Metformin Inhibits the Development and Metastasis of Colorectal Cancer

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Abstract

Background

Metformin is a commonly used drug for the treatment of diabetes. Accumulating evidence suggests that it exerts anti-cancer effects in many cancers, including colorectal cancer. However, the underlying molecular mechanisms of colorectal cancer metastasis remain unclear.

Methods

Colorectal cancer cell lines were treated with metformin, and cell proliferation, invasion, and migration were analyzed in vitro. The relationship between metformin and the AMPK–mTOR axis was assessed by western blot analysis and transfection with small interfering RNA. A colorectal cancer xenograft mouse model was used to observe the effects of metformin on liver metastasis. Immunohistochemical analysis was performed on liver metastatic tumors.

Results

In *in vitro* experiments, metformin significantly inhibited the proliferation, migration, and invasion only in HCT116 and SW837 cells, but not in HCT8 and Lovo cells. Only in HCT116 and SW837, a change in AMPK–mTOR expression was observed in a dose-dependent manner. In colorectal cancer xenograft mice, the liver metastatic rate (10% vs. 50%, p = 0.05) and the number of liver metastatic nodules (0.1/body vs. 1.2/body, p = 0.04) were significantly lower in the metformin group. Tumor proliferation and EMT were decreased and apoptosis was promoted only in metastatic liver tumors of mice treated with metformin.

Conclusion

The molecular mechanism of the anti-cancer effects of metformin involves repression of mTOR pathways via AMPK activation. Moreover, the differences in metformin sensitivity depend on the response of the AMPK–mTOR pathway to metformin. Our study provides a theoretical basis for the anti-metastatic treatment of colorectal cancer using metformin.

Introduction

Colorectal cancer (CRC) is one of the most common neoplasms in the world. Approximately 25% of patients initially present with metastatic CRC (synchronous metastases). [1] Despite recent advances in the medical treatment of metastatic CRC, the 5-year survival rate of CRC patients with unresectable metastatic disease is reported to be less than 10%. [2] [3] Therefore, it is important to develop novel approaches to prevent metastasis of CRC.

Metformin is a biguanide derivative widely used for the treatment of type 2 diabetes. Metformin exerts its effects by reducing hepatic glucose production and by increasing insulin sensitivity as well as glucose use by peripheral tissues. Recently, clinical studies of various cancer types have reported the anti-cancer

effects of metformin. [4] [5] Fransgaad et al. reported that metformin treatment was associated with 15% of all-cause mortality in CRC patients with diabetes compared with patients with insulin-treated diabetes. [6] Furthermore, a recent meta-analysis demonstrated that metformin was associated with increased overall survival and cancer-specific survival in CRC. [7] Therefore, the potential anti-cancer effects of metformin have gained great attention.

Previous experimental study has reported that the anti-cancer effects of metformin were induced by the activation of AMP-activated protein kinase (AMPK). [8] AMPK activation leads to a reduction in mammalian target of rapamycin (mTOR) signaling, protein synthesis, and cell proliferation. [9] [10] [11] Furthermore, the administration of metformin significantly reduces the expression of epithelial–mesenchymal transition (EMT) markers in various types of cancer cells. [12] [13] These findings suggest that metformin may be an optimal therapeutic agent for cancer treatment. However, there is a lack of experimental evidence of the anti-cancer effects of metformin, especially for metastatic disease.

The objective of this study was to clarify the inhibitory effect of metformin on liver metastasis of CRC. These data support the clinical efficacy of metformin for metastatic CRC and provide fundamental evidence to establish a novel therapy using metformin in the future.

Materials & Methods

Cell Lines

The human CRC cell lines HCT116 (American Type Culture Collection, Human colon carcinoma, CCL-247), SW837 (JCRB cell bank, Human rectal adenocarcinoma, JCRB9115), Lovo (American Type Culture Collection, Human colon adenocarcinoma, CCL-229), and HCT8 (American Type Culture Collection, Human colon adenocarcinoma, CCL-244) were used in these studies and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in standard culture conditions of humidified 5% CO_2 at 37°C. All tissue culture reagents were purchased from Sigma–Aldrich (St. Louis, MO).

