RESEARCH ARTICLE

Metformin Inhibits Migration and Invasion of Cholangiocarcinoma Cells

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Abstract

Background: Metformin is an oral anti-diabetic agent that has been widely prescribed for treatment of type II diabetes. Anti-cancer properties of metformin have been revealed for numerous human malignancies including cholangiocarcinoma (CCA) with anti-proliferative effects *in vitro*. However, effects on CCA cell migration and invasion have not been fully investigated. The present study aimed to explore the inhibitory effects of metformin on motility, migration and invasion of the CCA cell line HuCCT1, and examine molecular mechanisms underlying metformin effects. **Methods:** HuCCT1 cells were exposed to increasing doses of metformin. Viability and growth of HuCCT1 cells were assessed by MTS and colony formation assays, respectively. Motility, migration and invasion of metformin-treated HuCCT1 cells were determined *in vitro* using wound healing, transwell migration and matrigel invasion assays. Expression of signaling molecules and epithelial-mesenchymal transition (EMT) markers was assessed by Western blotting. **Results:** It was observed that metformin significantly decreased HuCCT1 cell viability and colony formation. The agent also markedly reduced wound closure, migration and invasion of HuCCT1 cells. Furthermore, metformin exposure resulted in decreased STAT3 activation and down-regulation of anti-apoptotic protein Bcl-2 and Mcl-1 expression. In addition, it upregulated the expression of E-cadherin, while downregulating that of N-cadherin, Snail, and MMP-2. **Conclusion:** These results demonstrated inhibitory effects of metformin on CCA cell migration and invasion, possibly involving the STAT3 pathway and reversal of EMT markers expression. They further suggest that metformin may be useful for CCA management.

Keywords: Cholangiocarcinoma- metformin- migration- invasion- STAT3- EMT

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor originating in the biliary tree, and is the second most common liver cancer after hepatocellular carcinoma (HCC) (Rizvi and Gores, 2013). The incidence of CCA, particularly the intrahepatic type appears to be increasing. Age-adjusted rates of CCA are found to be highest in Hispanic and Asian populations (2.8–3.3 per 100,000) (Razumilava and Gores, 2014), indicating that CCA remains a public health concern in many regions including Asian countries. CCA is typically diagnosed when tumor has reached advanced and incurable stages, and most patients have a poor prognosis. This epithelial cancer has high metastatic capability. Metastatic dissemination of CCA was detected in many organs such as brain (Chindaprasirt et al., 2012), colon (Wakahara et al., 2005), and stomach (Kim et al., 2009). Since tumor metastasis is associated with poor prognosis and short survival of patients, alternative therapeutic approaches that can effectively inhibit this complex process is urgently needed for CCA.

Metformin is an oral anti-diabetic drug belonging to the biguanide family. It has been used widely as the first line therapy for type II diabetes (Inzucchi et al., 2015). In recent years, metformin has received considerable attention due to its remarkable anti-cancer potential observed in many human tumors. A number of epidemiologic studies showed that metformin use in diabetic patients was associated with a decreased incidence of various cancers, including gastroenterological cancers (Lee et al., 2011), pancreatic cancer (Li et al., 2009), breast cancer (Bodmer et al., 2010), and CCA. Intrahepatic CCA risk was reduced (60%) in diabetic patients receiving metformin (Chaiteerakij et al., 2013). Moreover, an experimental study elucidated that metformin could inhibit CCA cell proliferation by inducing apoptosis and cell cycle arrest via targeting the AMPK/mTORC1. In addition, metformin also sensitized CCA cells to chemotherapeutic agents by targeting multiple signaling proteins (Ling et al., 2014). These evidence highlight anti-cancer effects of metformin in CCA; however, further investigations are still required

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to elucidate whether metformin can inhibit migration and invasion of CCA cells.

In this study, the effects of metformin on CCA cell proliferation, motility, migration and invasion were assessed *in vitro*. The molecular mechanisms underlying metformin activities were also explored. It was found that metformin could significantly inhibit proliferation, migration and invasiveness of HuCCT1, accompanied by suppression of STAT3 activation and reversal of EMT markers.

