Metformin facilitates BG45-induced apoptosis via an anti-Warburg effect in cholangiocarcinoma cells

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Abstract. Cholangiocarcinoma (CCA) is a highly lethal malignancy with an often late diagnosis and consequent poor prognosis. Chemotherapy is the only therapeutic strategy for most patients. Compared to normal cells, tumor cells preferentially metabolize glucose to lactate, even in aerobic conditions. Such metabolic alterations not only support the growth and invasion of tumor cells, but also promote their chemoresistance. The purpose of our study was to explore the role of metformin in regulating the metabolism of CCA, as well as to investigate whether metformin could act as a chemosensitizer of the HDAC3 inhibitor BG45, and therefore have potential for the treatment of CCA. Through bioinformatic analysis, we found that aberrant metabolism contributed to the proliferation of CCA cells. Seahorse XF96 Extracellular Flux Analyzer analysis and lactate production analysis showed that metformin could act as a suppressor of the Warburg effect in CCA cells. Western blotting showed that metformin decreased the expression of LDHA, which plays a key role in the Warburg effect. However, suppression of the Warburg effect was not sufficient to induce CCA cellular apoptosis. According to our previous research, which showed that an HDAC3 inhibitor (MI192) was involved in CCA apoptosis, we observed that metformin combined with BG45 (a novel specific HDAC3 inhibitor) effectively induced the apoptosis of CCA cells *in vitro*. Furthermore, *in vivo* experiments revealed that the combined treatment with metformin and BG45 markedly reduced CCA growth in a CCA xenograft model. Our data revealed that reversing the Warburg effect with metformin sensitizes cells to the antitumor effects of HDAC3 inhibitors. This provides a rationale for using the combination of metformin and BG45 as a new therapeutic strategy in the treatment of CCA.

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Introduction

Cholangiocarcinoma (CCA) is a highly malignant disease with a poor prognosis, and comprises approximately 3% of all gastrointestinal malignant tumors (1,2). The etiology of this malignancy is mostly unknown, while the incidence and mortality rate of the disease are increasing in many countries (3). Due to limited diagnostic methodologies, most patients are diagnosed at an advanced stage and are ineligible for surgical resection. As a result, the 5-year survival rate of CCA has remained at 10% for many years (2). Chemotherapy (cisplatin plus gemcitabine) has been the exclusive therapy for a significant percentage of CCA patients (4), but chemoresistance attenuates the efficacy of conventional chemotherapy, thus making it essential to identify novel curative targets for the treatment of this disease.

Metformin, a biguanide, has been commonly prescribed for decades as an anti-hyperglycemic agent in the treatment of type II diabetes mellitus (5). Although metformin has been extensively used as an anti-diabetic for 40 years, the first report indicating its antitumor effect in mammals was in 2001 (6), and the first study discussing the association between a reduced incidence of cancer in patients with type II diabetes and the use of metformin was published only about 10 years ago (7). Since then, more and more evidence has shown that metformin has antitumor properties and can be used as a

chemosensitizer (8-11). However, the antitumor and chemopreventive mechanisms of metformin have not yet been fully elucidated.

Tumor cells preferentially use glycolysis for energy production even in the presence of oxygen, which is the so-called Warburg effect (12). This metabolic alteration accumulates enough lactate and glycolytic intermediates to support tumor growth and invasion. Therefore, reversal of the Warburg effect is a potential therapeutic methodology for the treatment of cancer (13,14).

Epigenetic changes, including histone modifications, have been reported to play a crucial role in malignant disease (15). Histone deacetylases (HDACs) have emerged as new therapeutic targets in many cancers, as they can remove acetyl groups from histone to decrease gene transcription. HDACs can be divided into 4 classes: Class-I (HDAC1, 2, 3 and 8), Class-II (HDAC4, 5, 6, 7, 9 and 10), Class-III (SIRT1-7) and Class-IV (HDAC11) (16). Recent studies suggest that Class-I HDACs are upregulated in many malignancies, and that they inhibit the expression of specific tumor-suppressor genes through epigenetic modulation (17-19). Our previous studies demonstrated that high levels of HDAC3 expression and activity play a critical role in CCA, and that the inhibition of HDAC3 could induce apoptosis in CCA cells (20).

Several HDAC inhibitors have been developed in clinical trials for cancer treatment, and SAHA, as well as romidepsin, have been approved by the US Food and Drug Administration for the treatment of cutaneous T-cell lymphoma (21). Novel Class I HDAC inhibitors (4SC202, BG45 and SBHA) have shown efficacy in cancer cells with near-marginal toxicity (15,16,22). BG45, a selective HDAC3 inhibitor, has been demonstrated to be effective in the treatment of leukemia (16). However, it remains unknown as to whether BG45 can be used as a new treatment for CCA.

In the present study, we found that metformin could reverse the Warburg effect by downregulating the protein levels of LDHA, which was overexpressed in CCA; this could, in turn, make CCA cells vulnerable. Therefore, combining metformin with BG45 markedly inhibited the growth of cholangiocarcinoma via the induction of cellular apoptosis. Our findings strongly suggest that metformin combined with the HDAC3 inhibitor BG45 can be used as a therapeutic strategy for the treatment of CCA.

Materials and methods

Ethics, consent and permissions. The protocol for the animal experiments was reviewed and approved by the Ethics Committee of Medical Research, Nanjing Drum Tower Hospital, Affiliated Hospital of Nanjing University Medical School (Nanjing, China). Donors provided consent for any use of human samples for research and the study protocol was approved by the Ethics Committee of Medical Research, Nanjing Drum Tower Hospital, Affiliated Hospital of Nanjing University Medical School.

Bioinformatic analysis. The differentially expressed genes (DEGs) in CCA were analyzed using data from The Cancer Genome Atlas (TCGA). CCA RNA-Seq data were downloaded from the TCGA database using the GDC Data Portal (https://gdc-portal.nci.nih.gov/), which consisted of 9 normal samples and 9 paired CCA samples. KEGG pathway enrichment analysis was performed to detect the potential biological functions and pathways of these genes in CCA. The heat map was drawn using the gplots package in Bioconductor software (Bioconductor, org., version 3.6).

