Metformin inhibits proliferation and enhances chemosensitivity of intrahepatic cholangiocarcinoma cell lines

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Abstract. Metformin is an oral anti-hyperglycemic agent of the biguanide family, which is used first-line for type II diabetes with few side-effects. A recent epidemiological study that included 1,828 potential intrahepatic cholangiocarcinoma (ICC) patients showed that metformin use was significantly associated with a 60% reduction in ICC risk in diabetic patients, demonstrating the potential value of metformin in ICC management. In the present study, we firstly showed that metformin exhibited a dose- and time-dependent anti-proliferation effect on ICC cell lines, by mechanisms including apoptosis induction and cell cycle arrest. Metformin targeted the AMPK/mTORC1 pathway in ICC cells. Furthermore, metformin sensitized ICC cells to certain chemotherapeutic agents, such as sorafenib, 5-fluorouracil and arsenic trioxide (As2O3) by targeting the AMPK/mTOR/ HIF-1α/MPR1 pathway and ERK. As it is an inexpensive and widely used antidiabetic drug without severe adverse effects, metformin may be a prospective chemotherapeutic agent or a chemosensitizer in future ICC treatment.

Introduction

Metformin, a first-line oral anti-type II diabetes agent used worldwide, displays an antitumorigenesis effect, according to recent epidemic studies (1-3). As compared to insulin or sulfonylureas administration, metformin use may markedly reduce the cancer risk in patients with type II diabetes. Recent studies have confirmed the anti-proliferation effect on various human cancer cell types, such as pancreas (4), prostate (5), breast (6), stomach (7) and liver (8). Metformin inhibits the pro-proliferation effect of insulin receptor- and IGF receptor-dependent signaling by reducing insulin resistance. Furthermore, metformin activates AMP-activated protein kinase (AMPK) and subsequently inhibits activation of mammalian target of rapamycin (mTOR) to prevent proliferation of tumor cells, and activates p53 protein-inducing cell cycle arrest of tumor cells. Several studies have indicated that metformin can potentiate the effect of chemotherapeutic agents or reverse drug resistance in cancer cells (8-10). However, the mechanism of the anti-cancer effects of metformin remains unclear.

A recent epidemiological study that included 1,828 potential intrahepatic cholangiocarcinoma (ICC) patients described that metformin use was significantly associated with a 60% reduction in ICC risk in diabetic patients (11). Cholangiocarcinoma (CC) categorized as intrahepatic and extrahepatic cholangiocarcinoma (ECC) is highly lethal. ICC is the second most common type of primary liver cancer and its incidence and mortality rates have been rising in recent decades (12-15). Less than 30% of patients with ICC have the opportunity to have radical operation at diagnostic presentation. Apart from radical operation, some treatment approaches such as systemic chemotherapy, transarterial chemoembolization and radiofrequency ablation may be applied at advanced stages of ICC; however, none of the approaches can significantly improve the prognosis of ICC. Thus, new treatment strategies are needed for ICC.

In the present study, we showed that metformin inhibited the growth of ICC cells by inducing apoptosis and cell cycle arrest and inhibiting colony formation. Metformin inhibited growth by activation of the AMPK/mTOR complex 1 (mTORC1) pathway. Furthermore, metformin amplified the effect of chemotherapeutic agents such as gemcitabine, 5-fluorouracil, arsenic trioxide (As2O3) and sorafenib. Metformin might

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target the mTOR/hypoxia inducible factor 1α (HIF1α)/multi-drug resistance proteins 1 (MRP1) signaling pathway and extracellular signal regulated kinases 1/2 (ERK) to sensitize the ICC cells to chemotherapy.

Materials and methods

Cell cultures. The ICC cell lines RBE and HCCC-9810 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 µg/ml each of penicillin and streptomycin (Invitrogen, USA) in 5% CO₂ at 37°C.

Reagents. Metformin (1,1-dimethylbiguanide hydrochloride, #D150959-5G), gemcitabine (2'-Deoxy-2',2'-difluorocytidine, #G6423-10MG) and 5-fluorouracil (2,4-dihydroxy-5-fluorouracil, #D150959-5G), gemcitabine (2'-Deoxy-2',2'-difluorocytidine, #G6423-10MG) and 5-fluorouracil (2,4-dihydroxy-5-fluoropyrimidine, #F6627-1G) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sorafenib (C43H35ClF2N3O2C2H4S, CAS 475207-59-1) was purchased from Selleck Chemicals (Houston, TX, USA). The Cell Counting Kit-8 (CCK-8, KGA317), the Annexin V-FITC Apoptosis Detection Kit (KGA108) and the Cell Cycle Detection Kit (KGA512) were purchased from KeyGen Biotech (Nanjing, China).