Materials and methods

Cell line and Reagents

Human cholangiocarcinoma cell line HuCCT1 was obtained from the RIKEN BRC (Ibaraki, Japan). The cell line was cultured in RPMI-1640 (Gibco, Waltham, MA, USA) in the presence of 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Waltham, MA, USA) at 37°C in a 5% CO2 humidified incubator. Metformin (1,1-dimethylbiguanide hydrochloride) was procured from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against STAT3, phosphorylated STAT3 (p-STAT3), Bcl-2, Mcl-1, E-cadherin, N-cadherin, Snail, and MMP-2 were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA).

Cell viability assays

HuCCT1 cells suspended in 10% FBS-supplemented media were added to 96-well plates ($4x10^3$ cells/100 μL/well) and incubated overnight. Next day, HuCCT1 cells were treated with 0, 10, 20, or 50 mM metformin diluted in fresh media. After 24, 48, and 72 h incubation, MTS solution (Promega, Fitchburg, WI, USA) was added (20 μL/well) and incubated for 2 h at 37°C, after which the absorbance at 490 nm was measured using a microplate reader (Infinite 200 PRO, Tecan Trading AG, Zurich, Switzerland).

Colony formation assay

A total of $1x10^3$ cells were seeded in a 6-well plate. The cells were cultured in 10% FBS-supplemented media for 24 h. Then, the media was discarded and the cells were cultured in fresh media containing 0, 0.5, or 1 mM metformin for 2 weeks. Media containing each concentration of metformin was replaced every three days to maintain metformin activity during the course of treatment. Colonies formed on plates were fixed with methanol and stained with 0.5 % crystal violet. Number of colonies were analyzed and counted under light microscope.

Wound healing assay

HuCCT1 cells were seeded in a 6-well plate, cultured until reached approximately 90% confluency, and were scratched with a sterile pipette tip to generate a wound. The scratched cells were washed with phosphate-buffered saline (PBS) buffer to remove detached cells and were subjected to metformin treatment (0, or 20 mM) for 24 h. Wound closure was observed under microscope at 0 and 24 h of culture for comparison. The wound area at each time point was quantified by ImageJ.

Transwell migration and invasion assay

Cell suspension ($1x10^5$ cells in FBS-media) containing 0, 10 or 20 mM metformin were added into the upper cup of transwell chambers (24-well, 8-μm pore membrane; Corning Incorporated, NY, USA). The lower chamber was supplied with 600 μL of 10% FBS-supplemented media. After 15 h, non-migrating cells inside upper cup were removed by cotton swab. Cells migrating through the membrane to bottom surface of the cup were fixed in methanol, stained with 0.5% crystal violet, and subjected to microscopic analysis. Numbers of migrating cells were counted in ten randomly selected fields. Invasion assay was conducted using 24-well transwell chambers coated with 3 mg/ml matrigel (Corning Incorporated, NY). The invading cells were analyzed after 24 h.

Western blot analysis

The treated cells were collected and total protein was extracted in radioimmunoprecipitation assay (RIPA) lysis buffer (Cell Signaling Technology, Beverly, MA, USA) containing protease and phosphatase inhibitors (Cell Signaling Technology). Concentration of protein samples was measured by Bradford method. An equal amount of protein samples was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Bio-Rad), and blocked with 5% skim milk. The membranes were incubated with primary antibodies at 4°C overnight, washed, and incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 h at room temperature. Protein signals were detected using the ECL Prime detection system (GE healthcare), and β-actin was detected as a loading control.

Statistical analysis

All results were presented as mean ± standard deviation (SD). Comparison between control cells without metformin treatment and metformin-treated cells was performed with PASW Statistics 18 by using Student's t-test. P-value of < 0.05 was accepted as statistically significant difference.

