K-ras gene mutation as a predictor of cancer cell responsiveness to metformin

YU MA, FU-CHUN GUO, WEI WANG, HUA-SHAN SHI, DAN LI and YONG-SHENG WANG

Department of Thoracic Oncology, Cancer Center, State Key Laboratory of Biotherapy,
West China Hospital, Sichuan University, Chengdu, Sichuan 610041, P.R. China

Abstract. An increasing number of studies support the use of metformin, a common antidiabetic drug, as a novel anticancer therapeutic. However, its mechanism of action has yet to be identified. In the current study, metformin was observed to effectively inhibit the growth of the K-ras mutant but not wild-type tumors *in vivo*. The antitumor effects of metformin were mediated by the induction of apoptosis and inhibition of proliferation *in vivo*. In addition, metformin induced apoptosis in the K-ras mutant tumors, A549 and PANC-1, but not in the K-ras wild-type tumor, A431, *in vitro*. Similarly, at lower concentrations, metformin inhibited cell proliferation in the K-ras mutant, but not in the K-ras wild-type tumor cells *in vitro*. These observations indicate that tumors with K-ras mutations are sensitive to metformin therapy. In addition, metformin significantly arrested K-ras mutant and wild-type tumor cells in G1 phase *in vitro* and metformin downregulated two important downstream effectors of the Ras signaling pathway in K-ras mutant tumors. Metformin was concluded to function as a potential K-ras-targeting agent that has potential for cancer therapy.

Introduction

Previous epidemiological studies have hypothesized an association between diabetes and cancer. Firstly, the incidence of several types of cancer, including pancreatic, liver, breast, colorectal, urinary tract and of the female reproductive organs, is increased in diabetic patients (1). Secondly, cancer patients with diabetes have been reported to respond poorly to cancer chemotherapy and have a poorer prognosis than non-diabetic patients (2).

Correspondence to: Dr Yong-Sheng Wang, Department of Thoracic Oncology, Cancer Center, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, No. 1 Keyuan Road, 4th Gaopeng Avenue, Chengdu, Sichuan 610041, P.R. China
E-mail: wangys@scu.edu.cn

Previous studies investigating patients with type 2 diabetes have reported that metformin significantly reduces cancer incidence and improves the survival of these cancer patients (3). Metformin is the most widely used antidiabetic drug, functioning to reduce circulating glucose levels, increase insulin sensitivity and reduce insulin resistance-associated hyperinsulinemia (4). A number of studies have reported that metformin is a novel anticancer drug that may be used to treat specific types of cancer, including breast, ovarian, endometrial and pancreatic (5-8). However, Bodmer *et al* reported that metformin did not decrease the risk of lung cancer and colon cancer (9,10). This may be ascribed to the various genetic backgrounds of the numerous tumor types analyzed. For example, Buzzai *et al* reported that metformin selectively impairs p53-deficient tumor cell growth (11). To date, there has been extensive epidemiological and retrospective studies that support the antineoplastic effects of metformin (4). However, the precise mechanisms by which metformin exerts its anti-tumor effects remain unclear.

Ras proteins are prototypical G-proteins that have been shown to play key roles in signal transduction, proliferation and differentiation (12). Under normal conditions, Ras is activated transiently in a stimulus-dependent fashion. By contrast, mutated Ras proteins are constitutively active and thus, persistently stimulate growth or differentiation (13). Therefore, mutationally activated *Ras* genes are hypothesized to represent important targets for cancer therapy. K-, H-and N-Ras of the Ras family are central in regulating diverse cellular functions important for growth, differentiation and survival. Of the Ras proteins, K-ras is the most frequently mutated (14). A number of strategies have been developed to target K-ras and specific agents have been demonstrated to effectively treat cancer (15). However, agents that specifically target K-ras with no toxicity in normal cells have not yet been developed (16).

