Phenformin inhibits cell proliferation and induces cell apoptosis and autophagy in cholangiocarcinoma

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Abstract. Cholangiocarcinoma (CCA) is an aggressive malignant tumor and the prognosis of patients with advanced stage disease remains poor. Therefore, the identification of novel treatment agents for CCA is required. In the present study, the biological effects of the diabetes therapeutic agent, phenformin, in CCA cell lines was investigated. Cell Counting Kit-8 cell viability, cellular clone formation and subcutaneous tumor formation assays were performed, which revealed that phenformin inhibited CCA cell proliferation and growth both *in vitro* and *in vivo*. In addition, phenformin induced CCA cell apoptosis and autophagy. Phenformin partly activated the liver kinase B1 (LKB1)/5' AMP-activated protein kinase signaling pathway to exert its biological effects on CCA cell lines, as demonstrated by knockdown of LKB1, which reversed these effects. In conclusion, the present study demonstrated the biological effects of phenformin in CCA and suggested that phenformin may be a potential novel agent for CCA treatment.

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor that arises from the epithelial cells in intrahepatic or extrahepatic bile ducts, and is difficult to diagnose and has a poor prognosis (1-3). Although surgical resection and liver transplantation are potentially curative treatments, the majority of patients present with unresectable tumors and succumb to disease within a year of diagnosis (4). CCA is frequently resistant to chemotherapy as results of monotherapy and combination chemotherapy are often disappointing (5-7). Therefore, finding novel chemical agents for the treatment of CCA is required.

The diabetes therapeutic biguanide compound, phenformin, has been demonstrated to inhibit complex I of the mitochondria (8). In previous studies, phenformin was identified to inhibit tumor growth in xenograft, transgenic and carcinogen-induced mouse cancer models (9,10). As phenformin increases intracellular adenosine monophosphate (AMP) and adenosine diphosphate and subsequently induces the activation of liver kinase B1 (LKB1)/5' AMP-activated protein kinase(AMPK) signaling. The LKB1/AMPK axis may inhibit cancer cell proliferation and growth and functions as a tumor suppressive signaling pathway (8). Phenformin has the potential to be a novel agent that inhibits CCA cell proliferation and tumor growth.

In the present study, CCA cell lines were treated with phenformin and the biological effects were observed. Phenformin inhibited CCA cell proliferation and tumor growth and induced cell apoptosis and autophagy.

Materials and methods

Cell culture. BE and Huh28 human CCA cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and were maintained under standard culture conditions (37˚C and 5% CO_2) in RPMI 1640 supplement with 10% (v/v) FBS (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

RNA isolation and quantitative real-time polymerase chain reaction (RT-qPCR). Total RNA was purified from CCA cells or CCA adjacent tissues using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 1 μg RNA was reverse transcribed using SuperScript Reverse Transcriptase III (Invitrogen; Thermo Fisher Scientific, Inc.) in 37˚C for 1 h. RT-qPCR was performed using SYBR® Green Supermix (4473369; Applied Biosystems; Thermo Fisher Scientific, Inc.) on an Applied Biosystems 7500 PCR system with the following program: 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 31 sec, and finally 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec. The relative expression of each gene was

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analyzed by the comparative cycle threshold method ($2^{-\Delta\Delta Cq}$ method) (11) which was normalized to GAPDH. Primers used in the present study are described in Table I.

Western blotting. Cells were lysed in IP lysis buffer (catalog no. P0013, Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's protocol. Subsequently, the protein concentration was measured by BCA assay kit (Thermo Fisher Scientific, Inc.) and cell lysates were boiled in 5X SDS-PAGE loading buffer for 10 min and resolved by 8% SDS-PAGE prior to transfer onto a nitrocellulose membrane. The membrane was blocked by 5% (m/v) non-fat milk (BD Biosciences, Franklin Lakes, NJ, USA). The following primary antibodies were used: LKB1 (3047;1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated (p)-AMPK (2532; 1:1,000; Cell Signaling Technology, Inc.), AMPK (2532; 1:1,000; Cell Signaling Technology, Inc.) and GAPDH (60004-1-Ig; 1:5,000; ProteinTech Group, Inc., Chicago, IL, USA). All these antibodies were incubated at 4˚C overnight. Then the membranes were incubated with HRP-linked anti-rabbit IgG (7074; 1:2,000; Cell Signaling Technology, Inc.) or HRP-linked anti-mouse IgG (7076, 1:2,000; Cell Signaling Technology, Inc.) for 1 h at room temperature. Proteins were then visualized with an Enhanced Chemiluminescence kit (Thermo Fisher Scientific, Inc.).