Results

Metformin decreases cell viability and colony formation of HuCCT1

A previous study demonstrated that metformin exerts inhibitory effects against proliferation and colony formation of CCA cell lines (Ling et al., 2014). In this study, we also evaluated these effects of metformin on human CCA cell line HuCCT1. Cell viability of HuCCT1 treated with increasing concentrations of metformin (0, 10, 20, or 50 mM) was measured after 24, 48, and 72 h. As shown in Figure 1A, metformin significantly reduced HuCCT1 viability in a dose- and a time-dependent manners compared to untreated control (0 mM). Next, metformin effect on growth of HuCCT1 cells was investigated by colony formation assay. We found that low
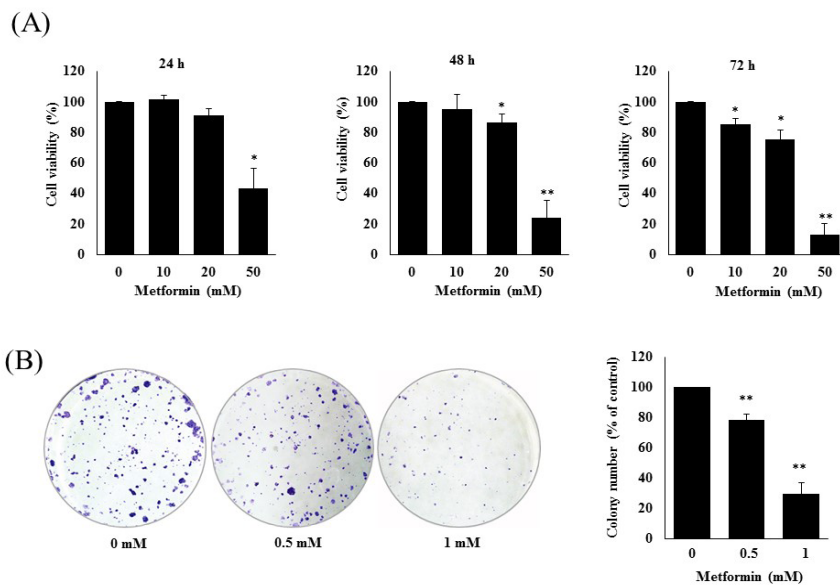
(A)

(B)

Figure 1. Effect of Metformin on Survival and Colony Formation of HuCCT1. (A) Metformin inhibits HuCCT1 cell viability. HuCCT1 cells were treated with increasing doses of metformin (0, 10, 20, or 50 mM) for 24, 48, and 72 h. Cell survival was determined by MTS assay. (B) Metformin reduces colony number of HuCCT1. The cells were grown in 6-well plates, and cultured in the presence of 0, 0.5, or 1 mM metformin. After 2 weeks, the colony number was counted and compared. Data are presented as mean ± standard deviation (SD) from three independent experiments. *P<0.05, **P≤0.01 versus untreated control (0 mM).

concentrations of metformin at 0.5 and 1 mM effectively decreased colony number of HuCCT1 in a dose-dependent manner (100%, 78.1%, and 29.2% for 0, 0.5, and 1 mM metformin, respectively) (Figure 1 B). These results support the anti-proliferative potential of metformin in CCA by reducing cell viability and growth. Metformin at concentrations of ≤ 20 mM did not show significant cytotoxicity, and were used in the subsequent experiments.

Metformin inhibits HuCCT1 cell migration and invasion

Metformin effect on motility of HuCCT1 cells was initially determined by wound healing assay. We observed that exposure of HuCCT1 cells to 20 mM metformin for 24 h significantly delayed cell motility to the wound area compared to untreated control (0 mM) (Figure 2
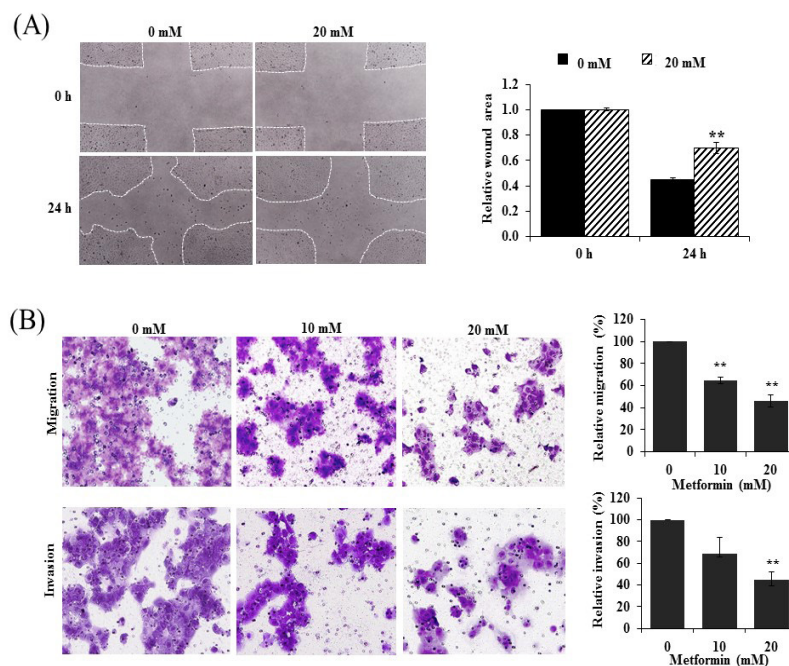
(A)

