Cheng (Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, USA), as described in previous studies (20,21). Cells were seeded into 6-well plates at a density of $1x10^6$ cells per well and transfected with 2.5 mg plasmid per well using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Following the addition of the plasmid at 37˚C for 6 h, the cells were washed and recovered for 30 h.

Western blotting. Cells were lysed in Triton lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology), and protein concentration was determined via the BCA method. Protein samples were denatured with 4X SDS-loading buffer at 100˚C for 8 min and an equal amount of protein (40 μg) was loaded and separated via SDS-PAGE on a 10% gel. After being transferred to a PVDF membrane, 5% non-fat milk was used to block non-specific sites at room temperature for 1 h. proteins were probed with specific antibodies (1:1,000) at room temperature for 2 h or 4˚C overnight. Then, the blots were incubated at room temperature for 1 h with the following secondary antibodies: IRDye 800CW Goat anti-Mouse (1:10,000; cat. no. 926-32210; LI-COR Biosciences) and IRDye 800CW Goat anti-Rabbit (1:10,000; cat. no. 926-32211; LI-COR Biosciences). Blots were then visualized in the Odyssey® CLx-1279 system (LI-COR Biosciences). ImageJ software (version 1.51; National Institutes of Health) was used to semi-quantify the western blotting results.

Lactate dehydrogenase (LDH) release assay. QBC939 and RBE cells were seeded into 96-well plates at a density of $4x10^4$ cells per well. LDH Release Assay kit (cat. no. C0017) was purchased from Beyotime Institute of Biotechnology. The ratio of cell death was detected according to the manufacturer's instructions.

Reverse transcription quantitative (RT-q)PCR analysis. Total RNA was isolated from transfected human CCA cells with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was generated using M-MLV reverse transcriptase (Promega Corporation) according to the manufacturer's protocol. RT-qPCR analysis was performed using SYBR Premix Ex Taq (Takara Bio, Inc.). PCR was performed as follows: 94˚C for 5 min, 35 cycles of 94˚C for 20 sec, 60˚C for 30 sec and 72˚C for 30 sec. RPL13A was used as an internal control for normalization. The primers sequences are presented in Table SI. The results were calculated by the $2^{-\Delta\Delta Cq}$ method (22).

PI3K activity. QBC939 and RBE cells were seeded into 96-well plates at a density of $4x10^4$ cells per well. The phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) concentration was detected by PI3-Kinase Activity ELISA:Pico kit (cat. no. K-1000S; Echelon Biosciences, Inc.), according to the manufacturer's instructions.

Statistical analysis. Data are presented as the mean ± SD of ≥3 independent repeats. SPSS software (version 17.0; SPSS, Inc.) was used for statistical analysis using ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

AKT involvement in Dox-treated human CCA cells. AKT signaling is reported to serve a key role in drug resistance in numerous types of cancer (23,24). In order to investigate the role of AKT, phosphorylation levels of AKT and S6 were detected in Dox-treated human CCA cells. Phosphorylation levels of AKT and S6, a classical downstream protein of AKT signaling, were decreased in Dox-treated human CCA cells (Fig. 1). These results indicated that AKT protected human CCA cells from Dox treatment.

AKT inhibition promotes Dox-induced apoptosis in human CCA cells. In order to investigate the role of AKT in Dox-treated human CCA cells, QBC939 and RBE cells were treated with Dox in the presence or absence of PI3K inhibitors LY294002 and PI828. The western blotting results demonstrated that LY294002 and PI828 pretreatment increased Dox-induced QBC939 and RBE cell apoptosis, as indicated by the increased expression of apoptosis-associated proteins (Fig. 2). The level of p-S6 was used as a marker of the effect of PI3K inhibitors. In addition, LDH analysis also indicated that AKT inhibition, via the inhibition of

