Therapeutic potential of the antidiabetic drug metformin in small bowel adenocarcinoma

TAIGA CHIYO[1], KIYOHITO KATO[1], HISAKAZU IWAMA[2], SHINTARO FUJIHARA[1], KOJI FUJITA[1], TOMOKO TADOKORO[1], KYOKO OHURA[1], ERI SAMUKAWA[1], YOSHIMI YAMANA[1], NOBUYA KOBAYASHI[1], TAE MATSUNAGA[1], NORIKO NISHIYAMA[1], MAKI AYAKI[1], TATSUO YACHIDA[1], ASAHIRO MORISHITA[1], HIDEKI KOBARA[1], HIROHITO MORI[1] and TSUTOMU MASAKI[1]

[1]Department of Gastroenterology and Neurology, and [2]Life Science Research Center, Faculty of Medicine, Kagawa University, Kita-gun, Kagawa 761-0793, Japan

Received October 5, 2016; Accepted March 15, 2017

DOI: 10.3892/ijo.2017.3971

Abstract. Small bowel adenocarcinoma (SBAC) accounts for 3% of all gastrointestinal tract tumors and approximately 0.5% of all cancer cases. Recent studies have indicated that the use of metformin, one of the most commonly prescribed antidiabetic drugs, is associated with a better prognosis for certain malignant diseases. However, there have been no reports on the effect of metformin in SBAC. In the present study, we evaluated the effect of metformin on human SBAC cell proliferation *in vitro* and *in vivo* and identified the microRNAs (miRNAs) associated with its antitumor effects. Metformin inhibited the proliferation of HuTu80 cells in a time- and dose-dependent manner. Importantly, metformin reduced the expression of cyclin D1, cyclin E, cyclin-dependent kinase 4, and phosphorylated retinoblastoma protein, which resulted in cell cycle arrest at the G0/G1 phase. This arrest was accompanied by activation of AMPKα and inhibition of mammalian target of rapamycin and p70s6k. Additionally, metformin reduced the levels of phosphorylated epidermal growth factor receptor and ROR2 as well as markedly altered miRNA expression in HuTu80 cells. Metformin also inhibited tumor growth *in vivo* in a xenograft mouse model. Our data suggest that metformin might have therapeutic potential in SBAC.

Introduction

Small bowel adenocarcinoma (SBAC) accounts for 3% of all gastrointestinal tract tumors and approximately 0.5% of all cancers. There are approximately 10,090 new cases and 1,330 deaths from SBAC annually in the USA (1). A diagnosis of SBAC is often difficult because of its rarity and the non-specificity of the presenting signs and symptoms. Therefore, the disease tends to remain undiagnosed until it reaches an advanced stage, which is associated with a poor prognosis. The majority of patients with SBAC are in stage III or IV at diagnosis (26 and 32% of patients, respectively), and the 5-year disease-specific survival of stage III and IV patients is 35 and 4%, respectively (2). Unfortunately, there is no standard chemotherapy for advanced-stage SBAC because of the absence of randomized trials comparing different chemotherapy regimens. Therefore, novel therapeutic approaches are needed for advanced SBAC to overcome and improve the prognosis of SBAC patients.

Metformin is an oral biguanide drug; it is one of the most commonly prescribed antidiabetic drugs and was introduced into clinical practice in the 1950s for the treatment of type 2 diabetes (3). Metformin reduces blood glucose levels by inhibiting hepatic gluconeogenesis. Previously, retrospective epidemiologic studies reported that the use of metformin decreases cancer incidence (4,5). Additionally, it reduces the risk of all cancers and contributes to lower cancer mortality (6-9) among patients with concurrent diabetes and cancer. Moreover, basic studies *in vitro* and *in vivo* have suggested the antitumor effects of metformin in various cancerous cells, such as those of prostate, breast, colon, stomach, esophagus, pancreas and liver (10-17). The antitumor effect of metformin is due to several mechanisms, including activation of the LKB1/AMPK pathway (12,18), induction of cell cycle arrest (10-14) and apoptosis (16), inhibition of the unfolded protein response, inhibition of protein synthesis, reduction in circulating insulin levels, activation of the immune system and eradication of cancer stem cells (18). In particular, the activation of the LKB1/AMPK pathway leads to the inhibition of mammalian target of rapamycin (mTOR), which decreases protein synthesis in cancer cells (18). Furthermore, metformin has been shown to affect microRNA (miRNA) expression in certain cancers; these miRNAs can function as either oncogenes or tumor suppressors (19). However, the therapeutic potential of metformin in cancer cell proliferation and tumor growth of SBAC is not well known.