Immunohistochemistry. We purchased the tissue microarray slides from Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). Staining intensity was graded as follows: absence of staining, 0; weak, 1; moderate, 2 and strong, 3. The scoring approach in the assessment of staining was as follows: 0 (no positive cells), 1 (<25% positive cells), 2 (25-50% positive cells), 3 (>50-75% positive cells), and 4 (>75% positive cells). The score for each tissue was calculated by multiplication of these two grades, and the range of this calculation was 0-12 (23).

Cell culture and reagents. HuCCT1 (JCRB, Osaka, Japan) and RBE (The Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Biological Industries, Cromwell, CT, USA), penicillin (100 U/ml; Invitrogen; Thermo Fisher Scientific) and streptomycin (100 U/ml; Invitrogen; Thermo Fisher Scientific, Inc.). Cells were all maintained at 37˚C with 5% CO_2. Metformin was purchased from Sangon Biotech Co., Ltd. (Shanghai, China) and BG45 was purchased from MedChem Express (Monmouth Junction, NJ, USA).

Cell transfection. HuCCT1 and RBE cells were transfected using Lipofectamine RNAiMax reagent (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. HDAC3 siRNA was produced as described previously (20). Briefly, 50 pmol siRNA and 0.5 ml Opti-MEM I Medium (Invitrogen; Thermo Fisher Scientific, Inc.) were mixed, and then 5.5 μl RNAiMax reagent was added to each well of a 6-well plate and incubated for 15 min. Next, the mixed reagent was added to a cell suspension containing $25x10^4$ cells in 1.5 ml RPMI-1640 with 10% FBS.

Western blotting. Cells were lysed with ice-cold RIPA buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1% NP-40 and 1 mM EDTA), mixed with a protease and phosphatase inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and phenylmethylsulfonyl fluoride (PMSF) (Biosharp, Hefei, China) for 15 min on ice. Extracted proteins were supplemented with loading buffer containing 5% 2-mercaptoethanol and then denatured at 100˚C for 10 min. The protein samples were separated by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 with 5% non-fat milk for 2 h at room temperature. Subsequently, membranes were incubated with specific primary antibodies overnight at 4˚C. All the primary antibodies were diluted in Tris-buffered saline containing 0.1% Tween-20 with 5% bovine serum

albumin (BSA; Biosharp, Hefei, China). The dilution ratio for β-actin and GAPDH was 1:5,000, and all the other antibodies were in 1:1,000. Next, the membranes were treated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3,000 dilution). The blots were visualized with ECL western blotting reagents (EMD Millipore), following the manufacturer's instructions. HRP-conjugated anti-mouse IgG (7076; Cell Signaling Technology, Inc.) and anti-rabbit antibodies (7074; Cell Signaling Technology, Inc.) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The following antibodies were used: HDAC3 (ab32369; Abcam, Cambridge, UK), cleaved caspase 3 (9664s; Cell Signaling Technology, Inc.), cleaved PARP (5625s; Cell Signaling Technology, Inc.), PKM2 (4053s; Cell Signaling Technology, Inc.), PDHA1 (ab92696; Abcam), LDHA (3582s; Cell Signaling Technology, Inc.), GLUT1 (12939; Cell Signaling Technology, Inc.), GAPDH (ab128915; Abcam) and β-actin (A5441; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

Apoptosis assay. Cellular apoptosis was detected with an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (556547; BD Biosciences, Franklin Lakes, NJ, USA), following the manufacturer's instructions. Briefly, HuCCT1 and RBE cells were seeded in 6-well plates for 24 h. Then cells were cultured in normal medium or treated with 10 mM metformin, 10 μM BG45, or the combination of both drugs. Next, the control and treated cells were collected and resuspended in 100 μl Annexin V binding buffer, and incubated with 5 μl FITC-conjugated Annexin V and 5 μl propidium iodide (PI) for 15 min in the dark. Annexin V binding buffer (400 μl) was then added to each tube. Samples were analyzed using a BD FACSCanto II flow cytometer (BD Biosciences) with CellQuest (BD Biosciences) version 3.3 software within 1 h.

Cell viability assay. Cell viability was measured using a Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) in 96-well plates ($2x10^3$ cells/well). Cells were seeded in 96-well plates. At 24 h, drugs were added and cells were cultured for 24, 48 or 72 h at 37˚C with 5% CO_2. After treatment, the medium was removed and 100 μl CCK-8 reagent was added to each well, according to the manufacturer's instructions. Then, the cells were incubated for 90 min at 37˚C with 5% CO_2. The absorbance of the samples at 450 nm was detected using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Relative cell viability (%) = (absorbance at 450 nm of the treated group-absorbance at 450 nm of the blank)/(absorbance at 450 nm of the control group-absorbance at 450 nm of the blank) x 100%.

Lactate production. HuCCT1 and RBE cells were seeded in 6-well plates at $2.5x10^5$ cells/well. At confluence, the cells were treated with or without 10 mM metformin for 24 h. Then the media and cells were collected separately for lactate production detection. The lactate production was detected with a lactate assay kit (K627; BioVision, Inc., Milpitas, CA, USA), according to the manufacturer's instructions. The absorbance values were measured at 450 nm with a spectrophotometer (BioTek Instruments, Inc.). The values were normalized to the cell number.

Mitochondrial oxidative phosphorylation analysis. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were detected in real time with an XF96 Extracellular Flux Analyzer from Seahorse Bioscience, Inc. (North Billerica, MA, USA), following the manufacturer's instructions. HuCCT1 and RBE cells were plated in 96-well XF cell culture microplates at $1x10^4$ cells/well and incubated for 24 h at 37˚C. Then, 1 mM metformin was added into each well of the plates. Before measurement, the medium was replaced with 175 μl/well XF-96 running medium (supplemented with RPMI-1640 without serum) and pre-incubated at 37˚C for 20 min in the absence of CO_2. For each analysis, different compounds that modulate mitochondrial respiration were injected into each well, according to standard protocols: for OCR, oligomycin, carbonylcyanide p-trifluoromethoxy-phenylhydrazone, rotenone and antimycin A; for ECAR, glucose, oligomycin and 2-deoxy-D-glucose. The cell number was used for data normalization. OCR is expressed as pmol/min (picomoles/minute). ECAR is expressed as mpH/min [milli-pH units (mpH) per minute].