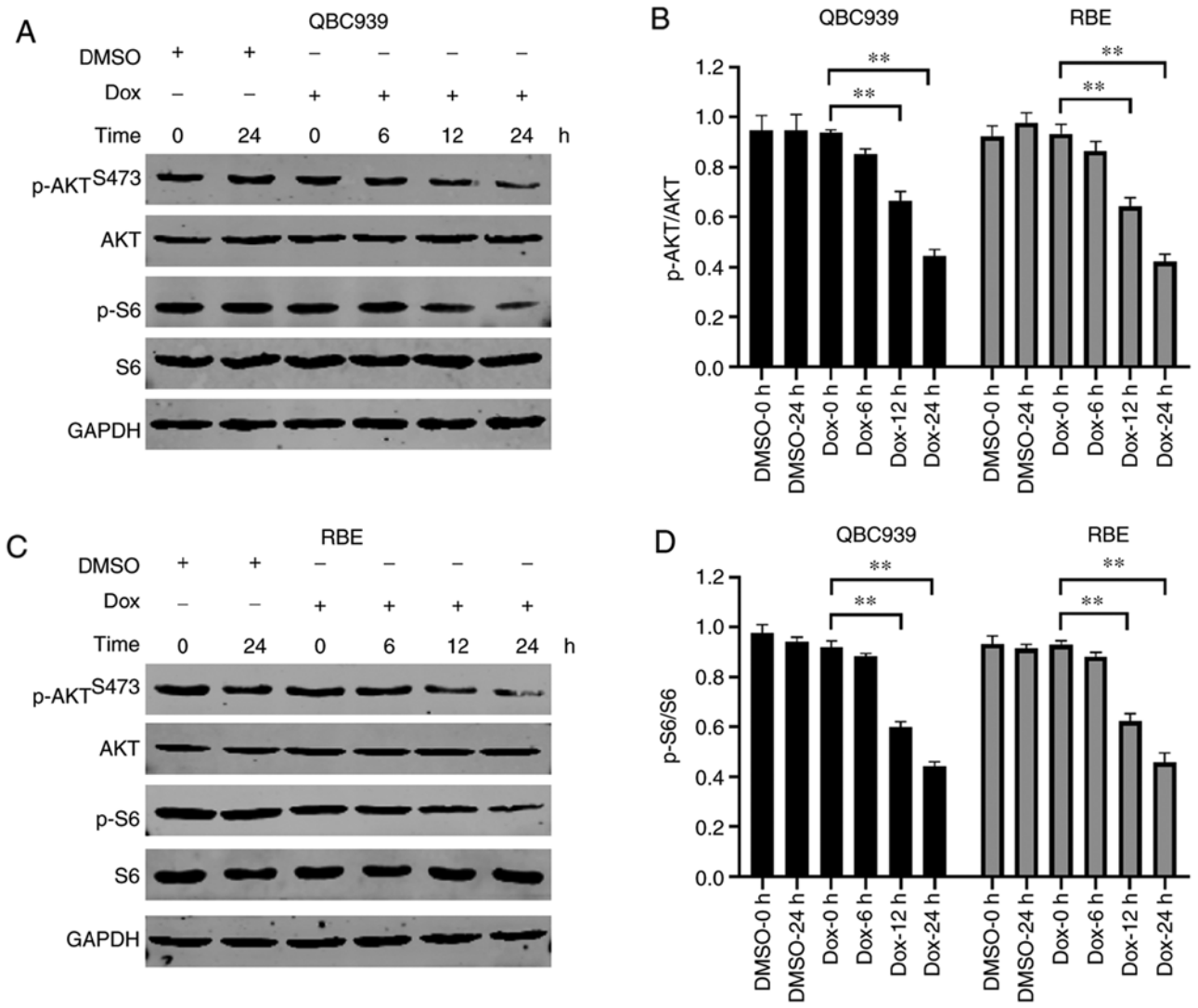

Figure 1. AKT involvement in Dox-treated human cholangiocarcinoma cells. (A) QBC939 and (C) RBE cells were treated with Dox (2 μM) for the indicated time periods, then cell lysates were subjected to western blotting. Ratios of (B) p-AKT/AKT and (D) p-S6/S6 were semi-quantified. **P<0.01. Dox, doxorubicin; p, phosphorylated.

AKT upstream kinase PI3K, promoted cell death following Dox treatment (Fig. S1). These results suggested that AKT protected human CCA cells against Dox-induced apoptosis.

GSK-3β inhibition promotes Dox-induced apoptosis in human CCA cells. Numerous studies have demonstrated that GSK-3β is a critical molecule involved in cell growth, proliferation and drug resistance (25,26). In order to determine the role of GSK-3β in Dox-induced CCA cell apoptosis, western blotting was used to detect the expression of p-GSK-3β. The level of p-GSK-3β was unchanged following Dox treatment (Fig. 3A). QBC939 and RBE cells were treated with Dox in the presence or absence of GSK-3β inhibitors BIO or CHIR99021. Immunoblotting results demonstrated that BIO pretreatment increased Dox-induced QBC939 and RBE cell apoptosis (Fig. 3B and D). Moreover, CHIR99021 treatment also increased Dox-induced QBC939 and RBE cell apoptosis (Fig. 3C and E). In order to further confirm the role of GSK-3β in Dox-mediated QBC939 and RBE cell apoptosis, three siRNAs targeting against GSK-3β (#1, #2, #3) were transfected into QBC939 cells, and knockdown efficiency was validated via RT-qPCR and western blotting. Although all of these siRNAs significantly decreased the expression of GSK-3β, the target sequence of #3 demonstrated the highest interference effect (Fig. 3F). Based on these results, #3 was selected for further investigation. The data showed that knockdown of GSK-3β expression significantly enhanced QBC939 and RBE cell apoptosis following Dox treatment (Fig. 3G and H). Moreover, LDH analysis also demonstrated that GSK-3β inhibition promoted cell death following Dox treatment (Fig. S2). These results indicated that GSK-3β protected human CCA cells against Dox-induced apoptosis.

GSK-3β inhibition suppresses AKT signaling in human CCA cells. In order to determine whether the AKT signaling pathway was associated with GSK-3β signaling in human CCA cells, the phosphorylation levels of AKT were detected in Dox-treated QBC939 and RBE cells following GSK-3β inhibition. BIO and CHIR99021 pretreatment significantly decreased expression levels of p-AKT (S473 and T308) in Dox-treated RBE and