Western blotting

Cells were lysed in radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors. Proteins were quantified by protein assay (Nano Drop 2000c, Thermo Fisher Scientific, Waltham, MA), and 20 μg protein was separated by SDS-PAGE and transferred to 0.2 μm nitrocellulose membrane. Membranes were blocked in TBS, 0.1% Tween 20, and 5% BSA for 2 h before overnight incubation with primary antibodies diluted to 1:1,000 in TBS, 0.1% Tween 20, and 5% BSA. Antibodies against phospho-mTOR, mTOR, phospho-AMPK, and AMPK were obtained from Cell Signaling Technology (Beverly, MA). The membranes were incubated for 1 h in HRP-conjugated secondary antibody diluted at 1:2,000–1:10,000 in TBS and 0.1% Tween 20. Immunoreactive protein was detected using MultiImage ll (Alpha Innotech, San Leandro, CA). [14] Western blot densitometry quantification was performed using ImageJ.

Cell proliferation assays

Cell proliferation was assessed by MTT assay. [15] Cells were plated into 96-well plates at a density of 4 × 10^4 cells/well. After 24 h incubation, 10 μl SP cell count reagent SF (Nacalai Tesque, Kyoto, Japan) was added to each well, and cells were further incubated for 2 h. The viable cell number was directly proportional to the production of formazan following solubilization. Color intensity was measured at 450 nm using a Sunrise R microtiter plate reader (Tecan, Mannedorf, Switzerland). All experiments were performed in triplicate. [16]

Cell migration assay

Cell monolayers were wounded with a plastic tip at 48 h after the initiation of metformin treatment. Cell migration was monitored for 24 h at 37°C and photographed using an EVOS FL Cell Imaging System (Life Technologies, Carlsbad, CA). [14] The original magnification was 10×.

Cell invasion assay

Cells were trypsinized, and 50,000 cells resuspended in DME with 0.2% FBS were added to rehydrated Matrigel-coated Cell Culture Inserts (Corning, Corning, NY) and seeded in 24-well companion plates with DME and 10% FBS. [17] After 24 h, the non-invading cells and Matrigel in the upper chambers were removed using a cotton tip. The cells invading to the lower surface and the filters were fixed in methanol for 5 min at room temperature, and the nuclei were stained with hematoxylin and eosin (HE). The invading cells were counted at room temperature using an BX 51 System microscope (Olympus, Tokyo, Japan) and images were analyzed using ToupView software (ToupTek).

Plasmid transfection

Human *AMPK* siRNA and control siRNA were purchased from Shanghai GenePharma (Shanghai, China). For siRNA transfection, a total of 1.5 × 10^5 cells/well was seeded into six-well plates and transfected with 30 pmol *AMPK* siRNA or control siRNA using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA) in accordance with the manufacturer's instructions. Cell proliferation and wound healing were measured at 72 h post-transfection. Metformin treatments were initiated after completion of 48 h transfection.

In vivo studies

Liver metastasis was generated using a previously described splenic injection model. [18] Briefly, 6-week-old female severe combined immunodeficient (SCID) mice were anesthetized using ethyl ether, and the spleen was exteriorized via a 5 mm incision in the left upper abdomen. HCT116 cells (2 × 10^6) in 100 μL PBS were slowly injected into the spleen and allowed to flush to the liver for 1 min. Then, the spleen was removed, and homoeostasis was assured by ligation with a suture. The mice were killed at 42 days post-injection. The dosage selected in the present study was based on a previous report in which metformin suppressed ACF formation in a mouse model of azoxymethane-induced colon cancer. [19] All the mice

were killed under ether anesthesia, and the livers were removed and weighed. Finally, surface liver metastases were counted under blinded conditions. All animal procedures were performed in the SCID mouse facility using protocols approved by the Keio Animal Care and Use Committee.