CCA cancer xenograft model. Nude mice were purchased from the Department of Laboratory Animal Science, Nanjing Drum Tower Hospital. HuCCT1 cells ($3x10^6$) in serum-free RPMI-1640 medium were subcutaneously injected into the right flank of the mice. Once palpable tumors were exhibited, mice were randomly assigned into 4 groups: control (100 μl natural saline, NS), metformin (200 mg/kg diluted in 100 μl NS), BG45 (20 mg/kg diluted in 100 μl NS), and a combination of both drugs (metformin at 200 mg/kg and BG45 at 20 mg/kg in 100 μl NS). Each group received an intraperitoneal injection 3 times/week for 4 weeks. Tumor volume (V) was calculated using the following formula: V = length x $width^2/2$. All the experiments involving animals were reviewed and approved by the Animal Welfare Committee of Nanjing Drum Tower Hospital.

Statistical analysis. Data are expressed as the mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was performed with Dunnett's multiple comparisons test (SPSS 17.0; SPSS, Inc., Chicago, IL, USA). The Chi-squared test and unpaired Student's t-test were performed for comparisons between two groups. Overall survival time was calculated by the Kaplan-Meier analysis. Staining scores were analyzed by the log-rank test. $P<0.05$ was considered to indicate a statistically significant difference. In figures, $^{*}P<0.05$ and $^{**}P<0.01$.

Results

Metabolic abnormalities are important features of CCA. Reprogramming of cellular metabolism is common in tumor cells, and is regulated by the expression of multiple genes, thus accelerating the malignant behavior of tumor cells (24). Therefore, we analyzed the differentially expressed genes (DEGs) in CCA using data from The Cancer Genome Atlas (TCGA). We confirmed the DEGs and detected the potential

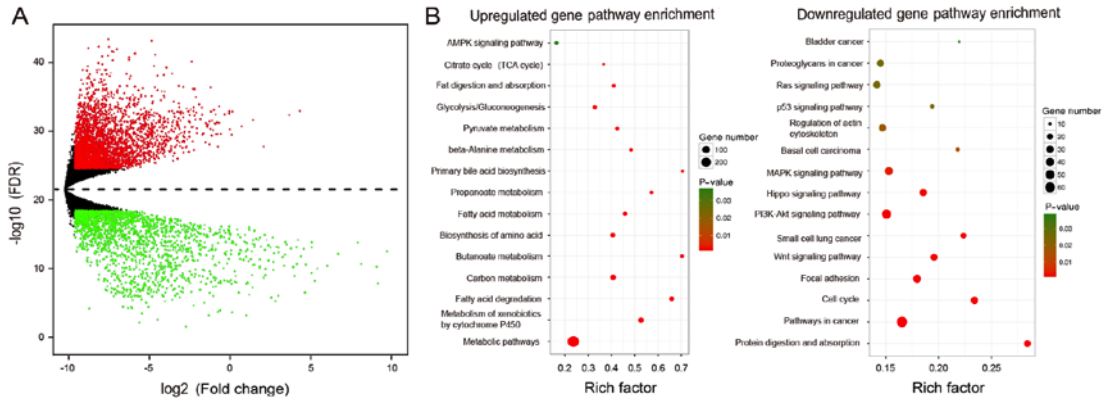

Figure 1. Metabolic abnormalities are important features of CCA. (A) Heat map of differentially expressed genes (DEGs) in cholangiocarcinoma (CCA) and paired normal liver samples. DEGs with a fold change (FC) >2 are shown in red; DEGs with FC ≤2 are in green (P<0.01), and the false discovery rate (FDR) was <0.05. (B) KEGG annotation pathways of high- and low-expression genes in CCA.