A previous study found that insulin induces activation of the *Ras* gene (4). In addition, other studies have reported that metformin reduces the levels of circulating insulin (17-19,20). Thus, we hypothesized that metformin exerts its antitumor effects by reducing activation of the *Ras* gene. In the current study, metformin was found to effectively inhibit the growth of K-ras-mutated tumors but not K-ras wild-type tumors. In addition, the antitumor effects of metformin were found to be mediated via induction of apoptosis and inhibition of proliferation. Downregulation of K-ras downstream signaling pathways

contributed to the inhibition of proliferation and induction of apoptosis. In conclusion, observations of the current study hypothesize the use of metformin to inhibit the growth of tumors with various molecular signatures and in particular with different K-ras backgrounds.

Materials and methods

Cells and reagents. K-ras mutant tumor cells [human A549 lung adenocarcinoma (21) and human PANC-1 pancreatic (22)] and K-ras wild-type tumor cells [human A431 epidermoid adenocarcinoma (23)] were purchased from American Type Culture Collection (Manassas, VA, USA) and were cultured according to the manufacturer's instructions. Metformin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and epidermal growth factor (EGF) was obtained from Shanghai PrimeGene Bio-tech Co., Ltd., (Shanghai, China).

Tumor growth assay. Seven-week-old female athymic (BALB/c, nu/nu) mice used for the *in vivo* NSCLC xenograft study were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). A549 (10^7), PANC-1 (10^7) and A431 cells (10^7) were injected subcutaneously into the flanks of the female nude mice and allowed to grow to a density of 100 mm^3. Animals were randomly divided into two groups of six mice. Control mice received daily intragastric administration of the vehicle solution for metformin, H_2O, while treated mice received daily intragastric administration of metformin (250 mg/kg).

Detection of Ki67 expression within tumors. Tumor tissue was removed from tumor-bearing nude mice following the final treatment and immunohistochemical analysis was performed with an anti-Ki67 antibody (1:100; Thermo Fisher Scientific Inc., Waltham, MA, USA) to determine the expression of Ki67.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay in tumors in situ. Tumor tissue was removed from tumor-bearing nude mice following the final treatment and apoptosis levels *in vivo* were determined using a TUNEL assay according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA).

Lactic acid assay. A549, PANC-1 and A431 cells were seeded at 2.5x10^5 cells/well in 6-well plates in the appropriate media overnight and treated with metformin at four concentrations (0, 1, 5 and 10 mM) for an additional 48 h. Supernatants of the cell culture media were harvested and the concentration of lactic acid was analyzed using a lactic acid assay according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Viable cells were counted and the lactic acid release/cell was calculated as follows: concentrations of lactic acid/cell densities.

Cell cycle analysis by flow cytometry. A549, PANC-1 and A431 cells were seeded at 2.5x10^5 cells/well in 6-well plates in the appropriate media overnight. Subsequently, cells were starved for 24 h and treated with 10% serum and four concentrations of metformin for 24 h. Cells were harvested, washed twice with PBS, fixed in a 70% methanol solution and stored at

4°C overnight. Following fixation, cells were resuspended and stained with PI and analyzed using flow cytometry (Beckman Coulter Inc., Brea, CA, USA).

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) cell proliferation assay. A549, PANC-1 and A431 cells were treated with varying doses of metformin (0, 0.5, 1, 2.5, 5 and 10 mM) for 72 h. MTT (5 mg/ml; Sigma-Aldrich) was added to the 96-well plates at 10 μl/well and the plates were incubated for an additional hour. The MTT reaction was terminated by the addition of 100 μl DMSO. Subsequently, MTT assay results were obtained by measuring the absorption of each well at 490 nm.

Apoptosis analysis by flow cytometry. A549, PANC-1 and A431 cells were seeded at 2.5x10^5 cells/well in 6-well plates in the appropriate media overnight and were treated with 10% serum and four concentrations of metformin for an additional 24 h. Cells were harvested, washed twice with PBS, resuspended and stained with PI and apoptosis levels were analyzed using flow cytometry (Beckman Coulter, Inc.).