Immunohistochemistry

The liver metastases obtained from xenografts were immediately fixed in 20% formalin. Blocking of endogenous peroxidase with 3% hydrogen peroxide was performed on dewaxed and rehydrated slides for 30 min. The sections were washed several times with PBS, blocked with 10% normal swine serum (Vector Laboratories, Burlingame, CA) at room temperature, and then incubated with primary antibodies at 4°C overnight. Antibodies against α-smooth muscle actin (α-SMA) and Ki-67 were purchased from Abcam (Cambridge, UK). After blocking with biotinylated anti-rabbit IgG blocking reagent (Vector Laboratories, Burlingame, CA), the sections were stained using the VECTASTAIN Elite ABC-HRP Kit (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. After rinsing in PBS, all sections were visualized with 0.05% 3,3'-diaminobenzidine. The sections were then counterstained with HE at room temperature for 1 min. Then, for each section, field at 200 × magnification were analyzed. All positive cells in the field were photographed and counted. Results were expressed as the average of positive cells and presented with the means ± standard error (n = 3).

Apoptosis detection by TUNEL assay

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining was conducted with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Billerica, MA) in accordance with the manufacturer's instructions. The sections were then counterstained with 4,6-diamidino-2-phenylindole (DAPI). Images of TUNEL staining were taken with the EVOS FL Cell Imaging System (Life Technologies, Carlsbad, CA). All TUNEL positive cells in the field were analyzed similarly as in the case of immunohistochemistry.

Statistical analysis

All data are expressed as means ± standard error (SE). Data analyses were performed by one-way and two-way ANOVA using Stata/SE 12.1 for Mac (Stata Corporation, College Station, TX). Statistically significant differences were considered at $p < 0.05$ and markedly significant differences were considered at $p < 0.01$.

Results

Inhibitory effect and chemosensitivity of metformin on cell proliferation

As shown in Figure 1A and 1B, significant dose-dependent inhibition of proliferation was observed in HCT116 and SW837 cells treated with metformin. In contrast, no significant effect was observed on HCT8 or Lovo cell proliferation, regardless of the increasing doses of metformin, as shown in Figure 1C and 1D. Metformin can phosphorylate AMPK and subsequently inhibit mTOR activity via the AMPK–

mTOR pathway. HCT116 and SW837 cells exhibited a decrease in p-mTOR levels that was inversely proportional to the increase in p-AMPK levels with metformin treatment, while HCT8 and Lovo cells showed no decrease in p-mTOR levels with metformin treatment. This finding suggested that metformin sensitivity varies among CRC cell lines, and the anti-proliferative effect of metformin is mediated by the suppression of mTOR caused by AMPK phosphorylation. We hypothesized that the AMPK–mTOR pathway plays a critical role in the differences in reactivity between the sensitivity of CRC to metformin and its insensitivity to metformin.

Differential inhibitory effect of metformin on cell migration and invasion

We also tested the effect of metformin on the invasive behavior of CRC cells. In the cell invasion assay, metformin significantly reduced the invasion of metformin-sensitive HCT116 and SW837 (Fig. 2A). However, metformin-insensitive Lovo also showed a significant decrease in the number of invaded cells (Fig. 2B). Overall, metformin elicited greater inhibition of cell motility in metformin-sensitive CRC cells than in metformin-insensitive CRC cells.

Transient AMPKknockdown inhibits proliferation and migration of metformin-sensitive CRC cells

To assess whether the anti-cancer effects of metformin treatment resulted from activation of AMPK and inhibition of mTOR, we transfected metformin-sensitive CRC cells with AMPK siRNA or non-specific control siRNA and then treated them with metformin. Specific knockdown of AMPK by the corresponding siRNA was confirmed by western blot analysis and densitometric analysis. The levels of p-mTORwere elevated in HCT116 cell lines (Fig. 3A). Although the elevation of p-mTOR expression was not observed in SW837, transfection with AMPK siRNA decreased the anti-proliferative activity of metformin compared with non-specific control siRNA. Additionally, metformin treatment significantly reduced the wound healing in HCT116 control cells but not in HCT116 cells transfected with AMPK siRNA (Fig. 4B). These findings suggest that inhibition of AMPK could abolish the anti-cancer effects of metformin in metformin-sensitive CRC cell lines. Therefore, it is suggested that activation of the AMPK–mTOR pathway in response to metformin plays an essential role in the anti-cancer effects of metformin against CRC.