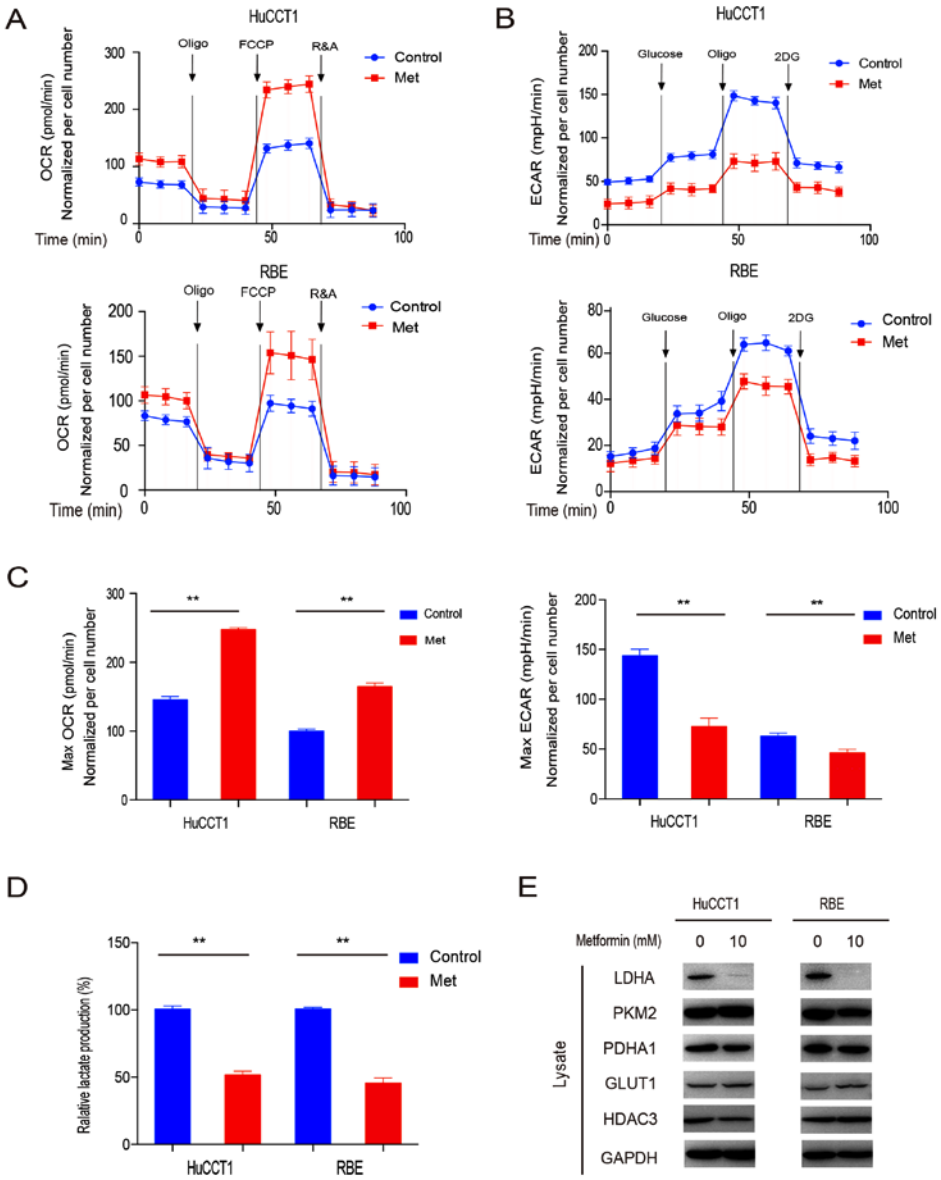

Figure 2. Metformin suppresses the Warburg effect through inhibition of LDHA. (A) The oxygen consumption rate (OCR) of cholangiocarcinoma (CCA) cells was detected at different time points after treatment with 1 mM metformin. OCR was calculated under oligomycin, carbonylcyanide p-trifluoromethoxy-phenylhydrazone (FCCP), and antimycin A/rotenone treatments. (B) The extracellular acidification rate (ECAR) of CCA cells was measured at different points following treatment with 1 mM metformin. ECAR was calculated under glucose, oligomycin and 2-deoxy-D-glucose (2-DG). (C) Max OCR (left) and ECAR (right) were calculated. (D) CCA cells were treated with 10 mM metformin and the relative lactate output was observed. (E) CCA cells were treated with 10 mM metformin and the indicated proteins were detected by western blotting. Data represent the mean ± SEM, n≥3. **P<0.01. OCR is expressed as pmol/min (picomoles/minute). ECAR is expressed as mpH/min [milli-pH units (mpH) per minute].

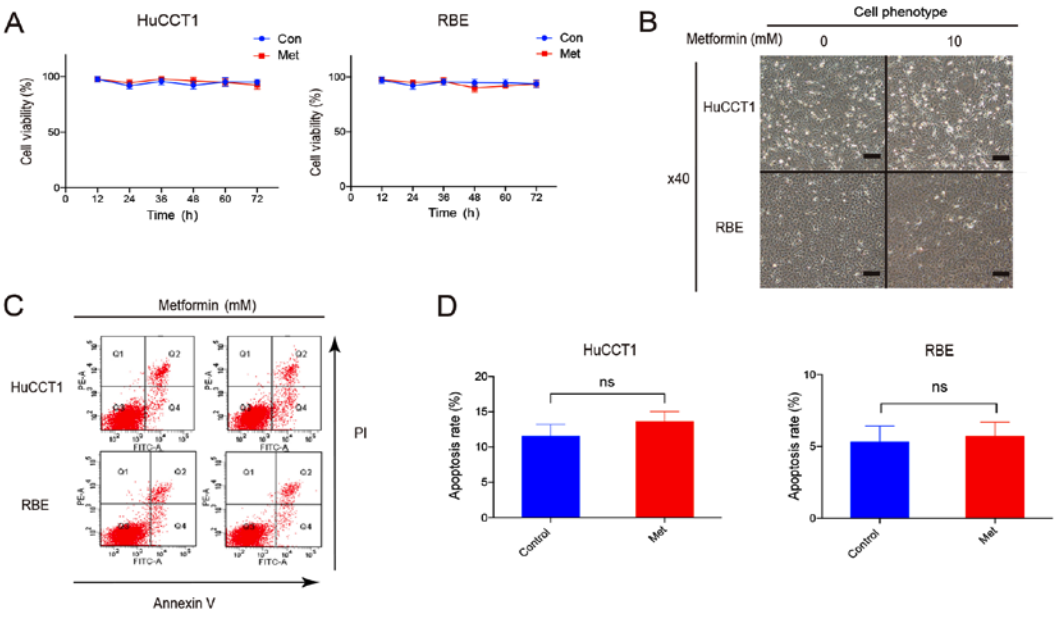

Figure 3. Metformin marginally affects the growth of CCA cells. (A) Cholangiocarcinoma (CCA) cell proliferation was analyzed via CCK-8 assay following treatment with 10 mM metformin. (B) CCA cells were treated with 10 mM metformin for 24 h and the morphological changes were observed. Scale bars, 100 μm. (C) CCA cells were treated with 10 mM metformin for 24 h; apoptotic cells were measured by flow cytometry and (D) quantified. Data represent the mean ± SEM, n≥3. ns, not significant.

biological functions and pathways of these genes in CCA through KEGG pathway analysis (Fig. 1A). We observed that the most relevant pathways in CCA were metabolic and tumor growth pathways (Fig. 1B). Together, these results indicate that metabolic reprogramming is an important feature of CCA; moreover, these results underscore the complex and unclear regulatory mechanisms.

Metformin suppresses the Warburg effect in CCA cells by decreasing LDHA. Metformin is a widely accepted first-line drug for the treatment of type 2 diabetes (5). Many studies have shown that metformin could serve as a potential cancer therapy, although little is known concerning the mechanism of its antitumor functionality (25,26). It has been reported that the antitumor effects of metformin are partially caused by altering cellular metabolism (14). We then investigated the effects of metformin on the cellular metabolic status. After employing the Seahorse bio-energy analyzer, we found that OCR was notably increased, while ECAR was markedly decreased after the use of metformin (Fig. 2A-C). This indicated the metabolic shift from glycolysis to oxidative phosphorylation in CCA cells following treatment with metformin.

To further validate the anti-Warburg effect properties of metformin, we measured the level of lactate production after metformin. We found a significant decrease in the lactate production of both CCA cell lines after the treatment, which confirmed the effect of metformin on reversing the Warburg effect (Fig. 2D).

To clarify the mechanisms underlying the fact that metformin suppressed the Warburg effect, we detected a number of candidate proteins that may be involved in the Warburg effect. We found that the expression of LDHA was notably decreased following the use of metformin, although this result was not observed with the other possible proteins PKM2, PDHA1, GLUT1 and HDAC3 (Fig. 2E). Collectively, these data suggested that metformin reversed the Warburg effect by decreasing LDHA in CCA cells.