Western blot analysis. A549, PANC-1 and A431 cells were seeded at 2x10^5 cells/well in 6-well plates in the appropriate media and treated with two concentrations of metformin (0 and 5 mM) for 24 h. Cell lysates were prepared and analyzed by western blot analysis as previously described (7). Antibodies against phospho-T172 adenosine monophosphate activated protein kinase (AMPK), mitogen-activated protein kinase (MAPK), phospho-T202/204 MAPK, AKT (protein kinase B), phospho-T308 AKT and p53 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibody against AMPK was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Peroxidase-labeled antibodies against mouse and rabbit were used as secondary antibodies (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China).

Statistical analysis. Data are presented as the mean ± SEM from a minimum of three independent experiments, unless otherwise noted. Statistical analyses were performed by Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Differential inhibition of xenograft tumor growth induced by metformin on various K-ras backgrounds. To gain an improved understanding of the effects of metformin treatment on cancer cell xenografts *in vivo*, mice were administered metformin orally. As shown in Fig. 1, treatment with metformin resulted in significant inhibition of human A549 and PANC-1 (both P<0.05) but not A431 xenograft tumor growth (P>0.05).

Metformin induces apoptosis and inhibits the proliferation of K-ras-mutated tumor cells in vivo. The effects of metformin on cell proliferation *in vivo* were determined by staining sections from each group with an anti-Ki67 antibody. As presented in Fig. 2A, treatment with metformin resulted in significant inhibition of cell proliferation in A549 and PANC-1
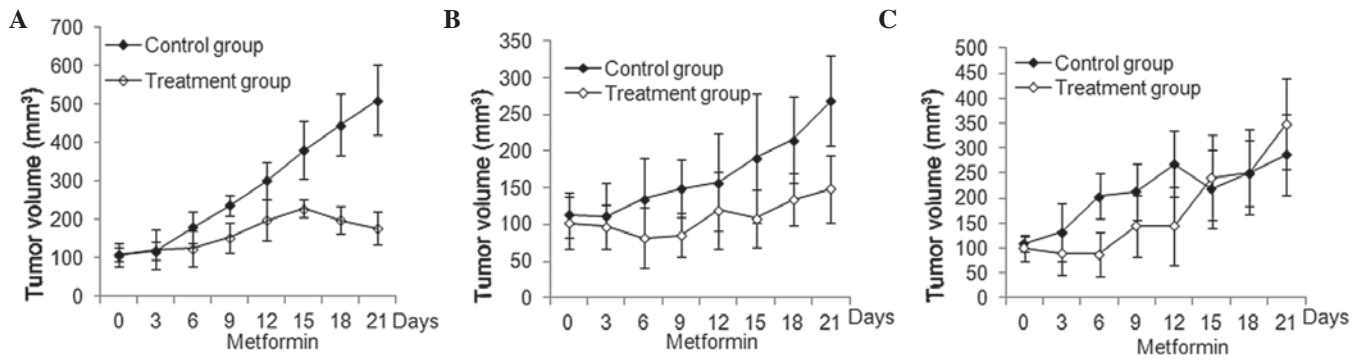
Figure 1. Effects of metformin on cancer cell xenografts *in vivo*. (A and B) Daily intragastric administration of metformin (250 mg/kg) resulted in significantly increased regression of established A549 and PANC-1 cell xenograft tumors (both P<0.05). (C) Increased regression was not observed in A431 cell xenograft tumors (P>0.05).
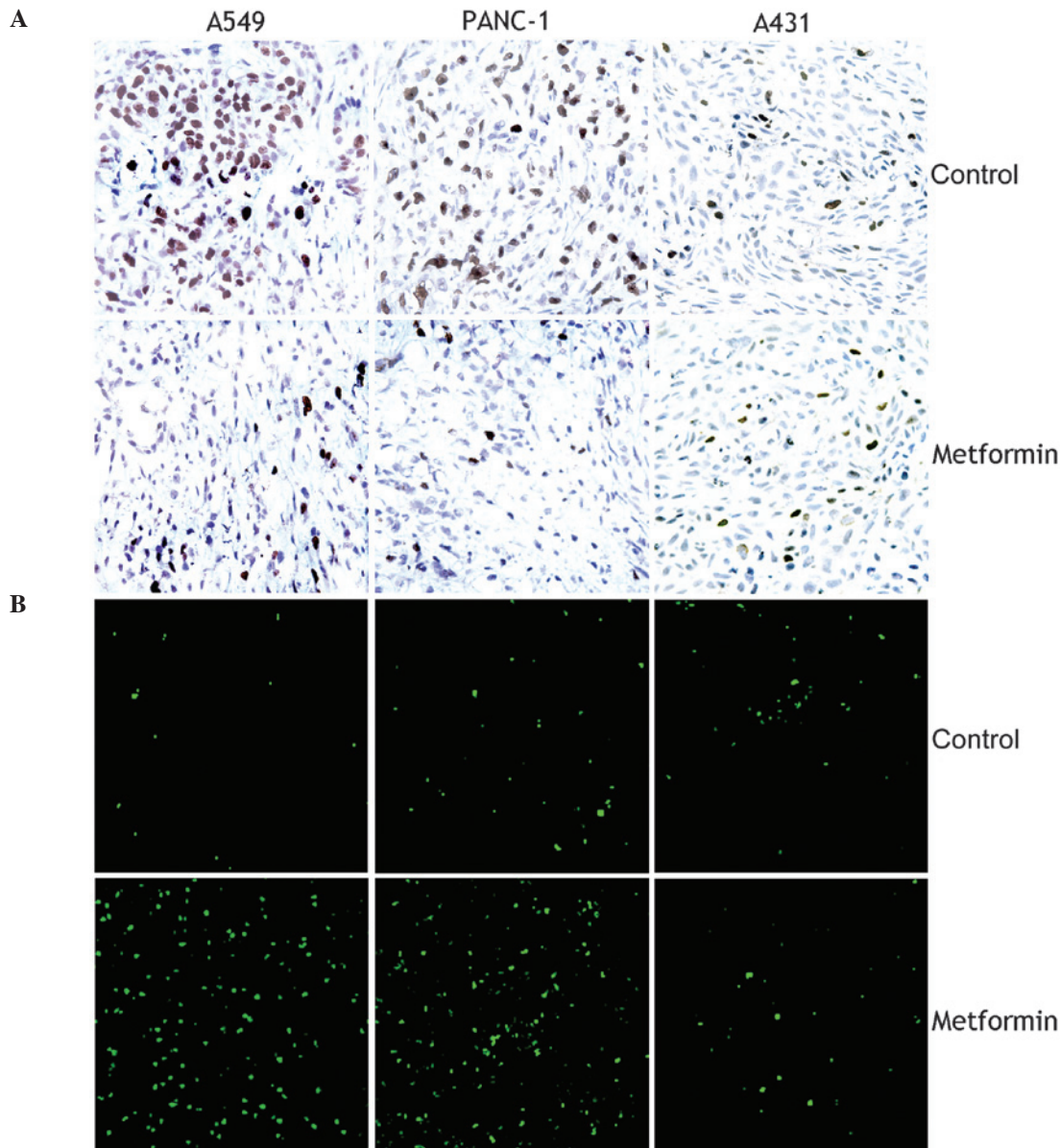