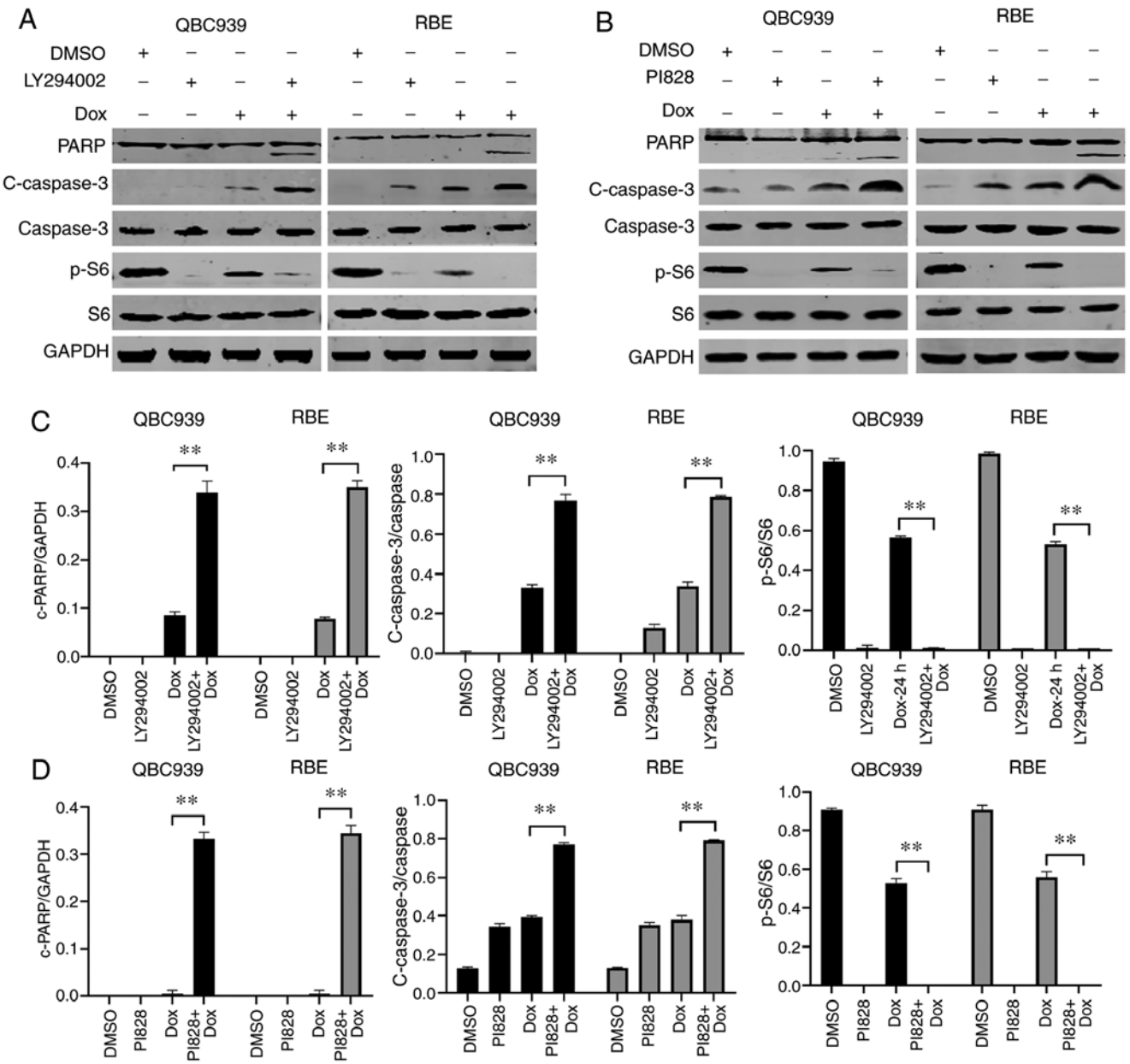

Figure 2. AKT inhibition promotes Dox-induced apoptosis in human cholangiocarcinoma cells. QBC939 and RBE cells were pre-treated with vehicle (DMSO) or AKT inhibitor (A) LY294002 (30 mM) or (B) PI828 (20 nM) for 1 h before Dox (2 μM) treatment for 24 h. Cell lysates were subjected to western blotting. Western blotting results of cells treated with (C) LY294002 or (D) PI828 were semi-quantified. **P<0.01. Dox, doxorubicin; C-caspase-3, cleaved caspase-3.

QBC939 cells (Fig. 4A and B). This suggested that GSK-3β inhibition suppressed the activation of the AKT signaling pathway. In addition, knockdown of GSK-3β expression levels by siRNA also significantly decreased the expression levels of p-AKT (S473 and T308) in Dox-treated QBC939 and RBE cells (Fig. 4C). Altogether, these data indicated that GSK-3β serves an important role in the activation of the AKT pathway in Dox-treated human CCA cells.

FAK protects human CCA cells against Dox-induced apoptosis via AKT signaling. In the present study, although it was demonstrated that the phosphorylation of T308 and S473 of AKT was regulated by Serine/Threonine kinase 3-PDK1 and mTOR complex 2 (mTORC2), respectively, in human CCA cells (Figs. S3 and S4), GSK-3β inhibition did not exhibit a notable effect on PDK1 and mTORC2 activation (Fig. S5). Since FAK has been reported to serve a key role in the regulation of AKT activity (27-29), the present study investigated the role of FAK in AKT activity in human CCA cells. The levels of p-AKT were detected in human CCA cells in the presence or absence of an FAK inhibitor. FAK inhibitor PF-573228 significantly decreased AKT phosphorylation (Fig. 5A). Moreover, inhibiting FAK promoted Dox-induced human CCA cell apoptosis (Figs. 5B and S6). In order to further confirm the role of FAK in AKT regulation in human CCA cells, myr-HA-AKT, the phosphorylation of which is independent of PIP_3, was transiently transfected into QBC939 and RBE cells. The results demonstrated that FAK inhibitor PF-573228 significantly decreased the expression of p-AKT (Fig. 5C). In addition, the concentration of PIP_3 was detected following inhibition of GSK-3β, PI3K or FAK in human CCA cells. The PI3K inhibitor, Wortmannin, decreased the concentration of PIP_3, whereas GSK-3β or FAK