Metformin marginally affects the growth of CCA cells. To explore the function of metformin in CCA, we detected the proliferation and apoptosis of CCA cells. Notably, we observed that a low concentration of metformin did not inhibit cell proliferation or induce cellular apoptosis, although the Warburg effect was suppressed (Fig. 3A-D). This suggested that metformin alone may only slightly affect the proliferation or apoptosis of CCA cells, although it suppresses the Warburg effect.

Metformin facilitates BG45-induced apoptosis. Several studies have reported that metabolic abnormalities can accelerate malignant behaviors, increase chemoresistance and inhibit tumor cell apoptosis (12,27). As we mentioned above, the present study demonstrated that metformin could regulate the energy utilization of CCA by decreasing the expression of LDHA. It is possible that reversing the Warburg effect in CCA cells with metformin could then increase tumor cell fragility and render them more susceptible to chemotherapy (14,28). Recently, we found that HDAC3 inhibitors are promising chemotherapeutic agents in the treatment of CCA (20). BG45, a novel HDAC3 selective inhibitor, has been validated as a therapeutic agent in multiple myeloma (16). However, our data showed that in CCA, the antitumor properties of BG45 were not significantly effective (Fig. 4A). As a result, we tested the combination treatment of metformin and BG45, and found that this combination inhibited cell viability (Fig. 4A and B). We further explored the mechanism of this combination-induced cell viability inhibition by performing flow cytometry. The results revealed that combined metformin and BG45

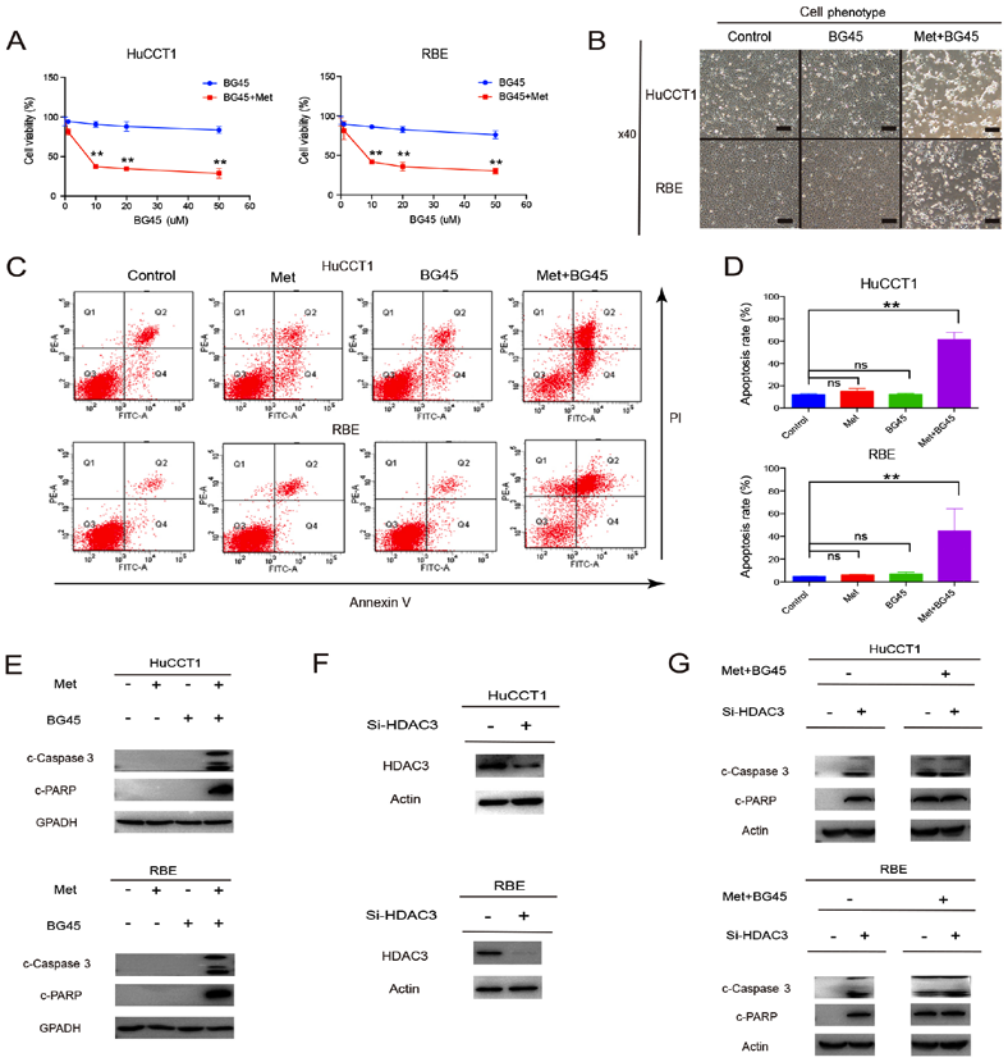

Figure 4. Metformin facilitates BG45-induced apoptosis. (A) Cholangiocarcinoma (CCA) cells were treated with BG45 0-60 μM alone or a combination of BG45 0-60 μM and metformin 10 mM for 24 h, then cell proliferation was quantified via CCK-8 assay. (B) CCA cells were treated with BG45 10 μM or a combination of BG45 10 μM and metformin 10 mM for 24 h, and cellular phenotype changes were observed. Scale bars, 100 μm. (C and D) After treatment with metformin 10 mM, BG45 10 μM, or a combination of both, CCA cells were analyzed using Annexin V/PI staining, and then apoptosis was measured by flow cytometry (left). The percentages of early and late apoptotic cells were quantified (right). (E) Cells were treated with 10 mM metformin, 10 μM BG45, or the combination of both drugs for 48 h, and then lysates were collected and apoptosis-associated markers were examined via western blot analysis. (F) HDAC3 was inhibited with siRNA, and then protein samples were collected and HDAC3 was detected via western blotting. (G) HDAC3 was inhibited with siRNA, cells were treated with 10 mM metformin and 10 μM BG45, protein samples were collected, and apoptotic markers were detected via western blotting. ns, not significant; **P<0.01.

led to a significant increase in the apoptosis of CCA cells, compared with single drug use alone (Fig. 4C and D). Consistent with the flow cytometry results, higher levels of cleaved caspase 3 and cleaved PARP were found in CCA cells treated with the combined treatment, compared to single drug treatment (Fig. 4E). We further inhibited HDAC3 using siRNA (Fig. 4F), and found that the expression levels of cleaved caspase 3 and cleaved PARP were significantly increased. However, the levels of these two apoptotic markers were not promoted following treatment with the combination of both drugs after HDAC3 inhibition (Fig. 4G).