Oral administration of metformin inhibits the liver metastasis of CRC cells in vivo

To examine whether metformin treatment of metformin-sensitive CRC cells affects growth and metastasis in vivo, we injected highly tumorigenic, metformin-sensitive HCT116 cells into SCID mice and monitored liver metastasis. As shown in Fig. 5, The number of mice with liver metastases was greater in the control group than in the metformin-treated group. Liver metastases were less frequently observed in the metformin-treated group (metformin-treated group 10% vs. control group 50%, $p = 0.05$). Furthermore, the number of metastatic nodules was significantly smaller in the metformin-treated group (metformin-treated group 1.2/body vs. control group 0.1/body, $p = 0.04$). These findings suggested that metformin inhibits the development of liver metastasis in metformin-sensitive CRC xenografts in SCID mice.

Metformin therapy inhibited tumor proliferation and EMT and promoted apoptosis in vivo

Immunohistochemical analysis of liver metastases collected from SCID mice treated with metformin and the control demonstrated positive reactions for the following markers: Ki-67, TUNEL, and α-SMA (Fig. 6A). There was significantly less Ki-67 expression in the metformin-treated groups compared with the control groups. This result indicated a decrease in cell proliferation and further supported the anti-proliferative effects of metformin treatment. Additionally, treatment with metformin increased cell apoptosis, as analyzed by TUNNEL assay. A significantly small number of TUNEL-positive cells were detected in the control group, whereas many TUNEL-positive apoptotic tumor cells were detected in the metformin group. Furthermore, the control group showed a higher proportion of α-SMA-expressing CRC cells compared with the metformin group. These results suggested that metformin treatment inhibits tumor proliferation and that EMT is beneficial for inhibiting the growth and metastasis of CRC.

Discussion

The findings of this study demonstrated that metformin induced the inhibition of liver metastasis, depending on the expression of phosphorylated mTOR. We found that the anti-cancer effects of metformin differed among different CRC cell lines. In this study, metformin inhibited the phosphorylation of mTOR via AMPK activation only in metformin-sensitive CRC cells. Additionally, metformin reduced cell motility by decreasing cell migration and invasion, thus inhibiting CRC metastasis. We also observed a significant decrease in liver metastasis in an animal xenograft model with metformin-sensitive HCT116 cells. These findings suggested that phosphorylation of mTOR via AMPK activation has an important role in the CRC metastasis inhibitory effect of metformin. The results of the current study provide fundamental evidence for a new therapeutic strategy with metformin in the treatment of CRC patients with metastatic liver disease.

Several studies investigating the clinical efficacy of metformin reported its preventive effect against CRC. Recently, a meta-analysis showed that metformin use significantly reduces colorectal adenoma and cancer incidence. [20] A phase 3 randomized control trial conducted in non-diabetic patients also demonstrated that metformin reduced the prevalence and number of metachronous colorectal adenomas or polyps. [21] Additionally, the synergistic effects of metformin with chemotherapeutic agents for CRC patients were also investigated. In a phase 2 trial, both metformin plus 5-FU and metformin plus irinotecan showed feasible anti-cancer effects in patients with refractory CRC. [22] [23] However, conflicting results were also reported. Fransgard et al. reported that there was no association between metformin treatment and recurrence-free or disease-free survival after surgery for colorectal cancer in their registry-based study of 25,785 patients. [24] Another population-based study conducted in England also did not support a protective association between metformin and cancer-specific survival in colorectal cancer patients. [25] The synergistic effect of metformin was also questioned. A subgroup analysis of RCTs reported that no relationship was found between metformin use and postoperative survival of resected stage III colon cancer patients receiving adjuvant oxaliplatin-based chemotherapy (FOLFOX/XELOX). [26] [27] This study indicated that varying sensitivity for metformin treatment among CRC cell lines might be the cause of these conflicting results. However, the clinical efficacy of metformin

in CRC treatment remains controversial. Hence, elucidation of the optimal indication and usage of metformin in CRC patients is urgently required.