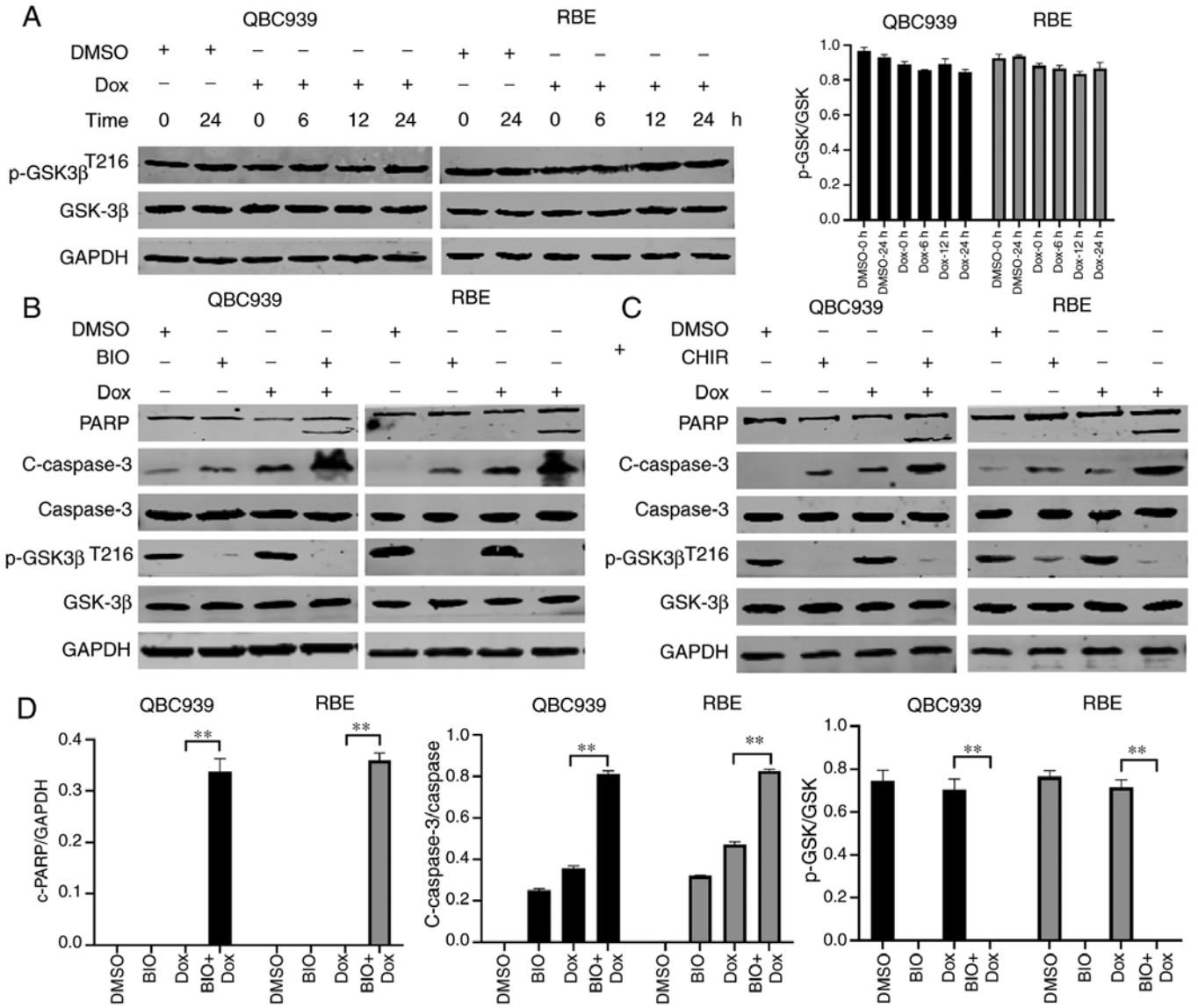

Figure 3. GSK-3β inhibition promotes Dox-induced apoptosis in human cholangiocarcinoma cells. (A) QBC939 and RBE cells were treated with Dox (2 μM) for the indicated time periods, then the cell lysates were subjected to western blotting and protein expression was semi-quantified. QBC939 and RBE cells were pre-treated with vehicle (DMSO) or GSK-3β inhibitor (B) BIO (5 μM) or (C) CHIR990219 (10 μM) for 1 h before Dox (2 μM) treatment for 24 h, then cell lysates were subjected to western blotting and the change in protein expression was semi-quantified. GAPDH was used as a loading control. Western blotting results of cells treated with (D) BIO were semi-quantified.

inhibition exhibited no notable effect on PIP_3 concentration in human CCA cells (Fig. S7). These data indicated that FAK inhibition promoted Dox-induced apoptosis by suppressing the activity of AKT independently of PI3K.

GSK-3β inhibition suppresses FAK signaling in human CCA cells. In order to investigate whether GSK-3β regulates AKT activation via FAK in human CCA cells, the phosphorylation levels of FAK were detected in QBC939 and RBE cells following GSK-3β inhibitor treatment. The results demonstrated that GSK-3β inhibitors BIO and CHIR99021 significantly decreased the expression of p-FAK in QBC939 and RBE cells (Fig. 6A). Knockdown of GSK-3β expression levels by siRNA also decreased the expression of p-FAK in QBC939 and RBE cells (Fig. 6B), and these protein levels were also downregulated in the presence of Dox with si-GSK-3β (Fig. 6C). Together, these data indicated that GSK-3β sustained AKT activation via FAK in human CCA cells.

Discussion

Although Dox is an important chemotherapeutic drug used to treat numerous types of tumors, including CCA, resistance to Dox is a significant impediment to successful chemotherapy (30-32). Therefore, it is crucial to develop a combination therapy to improve Dox efficacy. The present study demonstrated that the combination of GSK-3β inhibitor and Dox may be a potential therapeutic strategy for treating CCA.