Collectively, these results demonstrated that combined treatment using metformin and BG45 could significantly inhibit the proliferation of CCA cells by inducing apoptosis. Although metformin alone hardly induced cellular apoptosis at low concentrations, it did nonetheless facilitate HDAC3 inhibitor BG45-induced apoptosis.

Effects of metformin and BG45 on tumor xenografts. To further validate our findings *in vitro*, we evaluated the antitumor effect of the combined treatment *in vivo* using a CCA cell tumor xenograft model. We observed that the combined treatment group significantly inhibited tumor growth compared to the monotherapy groups (Fig. 5A and B). The weight loss of the mice was not found to be significant, which indicated that the combination therapy was safe *in vivo* (Fig. 5B). Altogether, these data revealed that the combination of metformin and BG45 could significantly induce cellular apoptosis and inhibit proliferation *in vivo*.

LDHA expression is upregulated in CCA tissues and indicates poor prognosis. By evaluating the expression of LDHA on the tissue microarrays from Shanghai Outdo Biotech Co., Ltd., we found that LDHA was significantly upregulated in tumor tissues compared to that noted in adjacent tissues (Fig. 5C and D).

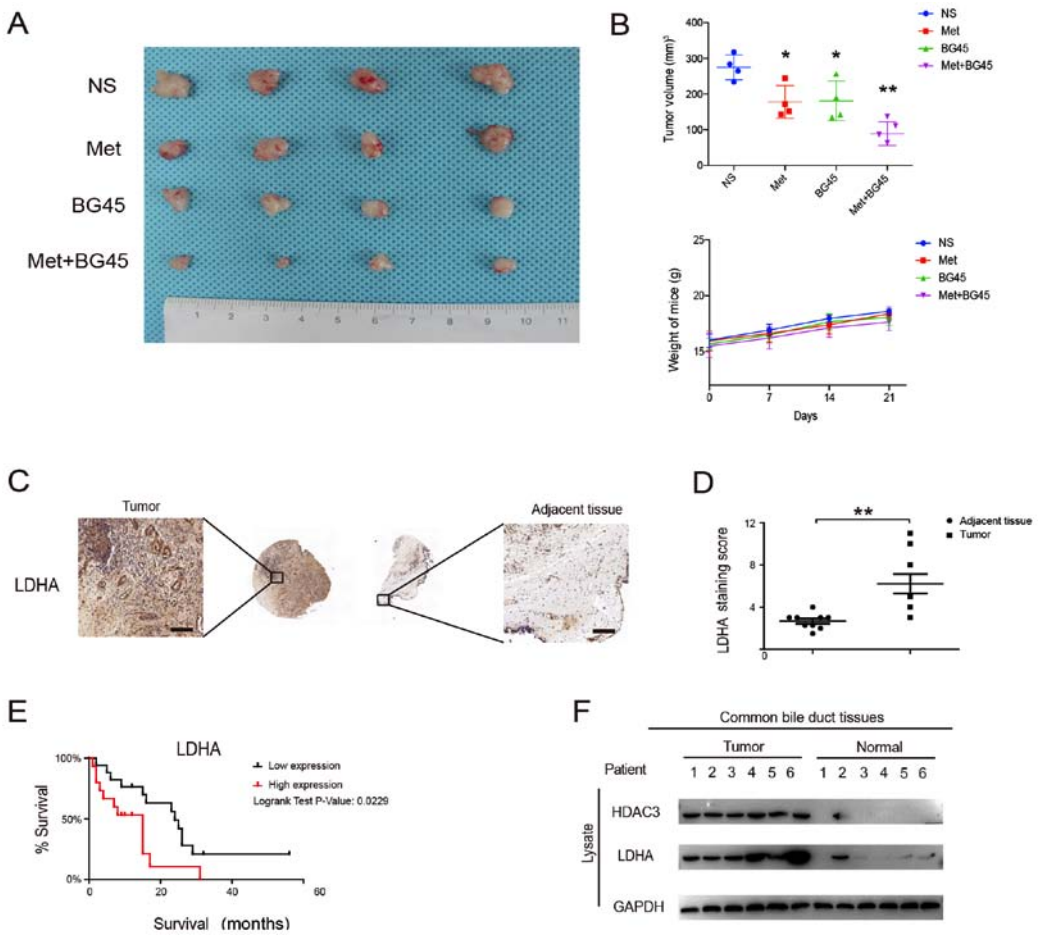

Figure 5. Effects of combined treatment with metformin and BG45 on nude mice, and LDHA expression in CCA tissues. (A) HuCCT1 cells were injected subcutaneously into the flanks of nude mice. When tumors were palpable, normal saline (NS), metformin, BG45, or a combination of both was administrated. Tumors were collected and photographed. (B) Tumor volumes of mice were measured after 3 weeks (upper). The body weights of mice were compared among the groups (lower). (C) LDHA protein levels in tumor and adjacent normal tissues from tissue microarrays were detected and (D) quantified. (E) Kaplan-Meier curves for overall survival in CCA patients with high protein expression and low protein expression (n=33). (F) HDAC3 and LDHA protein levels in normal tissues and CCA tissues from 12 donors were detected by western blotting. Data represent the mean ± SEM, n≥3. *P<0.05; **P<0.01.

Furthermore, we evaluated the clinical data of the tissue microarrays and found that LDHA protein was overexpressed in 68/127 cases (54.5%), and was associated with tumor size (Table I). Employing the 33 follow-up cases, we found that high LDHA protein expression in CCA reduced patient survival (P<0.001, log-rank test) (Fig. 5E). Next, we assessed the expression of HDAC3 and LDHA in fresh tissues, and found that HDAC3 and LDHA were markedly upregulated in tumor tissues compared to levels noted in normal tissues (Fig. 5F). Collectively, our data suggest that LDHA is overexpressed in CCA tissues and is associated with a worse prognosis.