(B)

Figure 2. Metformin Inhibits HuCCT1 Cell Motility and Invasion. (A) Microscopic appearance of wound healing in metformin-treated cells (20 mM), and control cells (0 mM) at 0 and 24 h. The wound areas were quantified by ImageJ and compared against that of control. (B) Metformin inhibits HuCCT1 migration and invasion. Representative images of migration (top panel) and invasion (bottom panel) of HuCCT1 cells treated with 0, 10, or 20 mM metformin. The inhibitory effects of metformin on cell migration and invasion was quantified by counting ten randomly selected fields under microscope. The results are expressed as mean ± SD from three independent experiments. **P≤0.01 versus control (0 mM).
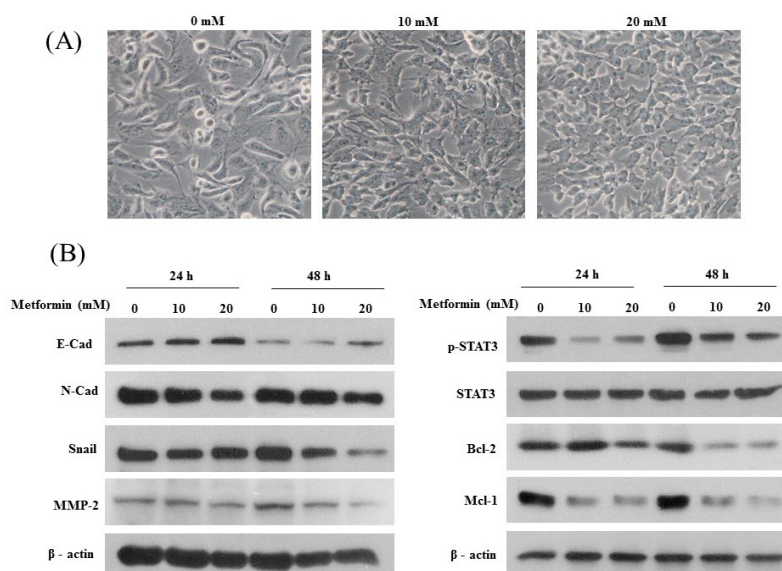
Figure 3. Metformin Effects on HuCCT1 Cell Morphology, EMT Markers, and STAT3 Activation. (A) Morphology of metformin-treated HuCCT1 compared to untreated control (0 mM) after 48 h. (B) HuCCT1 cells treated with 10 or 20 mM metformin for 24 and 48 h were subjected to western blot analyses. Metformin elevated expression level of E-cadherin (E-Cad), while reduced expression of N-cadherin (N-Cad), Snail, and MMP-2. Metformin also inhibited phosphorylated STAT3 (p-STAT3) and expression of Bcl-2 and Mcl-1. Detection of β-actin was included as a loading control.

A). Further investigation by transwell migration assay revealed that the agent substantially reduced the number of migrating cells to 64.8% (10 mM) and 46.4% (20 mM) compared to untreated control (Figure 2 B; top). Likewise, the invasion of metformin-treated HuCCT1 was remarkably decreased to 68.7% (10 mM) and 44.5% (20 mM) (Figure 2 B, bottom). The degrees of inhibition varied upon metformin doses, indicating its dose-dependent effect on cell migration and invasion. These results support that non-cytotoxic concentrations of metformin can inhibit migration and invasion of CCA cells.