Correspondence to: Dr Tsutomu Masaki, Department of Gastroenterology and Neurology, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan
E-mail: tmasaki@med.kagawa-u.ac.jp

Key words: small bowel adenocarcinoma, metformin, cell cycle, microRNAs, cyclin D1, AMPKα

In the present study, we demonstrated the antitumor effects of metformin in a human SBAC cell line and found that these effects were associated with cell cycle arrest by reducing cell cycle-related molecules. Moreover, we evaluated the expression of miRNAs related to the antitumor effect of metformin. These results suggest that metformin may provide a novel therapeutic approach for SBAC.

Materials and methods

Cell lines, reagents and antibodies. The human SBAC cell line HuTu80 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (533-69545; Wako, Osaka, Japan), and penicillin/streptomycin (100 mg/l; Invitrogen, Tokyo, Japan) in a 5% CO_2 atmosphere at 37˚C.

Metformin (1,1-dimethylbiguanide monohydrochloride) was purchased from Astellas Pharma, Inc. (Tokyo, Japan). Cell Counting kit (CCK)-8 was purchased from Dojindo Laboratories (Kumamoto, Japan).

The following antibodies were used: β-actin (Sigma-Aldrich, St. Louis, MO, USA; A5441); cyclin D1 (RB-9041), cyclin E (MS-870-p1) (Thermo Fisher Scientific, Waltham, MA, USA); Cdk2 (sc-163), Cdk4 (sc-749), Cdk6 (sc-177), retinoblastoma protein (Rb) (sc-50) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); phosphorylated retinoblastoma protein (pRb) (BD Biosciences, San Jose, CA, USA; 558385); AMPKα (#5832), phosphorylated AMPKα Thr172 (#2535), mTOR (#2983), phosphorylated mTOR Ser2448 (#5536), p70s6k (#2708), phosphorylated p70s6k Thr389 (#9205) (Cell Signaling Technology, Danvers, MA, USA); and secondary horseradish peroxidase-linked anti-mouse and anti-rabbit IgG antibodies (Cell Signaling Technology; used at 1:2,000). Chemiluminescence reagents for western blot imaging were purchased from Perkin-Elmer Inc. (Waltham, MA, USA).

Cell proliferation assay. Cell proliferation assays were performed using CCK-8 according to the manufacturer's instructions. Cells (5,000/well) were seeded into each well of a 96-well plate with 100 μl of DMEM supplemented with 10% FBS. After 24 h, the cells were treated with 0, 1, 5 or 10 mM metformin and then cultured for 24-72 h. CCK-8 reagent (10 μl) was added to each well, and the cells were incubated for 3 h. The absorbance was measured for each well at a wavelength of 450 nm using an automicroplate reader.

Flow cytometric analysis. To evaluate the mechanism of growth inhibition by metformin, cell cycle profiles were analyzed after treatment with metformin. HuTu80 cells ($1.0x10^6$ cells in a 100-mm dish) were treated with or without 10 mM metformin for 24-72 h. The cell cycle distribution was analyzed by measuring the amount of propidium iodide (PI)-labeled DNA in ethanol-fixed cells.

The fixed cells were washed with phosphate-buffered saline (PBS) and stored at -20˚C until flow cytometric analysis was performed. On the day of analysis, cells were washed and centrifuged using cold PBS, suspended in 100 μl of PBS and 10 μl of RNase A solution (250 μg/ml) and incubated for 30 min at 37˚C. Then, 110 μl of PI stain (100 μg/ml) was added to each tube, and the tubes were incubated at 4˚C for at least 30 min prior to analysis. Flow cytometry was performed by a Cytomics FC 500 flow cytometer (Beckman Coulter, Brea, CA, USA) with an argon laser (488 nm). The percentages of cells in different phases of the cell cycle were analyzed by FlowJo software (Tree Star, Inc., Ashland, OR, USA). All experiments were performed in triplicate to assess the consistency of the response.