It has been confirmed that abnormal activity of GSK-3β is involved in a number of disease processes, such as diabetes and cancer (33-36), but the role of GSK-3β in cancer chemotherapy is unclear. Although the expression of p-GSK-3β was not notably altered following Dox treatment, GSK-3β inhibition effectively promoted human CCA cell death following Dox treatment. Thus, it was hypothesized that GSK-3β protected human CCA cells against Dox-induced apoptosis.

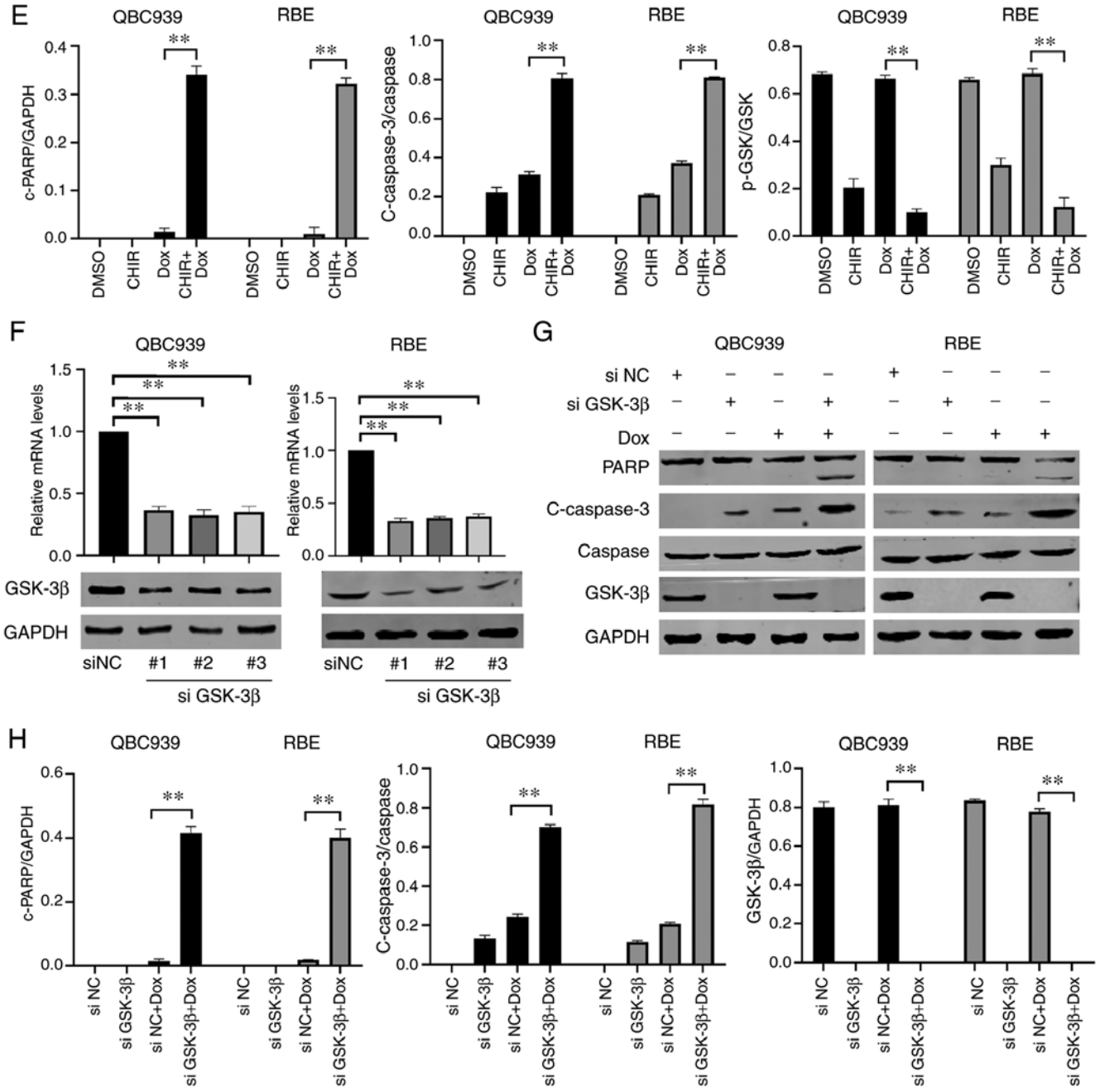

Figure 3. Continued. GSK-3β inhibition promotes Dox-induced apoptosis in human cholangiocarcinoma cells. Western blotting results of cells treated with (E) CHIR990219 were semi-quantified. NC and three GSK-3β-targeted siRNAs were transfected into QBC939 and RBE cells for 6 h, then allowed to recover for 30 h in normal media, and the cell lysates were subjected to (F) reverse transcription-quantitative PCR or western blotting analysis. (G) Following transfection with siRNAs targeting against NC and GSK-3β #3, QBC939 and RBE cells were treated with Dox (2 μM) for 24 h, and the cell lysates were subjected to western blotting and (H) protein expression was semi-quantified. **P<0.01. Dox, doxorubicin; BIO, 6-bromoindirubin-3'-oxime; NC, negative control; siRNA, small interfering RNA; C-caspase-3, cleaved caspase-3; p-, phosphorylated.

GSK-3β inhibition notably decreased the activity of AKT. As the AKT pathway serves a key role in promoting cancer cell survival following chemotherapeutic drug treatment (37,38), GSK-3β inhibition-mediated AKT suppression may be involved in the synergistic effects of GSK-3β inhibition on Dox-induced human CCA cell apoptosis. This hypothesis was supported by the present results, which demonstrated that PI3K inhibitors promoted Dox-induced apoptosis in human CCA cells. These data indicated that GSK-3β inhibition promoted Dox-induced cell death, at least in part via suppression of AKT signaling.