Figure 2. Effects of metformin on proliferation and apoptosis *in vivo*. (A) Metformin inhibited the proliferation of cancer cells *in vivo*. Proliferating tumor cells were detected using an anti-Ki67 antibody and positive cell densities were quantified by counting the number of cells/high power field (magnification, x400). There was a significant difference observed in the number of proliferating tumor cells between treated and control groups of A549 and PANC-1 cells (P<0.05) but not A431 cells *in vivo* (P>0.05). (B) Metformin induced apoptosis of cancer cells *in vitro*. Detection of apoptotic cancer cells using a TUNEL assay. The percentage of apoptotic cells was determined by counting the number of apoptotic cells divided by the total number of cells in the field (five high power fields/slide; magnification, x400). There was a significant difference in the percentage of apoptotic tumor cells between treated and control groups of A549 and PANC-1 cells (P<0.05) but not A431 cells *in vivo* (P>0.05). TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.
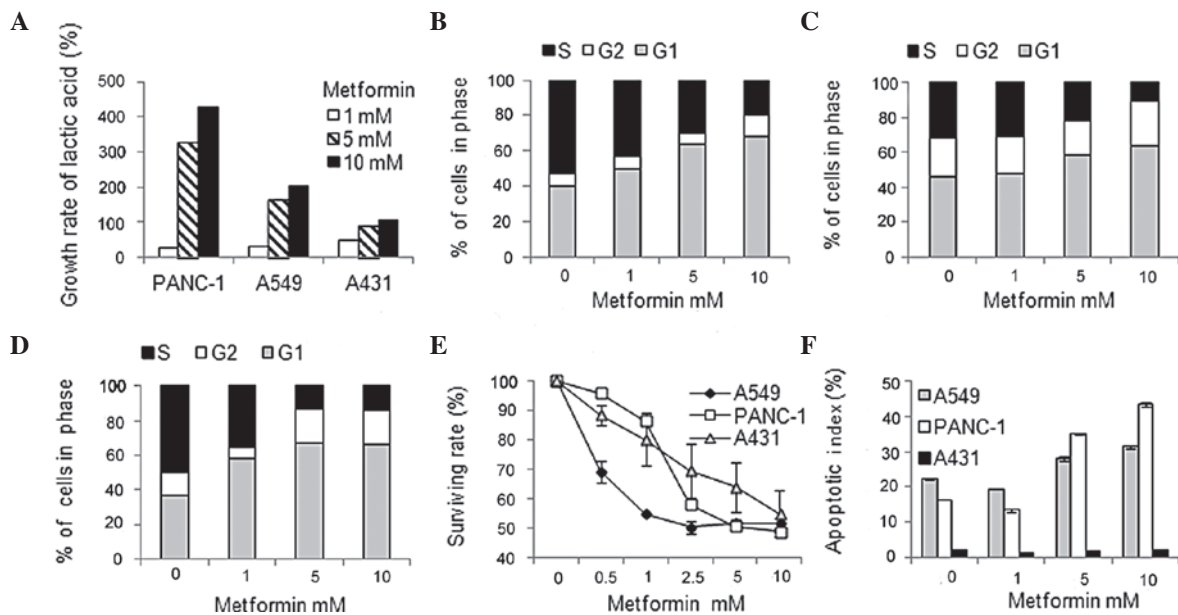
Figure 3. Effects of metformin on glycolysis, cell cycle progression, cell proliferation and apoptosis *in vitro*. (A) Metformin increased glycolysis in A549, PANC-1 and A431 cells. Lactic acid concentration was determined by a lactic acid assay and the lactic acid release of a single cell was calculated as the concentration of lactic acid/cell density. Metformin inhibited cell cycle progression by arresting the (B) A549, (C) PANC-1 and (D) A431 cells in G1 phase. (E) Cell cycle analysis was performed by flow cytometry. Metformin inhibited the proliferation of cancer cells in a dose-dependent manner. A549, PANC-1 and A431 cells were cultured in the presence of increasing concentrations of metformin for 72 h. The relative growth of the cells was determined by MTT assay. (F) Metformin induced apoptosis in A549 and PANC-1 cells but not in A431 cells and the percentage of apoptotic cells was determined by flow cytometry. MTT, methylthiazolyldiphenyl-tetrazolium bromide.

cell xenografts (both P<0.05) but not in A431 cell xenografts (P>0.05). Next, a TUNEL assay was performed to examine the effects of metformin on apoptosis *in vivo*. As shown in Fig. 2B, treatment with metformin resulted in significant induction of apoptosis in A549 and PANC-1 cell xenografts (both P<0.05) but not in A431 cell xenografts (P>0.05).

Metformin increases glycolysis and induces cell cycle arrest and apoptosis while inhibiting proliferation ex vivo. To understand the effects of metformin treatment on glycolysis, the concentration of lactic acid in the supernatants of the cell culture media was quantified and the lactic acid release/cell was analyzed following treatment with metformin. As presented in Fig. 3A, treatment with metformin resulted in elevated levels of glycolysis in the three cancer cell lines.