Discussion

CCA is a highly lethal disease, with an increasing incidence and mortality rate worldwide (1). Due to the lack of effective diagnostic methods, most patients with CCA are diagnosed at an advanced stage, and are thus ineligible for surgical resection (1). Although these patients can and do receive palliative chemotherapy (cisplatin and gemcitabine), the efficacy is limited due to drug resistance (2,4). Therefore, there remains an urgent need to develop new potential treatments for this malignancy.

In the present study, we showed that metformin could suppress the Warburg effect in CCA, which decreases aerobic glycolysis and promotes oxidative phosphorylation, thus making CCA cells vulnerable to chemotherapy. Moreover, we found that LDHA is more susceptible to metformin than other indicated proteins. Furthermore, we demonstrated that the combination of metformin and the HDAC3 inhibitor BG45 can be used as a novel curative therapeutic strategy in the treatment of CCA.

Through bioinformatic analysis, we found that metabolic and tumor proliferation pathways were most relevant in CCA. The Warburg effect, also known as aerobic glycolysis, refers to the phenomenon whereby cancer cells display a high level of glucose uptake and metabolism by glycolysis, even in the presence of normal oxygen levels (12). This leads to metabolic abnormalities in cancer cells and thereby promotes malignant behaviors, increases chemoresistance, and inhibits tumor cell apoptosis.

Metformin is a widely adopted therapy for type 2 diabetes. Recent studies have confirmed its antitumor properties, but the mechanisms require further elaboration (25,26). We found that low concentrations of metformin could change the metabolic status of tumor cells and reverse the Warburg effect through

Table I. Clinical characteristics and metabolic protein levels in patients with cholangiocarcinoma.

		LDHA expression		
	N	Low, n (%)	High, n (%)	P-value
Sex				0.499
Male	80	39 (49)	41 (51)	
Female	47	20 (43)	27 (57)	
Age (years)				0.393
≤70	99	44 (44)	55 (56)	
>70	28	15 (54)	13 (46)	
Size (mm)				0.012[a]
≤7	71	40 (56)	31 (44)	
>7	56	19 (34)	37 (66)	
Differentiation				0.186
Well	107	47 (44)	60 (56)	
Poor	20	12 (60)	8 (40)	
T stage				0.052
T1-T2	113	56 (50)	57 (50)	
T3	14	3 (21)	11 (79)	
Lymph node metastasis				0.535
Negative	102	46 (45)	56 (55)	
Positive	25	13 (52)	12 (48)	
Venous invasion				0.542
Negative	114	54 (47)	60 (53)	
Positive	13	5 (38)	8 (62)	
Nerve invasion				0.982
Negative	114	53 (46)	61 (54)	
Positive	13	6 (46)	7 (54)	

[a]P<0.05 indicates significance.

the inhibition of LDHA, which was overexpressed in CCA tissues and indicated a shorter survival time.

Notably, we found that metformin alone could hardly induce cellular apoptosis in CCA cells. It is widely accepted that multiple key pathways, as well as genes, converge to change cellular metabolism in order to support tumor growth and development (29). Therefore, it is difficult to induce cellular apoptosis under single-target inhibition.

Since metabolic reprogramming in tumor cells could possibly endow the cells with resistance to chemotherapies (30,31), we thought that low concentrations of metformin could alter the metabolic abnormalities of tumor cells, causing the cells to become fragile and sensitive to chemotherapy. Our previous data showed that HDAC3 is a potential chemotherapeutic target (20). However, the present study found that the new selective HDAC3 inhibitor, BG45, could hardly induce CCA cellular apoptosis. As a result, we utilized a combination of low-concentration metformin and BG45, and found that cell viability, as well as proliferation, was inhibited, and that the apoptosis rate was increased dramatically. These results were validated both *in vitro* and *in vivo*. In summary, we revealed that in CCA, metformin could be adopted as a chemotherapy sensitizer, which could enhance the antitumor properties of HDAC3 inhibitors.