To the best of our knowledge, the mechanism underlying GSK-3β regulation of the activation of AKT in human CCA cells has not previously been elucidated. PDK1 and mTORC2 are responsible for the phosphorylation of AKT at T308 and S473, respectively (39-43), the present study therefore investigated the role of GSK-3β in PDK1 and mTORC2 regulation. According to the current results, GSK-3β had no effect on PDK1 and mTORC2 in human CCA cells. Thus, the present study demonstrated that GSK-3β mediated AKT activation in human CCA cells independently of PDK1 and mTORC2.

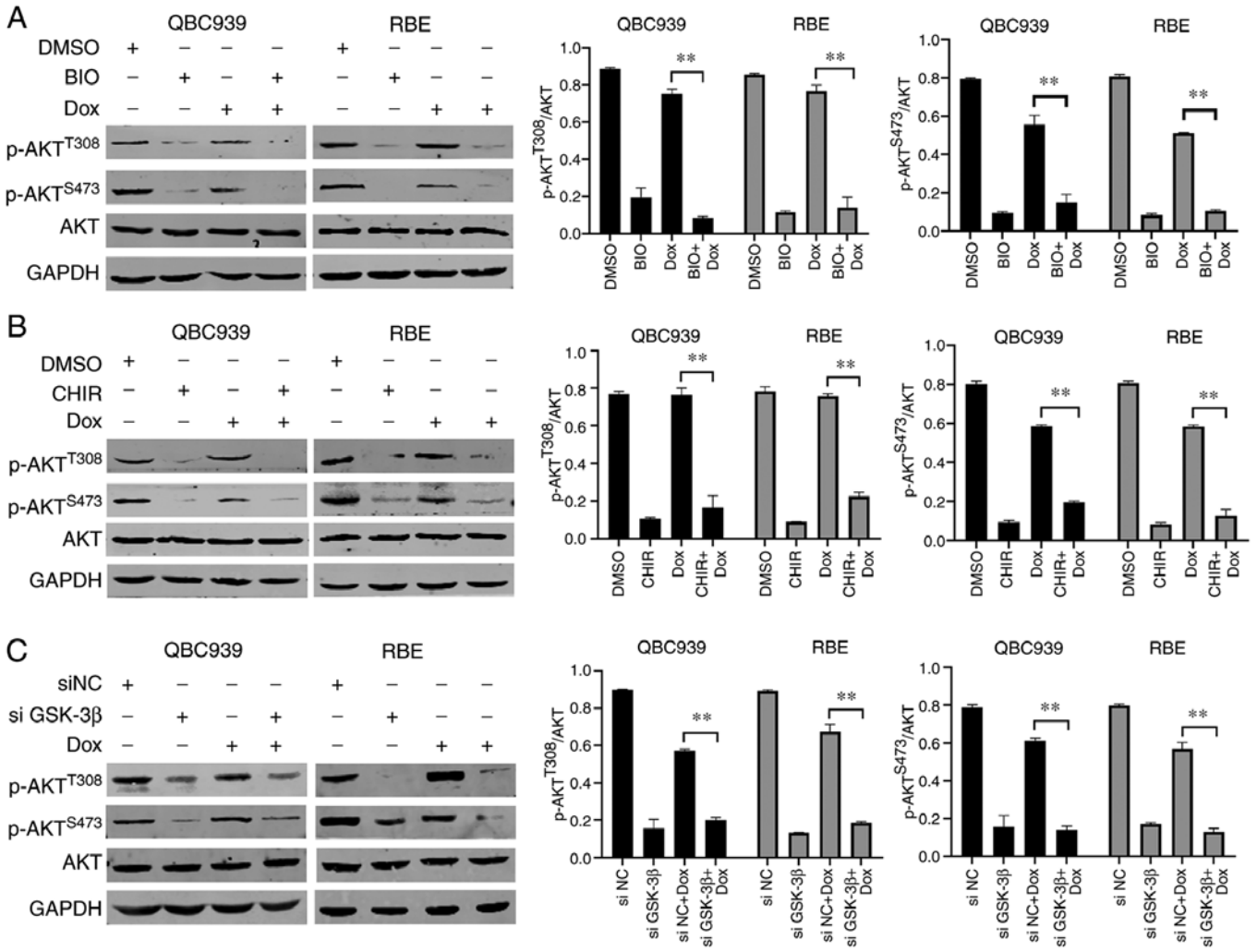

Figure 4. GSK-3β inhibition suppresses AKT signaling in human cholangiocarcinoma cells. Following pre-treatment with GSK-3β inhibitors (A) BIO (5 μM), (B) CHIR990219 (10 μM) or (C) GSK-3β siRNAs, QBC939 and RBE were treated with Dox (2 μM) for 24 h. Expression levels of p-AKT (S473), p-AKT (T308) and AKT were detected via western blotting and proteins were semi-quantified. GAPDH was used as the loading control. **P<0.01. BIO, 6-bromoindirubin-3'-oxime; siRNA, small interfering RNA; Dox, doxorubicin; p-, phosphorylated; NC, negative control.