Effects of metformin treatment on cell cycle progression were determined by flow cytometry analysis following treatment with metformin. As presented in Fig. 3B-D, metformin significantly blocked serum-induced entry into S phase in a dose-dependent manner, resulting in cell cycle arrest in G1 phase in the three cancer cell lines following 24 h metformin treatment.

To determine the effects of metformin treatment on cell proliferation, an MTT assay was performed following treatment with metformin (24). As presented in Fig. 3E, metformin inhibited cell growth in a dose-dependent manner in all three cancer cell lines following 72 h metformin treatment. The mean IC_{50} value was ~5 mM for A549 and PANC-1 cells and ~10 mM for A431 cells.

The effects of metformin treatment on apoptosis were investigated by flow cytometry analysis following treatment with metformin. As presented in Fig. 3F, metformin induced
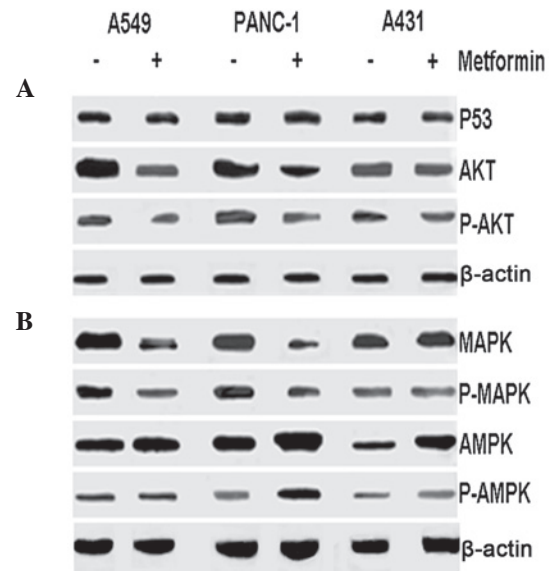

Figure 4. Effects of metformin on cell signaling pathways. (A) Metformin did not affect the protein expression of p53 in A549, PANC-1 and A431 cells. Metformin inhibited AKT downstream of Ras signaling pathways in A549 cells and PANC-1 cells but not in A431 cells. (B) Metformin inhibited MAPK in A549 cells and PANC-1 cells but not in A431 cells. Metformin activated AMPK in PANC-1 cells and A431 cells but not in A549 cells. Untreated control A549 cells, PANC-1 cells and A431 cells pretreated with 5 mM metformin for 24 h were stimulated with EGF (100 ng/ml) for 60 min before cell lysis. Then, the cell lysates were used for western blot analyses. AMPK. adenosine monophosphate activated protein kinase; MAPK, mitogen-activated protein kinases; AKT, protein kinase B.

apoptosis in A549 and PANC-1 cells. The percentage change ranged between 9.2 and 27.2%, depending on the cell line. However, metformin did not induce apoptosis in A431 cells.

Metformin inhibits the downstream signaling effectors of K-ras. Western blotting was performed on the three cell lines following treatment with metformin to determine its underlying anti-tumor mechanisms. As presented in Fig. 4, expression of p53 was not affected by metformin in A549, PANC-1 and A431 cells. Treatment with metformin resulted in decreases in MAPK, p-MAPK, AKT and p-AKT levels in A549 and PANC-1 cells but not in A431 cells. However, metformin treatment increased AMPK and p-AMPK in PANC-1 cells and A431 cells, but not in A549 cells.

Discussion

An increasing number of studies have implicated metformin as a potentially effective anticancer drug; however, the mechanism of action remains unclear. Observations of the current study indicate that metformin specifically targets K-ras to elicit its anticancer effects.