Antibodies. The following antibodies were used in western blot analysis: β-actin (sc-7778, diluted 1:1000) was from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA). HIF-1α (N-term) (AP4776a, diluted 1:1000), active Caspase-3 (AJ1131b, diluted 1:1000), Bcl-2 (AJ1082a, diluted 1:1000), CDK4 (AP7520b, diluted 1:1000) and Cyclin D1 (AP2612c, diluted 1:1000) were from Abgent (San Diego, CA, USA). AMPKα (Ab-172, #B0003, diluted 1:500) and phosphorylated AMPKα (Phospho-Thr172, #A0003, diluted 1:500), mTOR (#B7156, diluted 1:500) and phosphorylated mTOR (Phospho-Ser248, #A7156, diluted 1:500) were from Assay Biotech (Sunnyvale, CA, USA). MRPl (PA5-30594, diluted 1:500) was from Thermo Fisher Scientific Inc., (Rockford, IL, USA). Phosphorylated Raptor (Phospho-Ser792, #2083, diluted 1:1000), phosphorylated p7056 Kinase (Phospho-Thr389, #9234, diluted 1:1000), phosphorylated 4E-BP1 (Phospho-Thr37/46, #2855, diluted 1:1000), ERK (#4696, diluted 1:2000) and phosphorylated ERK (Phospho-Thr202/Tyr204, #4370, diluted 1:2000) were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Goat anti-rabbit and goat anti-mouse IgG, peroxidase-dense conjugated secondary antibodies (31460 and 31430, both diluted 1:2000) were purchased from Cell Signaling Technology,Inc. (Danvers, MA, USA). Goat anti-rabbit and goat anti-mouse IgG, peroxidase-dense conjugated secondary antibodies (31460 and 31430, both diluted 1:2000) were from Thermo-Pierce (Rockford, IL, USA).

Flow cytometric analysis. To evaluate the effects on cell cycle arrest and induction of apoptosis by metformin, the cells were examined using the Annexin V-FITC Apoptosis Detection Kit and the Cell Cycle Detection Kit according to the manufacturer's protocols. RBE and HCCC-9810 cells were seeded into 6-well plates (1x10⁴ and 2x10⁵ cells/dish for analysis of cell cycle arrest and apoptosis, respectively). For cell cycle analysis, after treatment with metformin (0, 1, 5, 10, 20, 40 mmol/l) for 48 h, a total of 1x10⁴ cells was pelleted by centrifugation and washed twice with PBS. Then, the cell pellets were resuspended in 500 µl of ice-cold 70% ethanol and incubated at 4°C overnight. The fixed cells were centrifuged and the pellets were washed with PBS. After incubation with 100 µl RNase A (10 µg/ml) for 30 min at 37°C in the dark, the cells were resuspended in 400 µl PI (50 µg/ml) and placed at 4°C in the dark for 30 min. The stained cells were analyzed using an Accuri C6 flow cytometer (Accuri Cytometers Inc., Ann Arbor, MI, USA). For the apoptosis analysis, the cells were trypsinized, washed with cold PBS and suspended in PBS. Then, the cells were stained using the Annexin V-FITC.
reaction reagent (5 µl of Annexin V-FITC, 5 µl of propidium iodide) at 37˚C for 30 min in the dark. The stained cells were analyzed using an Accuri C6 flow cytometer (Accuri Cytometers Inc).

Statistical analysis. SPSS 13.0 statistical software was used for the statistical analysis. Values are presented as the mean ± SD. Statistical analyses were performed using Student's t-test. The analysis of multiple groups was performed with ANOVA with an appropriate post hoc test.

Results

Metformin inhibits the proliferation of ICC cells. The effects of metformin on the proliferation of ICC cells were investigated in RBE and HCCC-9810 cell lines using CCK-8 assay. As shown in Fig. 1A-H, metformin significantly reduced cell viability in a dose- (0–40 mM) and time-dependent (24, 48, 72 h) manner in both RBE and HCCC-9810 cell lines. With the 5 mM metformin and 48-h incubation, the two cell lines showed statistical differences in relative cell viability.
compared to the control cells. The microscopic examination showed a significant decrease of cell density and change of cell morphology, which displayed a smaller and granulated shape in metformin treated cells (Fig. 1I). Taken together, the results show that metformin inhibits the proliferation of ICC cells.

Metformin promotes apoptosis and induces G0/G1 cell cycle arrest in ICC cells. The number of apoptotic cells and percentages of cell cycle distribution were examined after 48 h metformin incubation by flow cytometric analysis. A significant increase in the number of apoptotic cells and cells
undergoing G0/G1 cell cycle arrest was observed in RBE and HCCC-9810 cells with metformin treatment compared with the control cells (Figs. 2A and B; 3A and B). With 48 h metformin treatment, the percentages of early apoptotic and late apoptotic/necrotic were increased from 5.7±0.7% to 41.7±3.1% in RBE cells and 2.4±0.4% to 35.1±3.21% in HCCC-9810 cells, and the G0/G1 phase cells were increased from 53.79±3.59% to 79.09±2.79% in RBE cells and 57.81±3.56% to 70.40±2.81% in HCCC-9810 cells, depending on the metformin doses.