FAK, which regulates cell migration, invasion, adhesion, proliferation and survival, has been reported to serve a key role in cancer (28,29). Moreover, it has been demonstrated that FAK is a pivotal upstream regulator of AKT signaling in various types of tumors (44,45). In order to investigate whether GSK-3β regulated the activation of AKT via FAK in human CCA cells, the association between GSK-3β and FAK in human CCA cells was investigated. GSK-3β inhibition notably decreased the activation of FAK, indicating that GSK-3β may promote AKT activation via FAK in human CCA cells. The present results demonstrated that FAK inhibition decreased AKT activity in human CCA cells. Thus, it was hypothesized that GSK-3β induces AKT activation, at least in part, via FAK in human CCA cells. It has been reported that FAK promotes AKT activation via PI3K activity regulation (46). Moreover, FAK inhibition notably decreased the levels of AKT phosphorylation on S473 and had no effect on PI3K activity, confirming that FAK sustained AKT activation independently of PI3K. Taken together, these results indicated that GSK-3β inhibition promoted apoptosis following Dox treatment by suppressing the FAK/AKT pathway independently of PI3K activity in human CCA cells. Further investigation is required to investigate the association between GSK-3β and FAK in human CCA cells.

In summary, GSK-3β inhibition increased human CCA cell death following Dox treatment. The synergistic role of GSK-3β inhibition under Dox treatment was mediated, at least in part, by FAK/AKT pathway inactivation. These results provided a basis for the development of a novel combination treatment strategy against human CCA.

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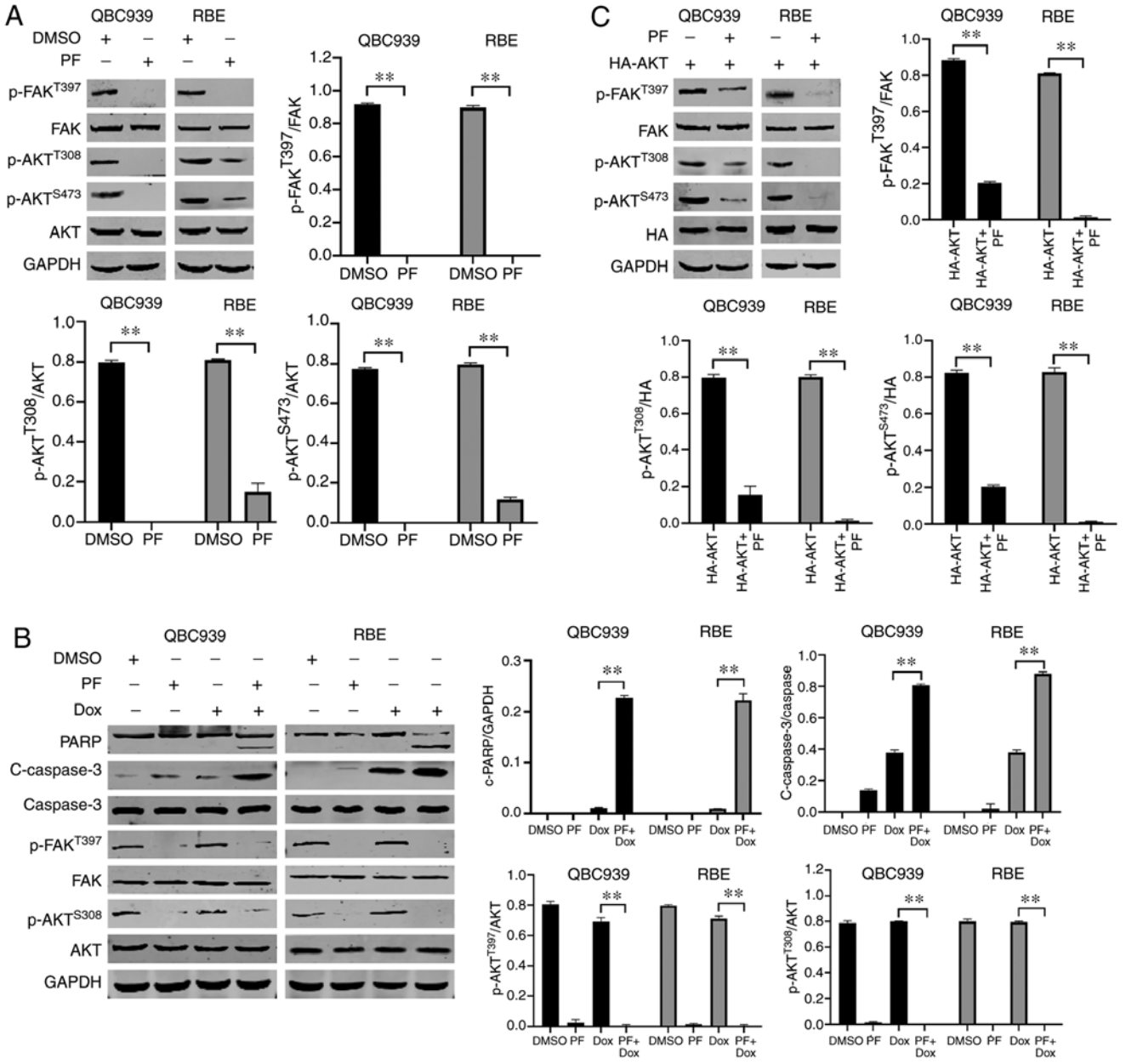

Figure 5. FAK protects human cholangiocarcinoma cells against Dox-induced apoptosis via AKT signaling. (A) QBC939 and RBE cells were treated with vehicle (DMSO) or FAK inhibitor PF-573228 (10 μM) for 12 h, then cell lysates were subjected to western blotting and protein expression was semi-quantified. (B) QBC939 and RBE cells were pre-treated with vehicle (DMSO) or FAK inhibitor, PF (10 μM), for 1 h before being treated with Dox (2 μM) for 24 h, then cell lysates were subjected to western blotting and protein expression was semi-quantified. (C) myr-HA-AKT expression plasmid was transfected into QBC939 and RBE cells for 6 h, then allowed to recover for 30 h in normal media before PF (10 μM) treatment for 12 h. Cell lysates were subjected to western blotting and protein expression was semi-quantified. **P<0.01. FAK, focal adhesion kinase; Dox, doxorubicin; PF, PF-573228; C-, cleaved; p-, phosphorylated; PARP, poly [ADP-ribose] polymerase.