To our knowledge, this is the first study demonstrating that metformin mediated inhibition of liver metastasis of CRC cells both in vitro and in vivo. On the basis of the results of this study, it is suggested that the anti-proliferative and anti-metastatic effects of metformin are associated with the inhibition of liver metastasis of CRC. Previously, the anti-proliferative effects of metformin combined with chemotherapeutics have been explored. [28] [29] However, few studies have focused on the anti-metastatic effects of metformin monotherapy. [30] The results of this study, which demonstrated the anti-metastatic effects of metformin in a xenograft model of orthotopic liver metastasis, support the use of metformin in clinical practice. Further studies investigating the therapeutic potential of metformin for CRC liver metastasis are required.

In this study, we found that the anti-proliferative effects of metformin varied amongst different CRC cell types. The anti-proliferative effect was a dose-dependent decrease in p-mTOR levels in inverse proportion to the increase in p-AMPK levels. It is suggested that the metformin sensitivity of CRC cells differs according to the degree of phosphorylation of AMPK and following dephosphorylation of mTOR caused by metformin. mTOR and its upstream PI3K–AKT signaling axis has gained attention as a potential therapeutic target for various types of cancers. At present, as a semi-synthetic rapamycin analog, the mTOR inhibitor everolimus is widely accepted as the treatment option for patients with clear-cell renal cell cancer, pancreatic neuroendocrine tumor, and breast cancer. However, clinical trials have failed to demonstrate the clinical efficacy of mTOR inhibitors in the treatment of CRC [31] [32] A possible explanation is that the presence of PIK3CA mutations is associated with a lack of benefit after mTOR inhibitor therapy. [33] [34] Metformin-sensitive HCT116 cells carry mutations in both PIK3CA and KRAS/BRAF, and this genetic difference may be important for metformin sensitivity in CRC patients. However, the distinct effect of metformin between metformin-sensitive and -insensitive CRC cell lines was not observed in invasion and migration assays. We speculate that there are anti-metastatic effects of metformin independent of the mTOR–AMPK pathway. This evidence also supported our hypothesis. Metformin can activate an AMPK-independent signaling pathway, which inhibits EMT through different mechanisms. [35] [36] Additionally, it was also reported that metformin inhibits EMT in rectal cancer cells by suppressing the TGF-β pathway. [37] These AMPK-independent pathways may be the reason of contraindicated results in wound healing assay and invasion assay for Lovo cell line in current study. Our results indicated both mTOR–AMPK-dependent and -independent mechanisms have critical roles in the inhibitory effect of metformin in CRC cell lines. Further investigation focused on the mechanisms of the anti-cancer effects of metformin is needed to identify CRC patients who can benefit from metformin treatment.

In this study, we first demonstrated that metformin inhibited CRC liver metastasis in vivo. Additionally, we found that decreased proliferation, increased apoptosis, and inhibition of EMT in liver metastasis are associated with the inhibitory effects of metformin. Previously, metformin showed inhibitory effects on the metastasis of CRC cells in several studies. Kang et al. reported that metformin reduced IL-6-induced

EMT in colon cancer cell lines. [38] Another group showed that metformin decreases EMT in CRC cells by regulating the SNAIL–*miR-34* and ZEB–*miR-200* system. [39] However, the exact underlying mechanism of metformin in anti-metastatic regulation, especially in CRC, remains elusive. There are no published findings showing that metformin induces apoptosis in CRC, and the absence of cell mortality after treatment with the drug has previously been observed in prostate and breast cancer cells. [40] [41] Nevertheless, we could observe a cytocidal effect of metformin in vivo that could be due to the microenvironment of liver metastasis. It is reported that CRC metastatic colonization of the liver occurs in the hypoxic microenvironment, and CRC cells have inadequate levels of ATP. [42] [43] Additionally, metformin may induce apoptosis only in nutrient-poor conditions. [44] Taken together, it is plausible that metformin has cytocidal effects on CRC cells that are induced by nutrient-poor conditions at liver metastasis sites. However, further detailed experiments are required.