**Figure 4.** Metformin inhibits colony formation of ICC cells. (A) RBE and HCCC-9810 cells were grown in 6-well plates (500 cells/well) in triplicates. After 48 h, cultures were replaced with fresh medium contain 0, 1, and 2.5 mM metformin and 2% FBS. After 2 weeks, the colonies were stained with 1% crystal violet and counted. (B) Bars represent the means ± SD from 3 independent experiments (*P<0.05, **P<0.01).

**Figure 5.** Metformin treatment increases AMPK phosphorylation and inhibits the activation of mTORC1 complex. (A) The phosphorylation of AMPK was upregulated and the phosphorylation of mTOR was downregulated depending on the dose of metformin (0-20 mM). (B) The phosphorylation of Raptor, p70 S6 kinase and 4E-BP1 was downregulated depending on the dose of metformin (0-20 mM). (C–G) The band intensities were quantified using Image Lab 5.0 software and are represented as the means ± SD from 3 independent experiments (*P<0.05, **P<0.01).
Furthermore, to confirm the metformin-induced apoptosis and G0/G1 cell cycle arrest in ICC cells, cleaved caspase-3, Bcl-2, cyclin D1 and CDK4 expression were monitored using western blot analysis in RBE and HCCC-9810 cells (Figs. 2C and D; 3C and D). Activation of the apoptosis promoter caspase-3 was related to the metformin-induced apoptosis. However, Bcl-2, known as an apoptosis inhibitor, was upregulated by treatment of metformin in a dose-dependent manner. Cyclin D1 and CDK4, which are responsible for the transition from G0/G1 to S phase, were downregulated after metformin treatment. Collectively, these results suggest that metformin promotes apoptosis and induces G0/G1 cell cycle arrest in ICC cells.

Metformin inhibits colony formation of ICC cells. We studied the ability of these two ICC cell lines to form colonies in 6-well plates in the presence or absence of metformin. The RBE cells were more sensitive to 1 mM metformin than HCCC-9810 cells (Fig. 4). At the concentration of 2.5 mM metformin, almost no colonies formed in either cell line.

Metformin targets the AMPK/mTORC1 pathway in ICC cells. To evaluate the specific effect of metformin on the AMPK/mTORC1 pathway, which is widely believed to be the most common target of metformin, western blot analysis was used to evaluate the AMPK/mTORC1 pathway in HCCC-9810 and RBE cells. Metformin treatment resulted in enhanced AMPK phosphorylation and reduced mTOR phosphorylation in a dose-dependent manner in ICC cells. Furthermore, the regulatory protein of mTOR (Raptor), which is identified as an mTOR binding partner, mediates mTOR signaling to downstream targets through binding to mTOR substrates.
including eIF4E-binding protein 1 (4E-BP1) and p70 S6 kinase. Metformin dose-dependently inhibited the phosphorylation of Raptor, 4E-BP1 and p70 S6 kinase. Taken together, these results suggest metformin targets the AMPK/mTORC1 pathway in ICC cells (Fig. 5).

**Metformin targets the HIF-1α/MRP1 pathway and ERK and sensitizes ICC cells to certain chemotherapeutic agents.** The combination of metformin with one of the following chemotherapeutic agents, sorafenib, gemcitabine, 5-fluorouracil and arsenic trioxide (As$_2$O$_3$) was more effective than certain agents alone (Fig. 6F and G). Markedly, metformin plus sorafenib or As$_2$O$_3$ significantly enhanced the inhibitory effect on RBE and HCCC-9810 cells induced by a single agent. However, metformin did not significantly sensitize ICC cells to gemcitabine, and alternatively sensitized ICC cells to 5-fluorouracil.

To explore the potential mechanisms related to the chemotherapy sensitization effect of metformin, HIF-1α/MRP1 and phosphorylation of ERK were analyzed in RBE and HCCC-9810 cells. Western immunoblot analysis revealed that metformin markedly suppressed the expression of HIF-1α protein and MRP1 protein and decreased the phosphorylation of ERK in a dose-dependent manner (Fig. 6A-E).