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Availability of data and materials

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

Authors' contributions

LL, YCX, RYD and YPL conceived and designed the experiments, analyzed the data and wrote the manuscript. YZ, CYD, XMX, TZ and YQZ carried out the experiments, and prepared and analyzed the figures and tables. BX and WJY obtained the study materials and reagents in preparation for the experiments, and participated in the design of the experiment and checked the statistics. All authors reviewed drafts of the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

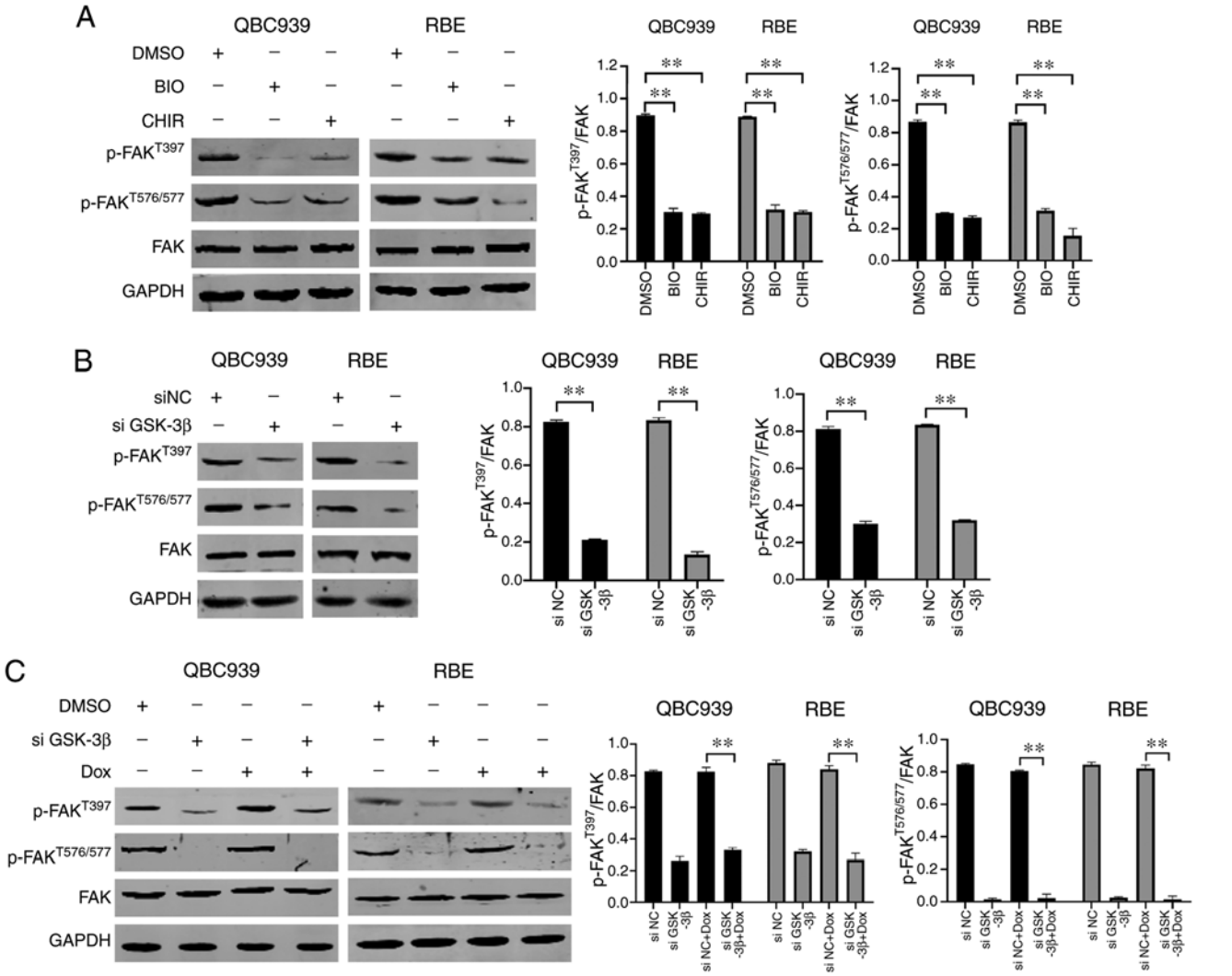

Figure 6. GSK-3β inhibition suppresses FAK signaling in human cholangiocarcinoma cells. QBC939 and RBE cells were treated with GSK-3β inhibitors (A) BIO (5 μM), CHIR990219 (10 μM) or (B) GSK-3β siRNAs for 12 h, then cell lysates were subjected to western blotting and protein expression was semi-quantified. (C) QBC939 cells were transfected with si-NC or si-GSK-3β and treated with Dox (2 μM) for 24 h, then cell lysates were subjected to western blotting and protein expression was semi-quantified. **P<0.01. FAK, focal adhesion kinase; BIO, 6-bromoindirubin-3'-oxime; siRNA, small interfering RNA; NC, negative control; Dox, doxorubicin; p-, phosphorylated.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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