Cell Counting Kit-8 (CCK-8) cell viability assays. Cells were seeded into a 96-well plate at $3x10^3$ cells per well with 100 μl culture medium containing 0 or 2 mM phenformin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and cultured at 37˚C and 5% CO_2 for 12, 24 or 48 h. The cell viability was quantified by addition 10 μl of CCK-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). After a 1.5 h incubation, the optical density (OD) was measured using a Power Wave XS microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA) at a wavelength of 450 nm.

Colony formation assays. A total of 500 cells/well were seeded in 6-well plates and incubated for 10 days at 37˚C and 5% CO_2. Colonies were fixed and stained with 0.5% crystal violet at room temperature and the number of colonies was counted.

Flow cytometry cell apoptosis assays. A total of $2x10^5$ cells/well were seeded into 6-well plates and treated with 0 or 2 mM phenformin in an incubator at 37˚C. After 24 h, cells were washed twice with 1X cold PBS, and stained with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacture's protocol. BD FACSCalibur (BD Biosciences) was used for the detection and CellQuest Pro software (BD Biosciences) for the analysis.

In vivo tumor formation assay. A total of 10 male BALB/c (nu/nu) mice (Shanghai SLAC Laboratory Animal Co., Ltd.; 5 weeks old, mean weight 15 g) were kept at 25˚C and 45% humidity with 12-h light/dark cycle with free access to sterile food and water. Then, $4x10^6$ RBE cells were subcutaneously injected into the right flank of the mice. After 2 weeks, the mice were intratumorally injected with 0 (physiological saline) or 2 mM phenformin (5 mice/group) every 4 days for

Table I. Sequences of primers and siRNAs.

Name	Sequence (5'-3')
BECN1	F, TGGACACGAGTTTCAAGATCC
	R, CTCCTGGGTCTCTCCTGGTT
ATG5	F, AAAGATGTGCTTCGAGATGTGT
	R, CACTTTGTCAGTTACCAACGTCA
ATG7	F, ATGATCCCTGTAACTTAGCCCA
	R, CACGGAAGCAAACAACTTCAAC
GAPDH	F, AGCCTCAAGATCATCAGCAATGCC
	R, TGTGGTCATGAGTCCTTCCACGAT
siRNA-NC	ACGUGACACGUUCGGAGAATT
siRNA-LKB1	CTGGTTGATACCCACTCAAAAAG

siRNA, short interfering RNA; *BECN1*, Beclin1 gene; *ATG5*, autophagy 5 gene; *ATG7*, autophagy 7 gene; NC, negative control; LKB1, liver kinase B1; F, forward; R, reverse.