This study had several limitations. First, SCID mice lack an immune system, which is thought to play a critical role in both the initiation and progression of cancer metastasis and may also modify the response to metformin therapy. Thus, further investigation using mouse models with an intact immune system may be needed. Second, comparisons of metformin and other m-TOR inhibitors were not conducted in this study. Rapamycin, an original inhibitor of mTOR, and its rapalogs mainly inhibit mTORC1 activity and are classified as first-generation mTOR inhibitors. However, recent studies have shown that, unlike rapamycin, metformin not only prevents phosphorylation of mTORC1 complex components, but also inhibits mTORC2 complex components. [45] Dual inhibition of both mTORC1 and mTORC2 may lead to more effective inhibition of cancer cell proliferation than blocking mTORC1 alone. [46] Further studies exploring the anti-cancer efficacy of metformin in detail are needed. Finally, in this study, metformin was supplied by oral administration. Therefore, it is suspected that the concentration of metformin in vivo differed from the concentration in vivo.

In conclusion, our data indicate that metformin inhibits the metastasis of CRC by upregulating AMPK and inhibiting mTOR expression in vivo. This finding is extremely important because the main cause of mortality in patients with CRC is the development of metastasis and the scarcity of therapeutic options. Further experiments will focus on the underlying mechanisms of the anti-cancer effects of metformin and its potential clinical application for the management of CRC.

Declarations

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Kiyoaki Sugiura. The first draft of the manuscript was written by Kiyoaki Sugiura. Koji Okabayashi revised previous version of the manuscript. All authors read and approved the final manuscript.

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Disclosure of potential conflicts of interest

The authors have no competing interests to declare that are relevant to the content of this article.

Research involving Animals

Approval was granted by the Animal Experiments Committee of Keio University. (No. 15025-(0))

Data availability

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Western blot analysis demonstrating the expression patterns of p-AMPK, AMPK, p-mTOR, and mTOR following metformin treatment. Densitometric analysis of AMPK and p-AMPK in (A) HCT116, (B) SW837, (C) HCT8, and (D) Lovo cells. Proteins were quantified and expressed as ratio of p-AMPK expression relative to AMPK expression. Equal loading of total proteins were ensured by β-actin. Data are presented as mean ± standard error of 2 independent experiments. *p < 0.05 versus corresponding control, **p < 0.01 versus corresponding control.

Figure 2

Assessment of cell proliferation by MTT assay following metformin treatment for 48 h in (A) HCT116, (B) SW837, (C) HCT8, and (D) Lovo cells. Data are presented as mean ± standard error of 2 independent experiments. *p < 0.05 versus corresponding control, **p < 0.01 versus corresponding control.

Figure 3

Wound healing (A) and invasion (B) assays were conducted in CRC cells treated with or without 10 mM metformin for 48 h. The values of wound healing assay shown are mean ± standard error of 3 independent experiments. The values of invasion assay shown are mean ± standard error of 2 independent experiments. *p < 0.05 versus corresponding control.

Figure 4

Assessment of cell proliferation by MTT assay (middle) and migration by wound healing assay (right) in (B) HCT116 and (C) SW837 cells transfected with AMPK or control siRNA. Protein levels were assessed by western blotting and densitometric analysis (left) at 72 h after transfection. *p < 0.05 versus corresponding control, **p < 0.01 versus corresponding control.

Figure 5

SCID mice were injected with HCT116 cells and treated with either a normal diet or metformin (100 mg/kg) via oral gavage for 42 days. Liver metastasis was assessed according to the number of mice with liver metastasis and metastatic nodules on the liver from each group. The values shown are mean ± standard error (n=10). * $p < 0.05$ vs. control group.

Figure 6

Immunohistochemistry of Ki-67, α-SMA, and TUNEL expression at liver metastatic sites of the metformin-treated or control groups. (A) Representative images of tumor sections stained with antibodies against Ki-67, α-SMA, and TUNEL (magnification ×200). (B) The numbers of positive cells in each immunohistochemistry (microscopic quantitative analysis). Data are presented with the means ± standard error (n = 3). * $p < 0.05$ vs. control group.