Using an *in vivo* mouse model, metformin was observed to only exert anticancer effects in K-ras mutant tumors, but not in K-ras wild-type tumors. To understand the mechanisms of these effects *in vitro*, the effects of metformin on glycolysis were investigated. As described by Warburg *et al*, tumor cells maintain a high glycolytic rate even in conditions of adequate oxygen supply (25). Therefore, interfering with aerobic glycolysis represents a potentially effective strategy to selectively target cancer cells. Metformin increased the rate of glycolysis in K-ras mutant and wild-type cell lines, indicating that the anticancer effects of metformin are mediated through pathways other than aerobic glycolysis.

Next, the effects of metformin on cell cycle progression and proliferation were investigated. Metformin was observed to significantly arrest the cells in G1 phase and inhibit the proliferation of K-ras mutant and wild-type tumors. These results did not highlight an explanation for the differential efficacy of metformin on K-ras mutant and wild-type tumors *in vivo*. Therefore, the effects of metformin on apoptosis were investigated and it was observed that metformin induces apoptosis in K-ras mutant tumors but not in K-ras wild-type tumors. This indicates that metformin is likely to inhibit tumor growth of K-ras mutant tumors by increasing apoptosis and inhibiting proliferation, which is consistent with the *in vivo* results. The results indicate that the anticancer activity of metformin is primarily due to the induction of apoptosis and that metformin is cytostatic and cytotoxic to K-ras mutant tumors. To gain a full understanding of the underlying mechanisms by which metformin induces apoptosis, p53 expression, an essential component of apoptosis induction was investigated. Expression of p53 was not affected by metformin treatment in all three cancer cell lines and observations made by Buzzai *et al* support the hypothesis that p53 is unnecessary for apoptosis induction by metformin (11).

In the current study, metformin was observed to target important downstream effectors of the Ras signaling pathway in cancer therapy, including AKT. Akt/PKB is a downstream target of PI3-K that plays a significant role in the regulation of apoptosis by activating proapoptotic proteins, including Bad and caspase 9 (26). Following metformin treatment, AKT levels were identified to decrease in A549 and PANC-1 cells but not in A431 cells. These observations indicate that metformin induces apoptosis by inhibiting AKT rather than via the activation of p53. A previous study reported that metformin may exert antitumor effects via the downregulation of AKT (27).

An additional important downstream effector of the Ras signaling pathway is MAPK, which mediates the anticancer effects of metformin. MAPK activation results in the transcription of a number of genes associated with proliferation (28). Metformin treatment was observed to reduce MAPK and p-MAPK levels in A549 and PANC-1 cells. However, although metformin did not downregulate MAPK in A431 cells, the proliferation of these cells was inhibited, indicating that alternative mechanisms underlie the anti-proliferative effect of metformin in these cells.

It has been widely reported that metformin may function as an AMPK activator to target the LKB1/AMPK signaling pathway in cancer therapy (29). Thus, the impacts of metformin on AMPK activity in K-ras mutant and wild-type cell lines were determined. AMPK is a cellular energy sensor that inhibits tumorigenesis via the regulation of cell growth, cell proliferation, autophagy, stress responses and cell polarity (30). Treatment with metformin was observed to activate AMPK in PANC-1 and A431 cells but not in A549 cells. This indicates that activation of AMPK is a key mediator of the anti-proliferative mechanisms of metformin in PANC-1 and A431 cells. Therefore, the current study demonstrates an inhibition of proliferation by metformin via the downregulation of MAPK or the activation of AMPK. Once the *LKB1* gene is mutated, metformin may only inhibit proliferation by downregulating MAPK. These observations are consistent with previous studies (31-33).

In summary, the present study indicates that metformin targets K-ras and may therefore represent a drug target for the treatment of cancer. Mutational activation of *Ras* genes is associated with 33% of human cancers, hence, Ras is considered to be an important target for cancer therapy (34). However, an ideal agent that specifically targets K-ras has not yet been developed (14,16). Metformin is orally active, with a relatively favorable toxicity profile and is low in cost. The promising results of our preclinical study are consistent with previous studies analyzing metformin treatment in clinical settings (35). Since the current observations are preliminary, in depth molecular profiling of the patient's tumor must be performed prior to cancer treatment with metformin.

Acknowledgements

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