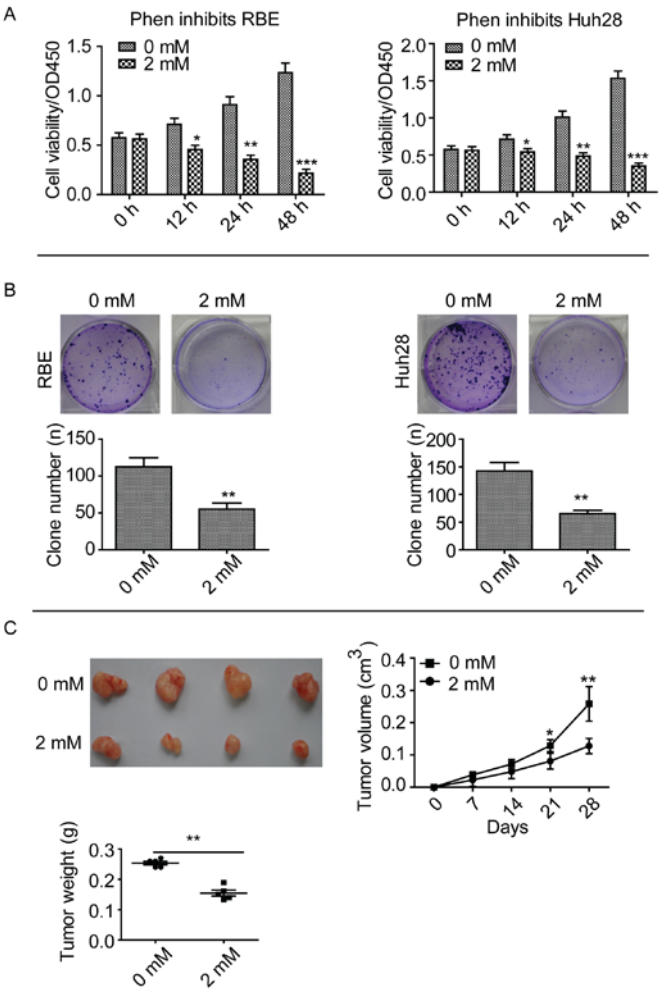


Figure 1. Phenformin inhibits CCA cell line proliferation and growth. (A) Phenformin inhibits the proliferation of RBE and Huh28CCA cell lines. (B) Cellular clone formation assays indicated that phenformin inhibited RBE and Huh28 cell growth. (C) Subcutaneous tumor formation assays suggested that phenformin inhibited RBE cell proliferation and growth *in vivo*, n=5. *P<0.05, **P<0.01 and ***P<0.0001 0 mM phenformin. *In vitro* assays were performed in triplicate. CCA, cholangiocarcinoma; Phen, phenformin.

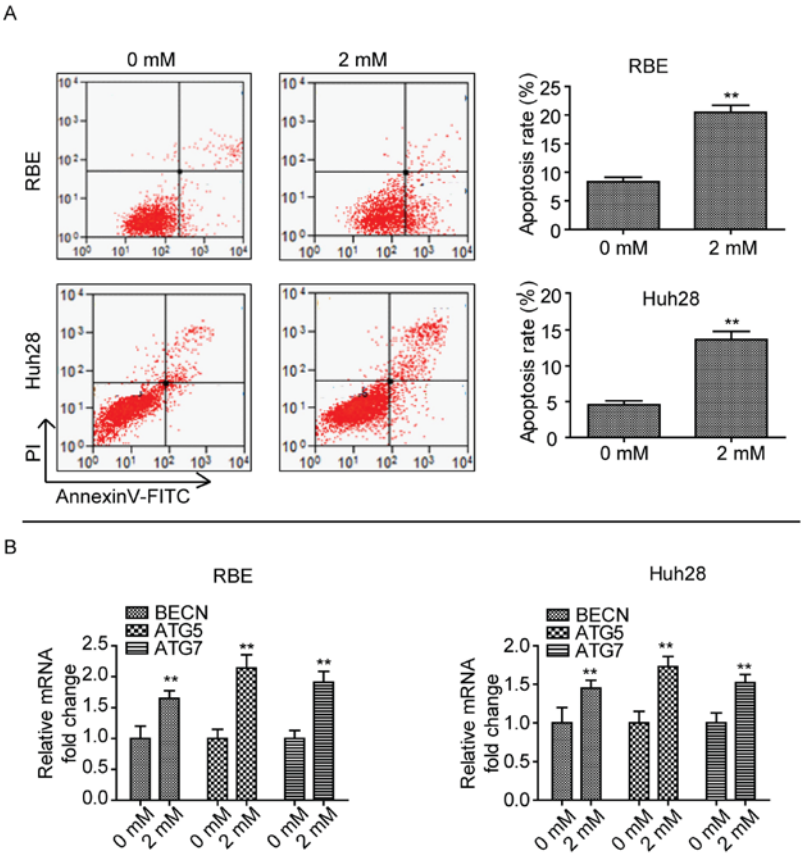


Figure 2. Phenformin promotes cholangiocarcinoma cell line apoptosis and autophagy. (A) Annexin V-FITC/PI cell apoptosis assays revealed that phenformin induces apoptosis in RBE and Huh28 cells. (B) mRNA expression levels of autophagy-associated genes, *BECN1*, *ATG5* and *ATG7*, were elevated following 24 h phenformin treatment in RBE and Huh28 cells. **P<0.01 vs 0 mM phenformin. Assays were performed in triplicate. FITC, fluorescein isothiocyanate; PI, propidium iodide; *BECN1*, Beclin1 gene; *ATG5*, autophagy 5 gene; *ATG7*, autophagy 7 gene.

a month. Tumor sizes were measured once a week and mice were sacrificed for the analysis of tumor burden after 4 weeks. The procedures in the present study were approved by the Ethics Committee of the Second Military Medical University (Shanghai, China).

siRNA treatment. The sequences for LKB1 siRNAs are described in Table I. A total of $3x10^5$ RBE and Huh28 cells were transfected with 100 nM LKB1 siRNA or with 100 nM negative control siRNA (Table I) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 24 h these transfected cell lines were used to perform the subsequent assays.