Metformin alters EMT markers, STAT3 activation, and pro-survival proteins

Cell morphology of HuCCT1 was altered following the treatment with 10 and 20 mM metformin. Majority of metformin-exposed cells became shrank and less elongated (Figure 3 A). This morphological change led us to hypothesize that expression of molecules mediating cell adhesion could be affected. To test this, we explored expression levels of EMT markers by western blot analysis and observed that expression level of E-cadherin was increased, whereas that of N-cadherin and Snail were decreased in metformin-treated cells. In addition, expression of MMP-2, the extracellular matrix degrading enzyme, was also reduced (Figure 3 B, left). Furthermore, to elucidate molecular mechanisms that underlie metformin activities observed in our study, STAT3 signaling pathway, which plays a pivotal role in tumor growth, migration, and invasion (Huang, 2007; Kamran et al., 2013), was examined by western blot. We observed that phosphorylated STAT3 (p-STAT3; Y705) was markedly decreased following metformin exposure. Concurrently, expression of STAT3 targets consisting of Bcl-2 and Mcl-1 was also downregulated (Figure 3

B, right).

Discussion

In recent years, growing attention has been drawn on metformin because of its promising anti-cancer properties against a variety of human cancers. In CCA, Chaiteerakij et al., (2013) showed that metformin use was significantly associated with reduced risk of intrahepatic CCA in diabetic patients. Soon after that, Ling et al., (2014) examined metformin effects on CCA cell lines and observed that metformin not only inhibited cell proliferation and colony formation, but also increased sensitivity of CCA cells to certain chemotherapeutic drugs. In the present study, we confirmed the anti-proliferative effect of metformin on HuCCT1, and further elucidated that metformin could potently inhibit migration and invasion of HuCCT1 cells. STAT3 plays important roles in tumor development and progression, and its expression was associated with shorter survival of CCA patients (Dokduang et al., 2014). Studies showed that metformin exposure significantly reduced cancer cell proliferation, migration and invasion by inhibiting STAT3 signaling pathway (Deng et al., 2012; Pan et al., 2015). Here, we also demonstrated that phosphorylation of STAT3 and expression of STAT3's targets, including Mcl-1 and Bcl-2 were inhibited by metformin exposure. Disruption of STAT3 and anti-apoptotic/pro-survival pathway is most likely associated with inhibition of cell viability and growth observed in metformin-treated HuCCT1.

Epithelial–mesenchymal transition (EMT) is a cellular metamorphosis that leads epithelial cells to acquire mesenchymal-like phenotypes and become motile. EMT is a crucial process that occurs during several stages of normal embryonic development, wound healing, and

cancer progression (Turley et al., 2008). Reactivation of EMT program in tumor enables malignant cells to undergo metastatic. CCA displays EMT feature via epidermal growth factor (EGF)-EGF receptor signaling pathway which favors tumor invasiveness (Claperon et al., 2014). EMT program is regulated by numerous EMT transcription factors. Of those, Snail has been defined as one of the well-established drivers of EMT programs, and its expression is governed by STAT3 (Wendt et al., 2014). Interestingly, we found that metformin altered expression pattern of EMT markers by inhibiting the expression of mesenchymal-associated molecules such as Snail and N-cadherin. In contrast, metformin upregulated the expression of epithelial junctional protein E-cadherin. In addition, metformin could also repress the expression of MMP-2. Thus, these results suggest that metformin treatment reverses EMT and downregulates the proteolytic enzyme MMP-2, resulting in suppression of CCA cell migration and invasion.

In conclusion, we elucidated the suppressive effects of metformin on migration and invasion of CCA, which is highly metastatic. Metformin is an inexpensive drug, and its use has been proved safe without severe adverse effects in human. Thus, our findings suggest that use of metformin might be beneficial for CCA patients and might be a potential therapeutic agent for the treatment of CCA.

Statement conflict of Interest

The authors declare that they have no conflict of interest

Acknowledgements

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