**Discussion**

ICC is an aggressive malignancy, the incidence and mortality rates of which are increasing worldwide. ICC is difficult to diagnose at an early stage and is associated with a low surgical resection rate. Management of ICC has not achieved significant improvements in recent decades. Metformin is an oral anti-hyperglycemic agent of the biguanide family, which is used first-line for type II diabetes with few side-effects. Recent data also described the anti-proliferation effect of metformin in numerous cancer cells. A recent epidemiological study that included 1,828 potential ICC patients described that metformin use was significantly associated with a 60% reduction in ICC risk in diabetic patients, demonstrating the potential value of metformin in ICC management. Thus, we investigated the anti-proliferation effect and the mechanisms by which metformin affects the ICC cell lines RBE and HCCC-9810. In addition, we evaluated the pro-sensitive effect of metformin in chemotherapy in ICC cells, the mechanisms of which were also explored.

In the present study, we showed that metformin exhibited a dose- and time-dependent anti-proliferation effect on ICC cell lines RBE and HCCC-9810. Metformin exerted inhibitory effects on the clonogenicity and promoted apoptosis and induced G0/G1 cell cycle arrest in RBE and HCCC-9810 cells, which were consistent with the cell viability variation. The two ICC cell lines showed similar sensitivity to metformin. Notably, expression of Bcl-2, which is known to block apoptosis, was upregulated by treatment of metformin in a dose-dependent manner in our study. To the best of our knowledge, most of the previous studies reported that metformin reduced expression of Bcl-2 (4,16,17). We hypothesize that the ICC cells resist the metformin-induced apoptosis via, paradoxically, upregulation of Bcl-2 protein and, on the other hand, Bcl-2 conversely acts as an apoptosis inhibitor or a promoter by certain mechanisms (18). Our future studies will focus on the exact mechanism by which metformin affects expression of Bcl-2 in ICC cells.

The AMPK/mTORC1 pathway is the most widely believed target of metformin. Metformin inhibited the activation of the mTORC1 by activating AMPK in RBE and HCCC-9810 cells in our study. Although it remains controversial whether activation of mTOR pathway promotes development of cholangiocarcinoma or predicts poor prognosis in patients with cholangiocarcinoma (19,20), our results are in line with numerous other reports describing the inhibitory effect of metformin on cancer cells (6,10,21).

In addition, we detected the inhibitory effect of combination treatment of metformin with sorafenib, gemcitabine, 5-fluorouracil or As$_2$O$_3$, which have been clinically used for cholangiocarcinoma treatment. Metformin significantly sensitized RBE and HCCC-9810 cells to sorafenib and As$_2$O$_3$, while metformin alternatively sensitized ICC cells to 5-fluorouracil and did not statistically sensitize ICC cells to gemcitabine. This phenomenon may be explained by the fact that RBE and HCCC-9810 cells were highly sensitive to gemcitabine in our study. In particular, metformin more significantly amplified the inhibitory effect of As$_2$O$_3$ than other agents in ICC cells, the mechanism of which we will explore in future studies. To approach the potential mechanisms related to the chemotherapy sensitization effect of metformin, we further investigated how metformin suppressed the expression of HIF-1α and MRP1, which are associated with the multidrug resistance of cancer cells, and decreased the phosphorylation of ERK, which is critical in regulating therapy response of cancer cells (22-24). Recent studies suggested that metformin could improve oxygenation and suppress HIF-1α accumulation in tumor- or diabetic-related diseases through the activation of the AMPK/mTOR pathway and the repression of oxygen consumption (25-28). HIF-1, a basic helix-loop-helix transcription factor, plays a significant role in regulating the transcription of various target genes in response to hypoxia (29). HIF-1α is an oxygen-regulated subunit that mediates the essential function of HIF-1. The overexpression of HIF-1α may contribute to the pathogenesis of tumor resistance to chemotherapy (30,31). MRP1 is regarded as energy-dependent membrane efflux pumps and are widely believed to be transcriptionally regulated by HIF-1α in multiple human tumors (32-35). Consequently, our results indicated that metformin might target the AMPK/mTOR/HIF-1α/MRP1 sensitizing ICC cells to chemotherapeutic agents. Furthermore, decreasing the phosphorylation of ERK, which is also the target of sorafenib and As$_2$O$_3$ (36-38), might compose the mechanisms by which metformin performed as a chemosensitizer in our study.

In conclusion, our results revealed that metformin inhibits ICC cell proliferation and sensitizes ICC cells to certain chemotherapeutic agents, possibly by mechanisms including apoptosis induction and cell cycle arrest, and targeting the AMPK/mTORC1, AMPK/mTOR/HIF-1α/MRP1 pathway and ERK. Further studies are required to investigate how metformin increases the expression of Bcl-2 and the impact exerted by Bcl-2 on ICC cells. As it is an inexpensive and widely used antidiabetic drug without severe adverse effects, metformin may be a prospective chemotherapeutic agent or a chemosensitizer in ICC treatment.
Metformin inhibits intrahepatic cholangiocarcinoma cell lines

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References