Statistical analysis. Data are expressed as the mean ± standard deviation; two-way analysis of variance followed by the LSD post-hoc test was performed for comparisons between groups. GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Phenformin inhibits CCA cell line proliferation and growth in vitro and in vivo. An inhibitor of mitochondrial complex 1, phenformin, has been demonstrated to inhibit tumor growth in lung cancer and delay tumor progression in a lymphoma mouse model (8). In the present study, CCK-8 assays were performed after treating two CCA cell lines, RBE and Huh28, with 2 mM phenformin. Phenformin significantly inhibited the proliferation of these cell lines compared with cells treated with 0 mM, after 12, 24 and 48 h (Fig. 1A). Clone formation assays revealed that phenformin significantly inhibited the ability of the cell lines to form clones compared with cells treated with 0 mM phenformin (P<0.01; Fig. 1B). Subcutaneous tumor formation assays revealed that mice injected with 2 mM phenformin exhibited a significantly smaller RBE tumor volume compared with the 0 mM control group after 28 days (P<0.01; Fig. 1C). These results suggested that phenformin inhibits CCA cell proliferation and growth *in vitro* and *in vivo*.

Phenformin induces CCA cell apoptosis and autophagy. As phenformin inhibited cell proliferation, the role of phenformin in CCA cell apoptosis and autophagy was determined. When cells were treated with 2 mM phenformin for 24 h, a significant amount of cell death was observed compared with cells treated with 0 mM phenformin (P<0.01; Fig. 2A). In addition, the mRNA expression levels of autophagy-associated genes, Beclin-1 (*BECN1*), autophagy (*ATG)5* and *ATG7* (12) were upregulated after treatment with 2 mM phenformin in the two cell lines compared with cells treated with 0 mM phenformin (Fig. 2B). These findings suggested that phenformin induces CCA cell apoptosis and autophagy.