Our findings suggest that metformin could reverse the Warburg effect through inhibition of LDHA. This sensitizes cells to the antitumor effects of HDAC3 inhibitors and induces cellular apoptosis both *in vitro* and *in vivo*. Our study provides support for the use of metformin with BG45 as a novel therapeutic strategy in CCA treatment.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MZ, LW, XZ designed the study; DT, LX, QZ, YL and YP did the cell experiments; YL, YY, YW, LZ and DT collected the tissue samples; QZ, DT and YL performed the protein analysis; LZ, DT, LX, MZ, RGD drafted the manuscript and performed the immunohistochemistry experiment; RGD did the language editing; MZ, LW and XZ supported the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol for the animal experiments was reviewed and approved by the Ethics Committee of Medical Research, Nanjing Drum Tower Hospital, Affiliated Hospital of Nanjing University Medical School (Nanjing, China). Donors provided consent for any use of human samples for research and the study protocol was approved by the Ethics Committee of Medical Research, Nanjing Drum Tower Hospital, Affiliated Hospital of Nanjing University Medical School.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Razumilava N and Gores GJ: Cholangiocarcinoma. Lancet 383: 2168-2179, 2014.
2. Rizvi S and Gores GJ: Pathogenesis, diagnosis, and management of cholangiocarcinoma. Gastroenterology 145: 1215-1229, 2013.
3. Bridgewater J, Galle PR, Khan SA, Llovet JM, Park JW, Patel T, Pawlik TM and Gores GJ: Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. J Hepatol 60: 1268-1289, 2014.
4. Valle JW, Lamarca A, Goyal L, Barriuso J and Zhu AX: New horizons for precision medicine in biliary tract cancers. Cancer Discov 7: 943-962, 2017.
5. Inzucchi SE, Lipska KJ, Mayo H, Bailey CJ and McGuire DK: Metformin in patients with type 2 diabetes and kidney disease: A systematic review. Jama 312: 2668-2675, 2014.
6. Schneider MB, Matsuzaki H, Haorah J, Ulrich A, Standop J, Ding XZ, Adrian TE and Pour PM: Prevention of pancreatic cancer induction in hamsters by metformin. Gastroenterology 120: 1263-1270, 2001.
7. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR and Morris AD: Metformin and reduced risk of cancer in diabetic patients. BMJ 330: 1304-1305, 2005.
8. Camacho L, Dasgupta A and Jiralerspong S: Metformin in breast cancer-an evolving mystery. Breast Cancer Res 17: 88, 2015.
9. Ezewuiro O, Grushko TA, Kocherginsky M, Habis M, Hurteau JA, Mills KA, Hunn J, Olopade OI, Fleming GF and Romero IL: Association of Metformin Use with outcomes in advanced endometrial cancer treated with chemotherapy. PLoS One 11: e0147145, 2016.
10. Griss T, Vincent EE, Egnatchik R, Chen J, Ma EH, Faubert B, Viollet B, DeBerardinis RJ and Jones RG: Metformin antagonizes cancer cell proliferation by suppressing mitochondrial-dependent biosynthesis. PLoS Biol 13: e1002309, 2015.
11. Ling S, Xie H, Yang F, Shan Q, Dai H, Zhuo J, Wei X, Song P, Zhou L, Xu X and Zheng S: Metformin potentiates the effect of arsenic trioxide suppressing intrahepatic cholangiocarcinoma: Roles of p38 MAPK, ERK3, and mTORC1. J Hematol Oncol 10: 59, 2017.
12. Vander Heiden MG, Cantley LC and Thompson CB: Understanding the Warburg effect: The metabolic requirements of cell proliferation. Science 324: 1029-1033, 2009.
13. Green DR, Galluzzi L and Kroemer G: Cell biology. Metabolic control of cell death. Science 345: 1250256, 2014.
14. Liu X, Romero IL, Litchfield LM, Lengyel E and Locasale JW: Metformin targets central carbon metabolism and reveals mitochondrial requirements in human cancers. Cell Metab 24: 728-739, 2016.
15. Zhijun H, Shusheng W, Han M, Jianping L, Li-sen Q and Dechun L: Pre-clinical characterization of 4SC-202, a novel class I HDAC inhibitor, against colorectal cancer cells. Tumor Biol 37: 10257-10267, 2016.
16. Minami J, Suzuki R, Mazitschek R, Gorgun G, Ghosh B, Cirstea D, Hu Y, Mimura N, Ohguchi H, Cottini F, *et al*: Histone deacetylase 3 as a novel therapeutic target in multiple myeloma. Leukemia 28: 680-689, 2014.
17. Lakshmaiah KC, Jacob LA, Aparna S, Lokanatha D and Saldanha SC: Epigenetic therapy of cancer with histone deacetylase inhibitors. J Cancer Res Ther 10: 469-478, 2014.
18. Harting K and Knöll B: SIRT2-mediated protein deacetylation: An emerging key regulator in brain physiology and pathology. Eur J Cell Biol 89: 262-269, 2010.
19. Wang LT, Liou JP, Li YH, Liu YM, Pan SL and Teng CM: A novel class I HDAC inhibitor, MPT0G030, induces cell apoptosis and differentiation in human colorectal cancer cells via HDAC1/PKCδ and E-cadherin. Oncotarget 5: 5651-5662, 2014.
20. Yin Y, Zhang M, Dorfman RG, Li Y, Zhao Z, Pan Y, Zhou Q, Huang S, Zhao S, Yao Y and Zou X: Histone deacetylase 3 overexpression in human cholangiocarcinoma and promotion of cell growth via apoptosis inhibition. Cell Death Dis 8: e2856, 2017.
21. Zhang QL, Wang L, Zhang YW, Jiang XX, Yang F, Wu WL, Janin A, Chen Z, Shen ZX, Chen SJ and Zhao WL: The proteasome inhibitor bortezomib interacts synergistically with the histone deacetylase inhibitor suberoylanilide hydroxamic acid to induce T-leukemia/lymphoma cells apoptosis. Leukemia 23: 1507-1514, 2009.
22. Richon VM, Emiliani S, Verdin E, Webb Y, Breslow R, Rifkind RA and Marks PA: A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. Proc Natl Acad Sci USA 95: 3003-3007, 1998.
23. Xiao Y, Wang J, Qin Y, Xuan Y, Jia Y, Hu W, Yu W, Dai M, Li Z, Yi C, *et al*: Ku80 cooperates with CBP to promote COX-2 expression and tumor growth. Oncotarget 6: 8046-8061, 2015.
24. Shibata T and Aburatani H: Exploration of liver cancer genomes. Nat Rev Gastroenterol Hepatol 11: 340-349, 2014.
25. Alimova IN, Liu B, Fan Z, Edgerton SM, Dillon T, Lind SE and Thor AD: Metformin inhibits breast cancer cell growth, colony formation and induces cell cycle arrest in vitro. Cell Cycle 8: 909-915, 2009.
26. Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, Tanti JF, Le Marchand-Brustel Y and Bost F: The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene 27: 3576-3586, 2008.
27. Zhou Y, Tozzi F, Chen J, Fan F, Xia L, Wang J, Gao G, Zhang A, Xia X, Brasher H, *et al*: Intracellular ATP levels are a pivotal determinant of chemoresistance in colon cancer cells. Cancer Res 72: 304-314, 2012.
28. Iliopoulos D, Hirsch HA and Struhl K: Metformin decreases the dose of chemotherapy for prolonging tumor remission in mouse xenografts involving multiple cancer cell types. Cancer Res 71: 3196-3201, 2011.
29. Vander Heiden MG: Targeting cancer metabolism: A therapeutic window opens. Nat Rev Drug Discov 10: 671-684, 2011.
30. Shukla SK, Purohit V, Mehla K, Gunda V, Chaika NV, Vernucci E, King RJ, Abrego J, Goode GD, Dasgupta A, *et al*: MUC1 and HIF-1alpha signaling crosstalk induces anabolic glucose metabolism to impart gemcitabine resistance to pancreatic cancer. Cancer Cell 32: 71-87.e7, 2017.
31. Herranz D, Ambesi-Impiombato A, Sudderth J, Sánchez-Martín M, Belver L, Tosello V, Xu L, Wendorff AA, Castillo M, Haydu JE, *et al*: Metabolic reprogramming induces resistance to anti-NOTCH1 therapies in T cell acute lymphoblastic leukemia. Nat Med 21: 1182-1189, 2015.