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.

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 asromidepsin, 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) (Biocharp, 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
M. C. Cetinkaya, M. Ergün, M. Yılmaz, G. H. Gür, I. I. Dursun, F. V. Kaplan

Results

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

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 CO₂. 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 mP/min [milli-pH units (mP) 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].
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).

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

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*P<0.05 indicates significance.
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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 University Medical School (Nanjing, China). Donors provided ethical approval and consent to participate.

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