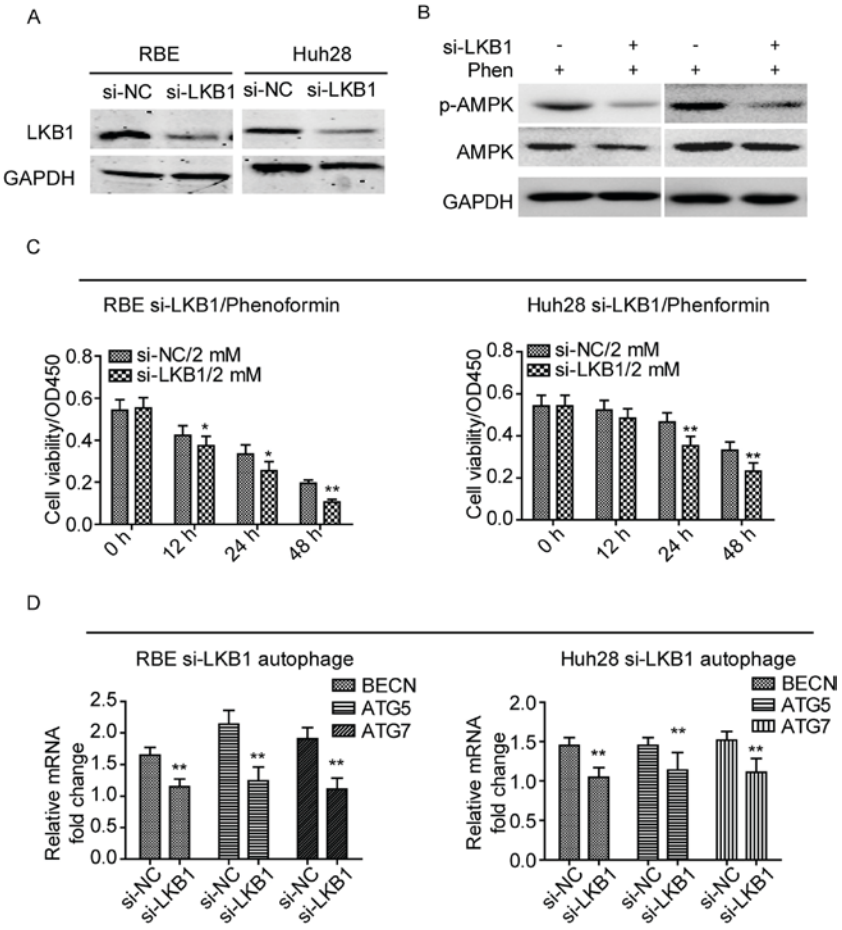


Figure 3. Phenformin exerts its effects via the LKB1/AMPK axis in cholangiocarcinoma cell lines. (A) Protein expression levels of LKB1 were reduced following transfection of cells with an LKB1-specific siRNA. (B) Knockdown of LKB1 inhibited the activation and phosphorylation of AMPK in the two cell lines following treatment with 2 mM phenformin. (C) Knockdown LKB1 significantly inhibited the cell viability of the two cell lines. (D) mRNA expression levels of autophagy-associated genes, *BECN1*, *ATG5* and *ATG7,* were reduced following knockdown of LKB1 in cell lines treated with phenformin for 24 h. Assays were performed in triplicate. *P<0.05 and **P<0.01 vs. siRNA-NC. NC, negative control; *BECN1*, Beclin1 gene; *ATG5*, autophagy 5 gene; *ATG7,* autophagy 7 gene; LKB1, liver kinase B1; AMPK, 5' AMP-activated protein kinase; Phen, phenformin; siRNA; short interfering RNA.

Phenformin exerts its effects via the LKB1/AMPK axis in CCA. A previous study revealed that phenformin induces apoptosis in LKB1-deficient non-small cell lung cancer cell lines (13). This finding suggests that phenformin may activate the LKB1/AMPK signaling pathway to inhibit CCA cell proliferation and induce apoptosis and autophagy. In the present study, the protein expression levels of LKB1 was reduced in RBE and Huh28 cell lines following knockdown of LKB1 using a specific siRNA, compared with cells treated with negative control siRNA (Fig. 3A). The expression levels of the target of LKB1, p-AMPK, following knockdown of LKB1 in the cell lines was measured after treatment with phenformin. Knockdown of LKB1 reduced the expression levels of p-AMPK when treated with phenformin for 12 h (Fig. 3B). In addition, the cell viability was reduced in LKB1 knockdown cells with phenformin treatment, as determined by a CCK-8 cell viability assay (Fig. 3C). The mRNA expression levels of autophagy-associated genes, *BECN1*, *ATG5* and *ATG7*, were reduced in LKB1 knockdown cell lines treated with phenformin, compared with cells transfected with negative control siRNA (P<0.01; Fig. 3D). These results suggested that phenformin exerts its effects via activation of the LKB1/AMPK signaling pathway.

Discussion

The present study investigated the biological effects of phenformin in CCA cell lines and demonstrated that phenformin inhibited CCA cell proliferation and growth and induced cell apoptosis and autophagy.

Phenformin is a biguanide compound and paralog to metformin, which is widely used in as a therapy for type 2 diabetes worldwide (14). Phenformin has been previously demonstrated to exhibit potential anti-cancer properties. Shackelford *et al* (13) revealed that phenformin inhibits LKB1-deficient non-small cell lung cancer progression. Liu *et al* (15) reported that phenformin induces cell cycle alterations and apoptosis in breast cancer. Cancer cells exhibit alterations in cellular metabolism to enable their continued growth and proliferation (16,17). Phenformin is a biguanide compound that inhibits complex 1 of mitochondria, resulting in increased intracellular AMP and ADP that induces the LKB1/AMPK signaling loop (8). AMPK is a conserved sensor of intracellular energy levels (18) and phenformin treatment and the activation of the LKB1/AMPK signaling pathway may significantly inhibit anabolic pathways and cell proliferation (19). In the present study, phenformin significantly

inhibited CCA cell proliferation and induced cell apoptosis and autophagy. These effects may be induced by the LKB1/AMPK signaling pathway in CCA cell lines.

Wang *et al* (20) reported that LKB1 is frequently downregulated in CCA tissues. The findings of the present study suggested that silencing of LKB1 in combination with phenformin led to these cells being more susceptible to death. The current findings suggest that Phenformin has the potential to be a therapeutic agent for CCA treatment.

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