Gel electrophoresis and western blot analysis. HuTu80 cells treated (or not) with 10 mM metformin were cultured for 24-72 h. The cells were lysed in lysis buffer (PRO-PREP; Intron Biotechnology, Inc., Seongnam, Korea). The cell lysate was centrifuged at 13,000 x g at 4˚C for 5 min, and the remnant was removed. The protein concentrations were analyzed by a NanoDrop 2000 fluorospectrometer (Thermo Fisher Scientific). After adding 2X SDS sample buffer, the samples were heated to 95-100˚C for 5 min and then cooled on ice. The samples were electrophoresed using 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) (20), and the proteins were transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies after blocking. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (21). The immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Perkin-Elmer Co., Waltham, MA, USA) on X-ray film. All experiments were repeated three times.

Xenograft model analysis. Animal experiments were performed according to the guidelines of the Committee on Experimental Animals of Kagawa University (Kagawa, Japan). Male athymic mice (BALB/c-nu/nu; 6 weeks old; 20-25 g) were purchased from Japan SLC (Shizuoka, Japan). The mice were maintained under specific pathogen-free conditions using a laminar airflow rack. The animals had continuous free access to sterilized food (gamma-ray-irradiated food, CL-2; Clea Japan, Inc., Tokyo, Japan) and autoclaved water. A total of 16 mice were subcutaneously inoculated with HuTu80 cells ($5x10^6$ cells/animal) in the flank. Five days later, the xenografts were identifiable as a 3-mm (maximum) diameter mass; the mice were randomly subdivided into two groups of 8 mice. Then, 1 mg/body per day metformin was injected 5 times a week intraperitoneally (i.p.) for 2 weeks in the metformin-treated group. The control group was administered PBS alone for 2 weeks. After the initiation of the metformin administration, tumor growth was monitored daily by the same investigators (T. Chiyo and T. Masaki). Tumor size was measured twice per week by measuring the two largest perpendicular dimensions. The tumor volume was calculated as follows: Tumor volume (mm^3) = [tumor length (mm) x tumor width (mm) ^{2}]/2 (22). All mice were sacrificed on day 15 after treatment. All animals were alive until this time.

Antibody arrays of phosphorylated receptor tyrosine kinases (p-RTKs). Human p-RTKs were assayed using Human Phospho-RTK array kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. HuTu80 cells ($1.0x10^6$ cells in a 100-mm dish) were treated with or without

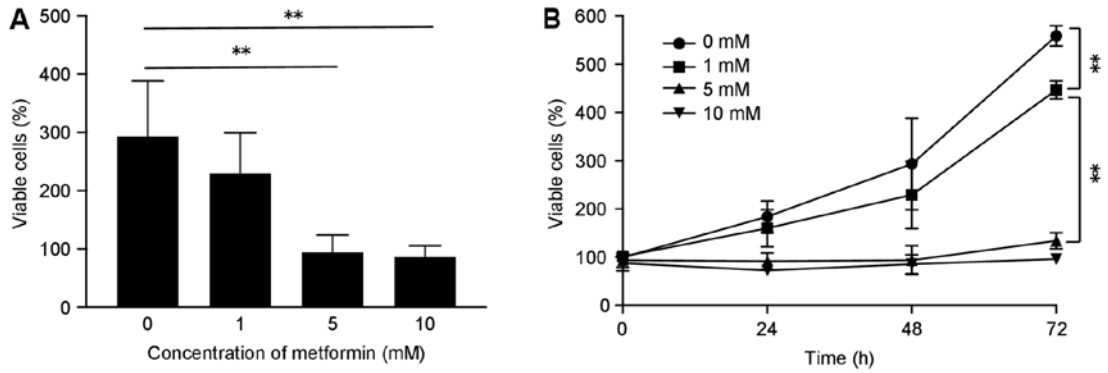

Figure 1. Metformin inhibits HuTu80 cell proliferation. (A) HuTu80 cells (5,000/well) were seeded in 96-well plates. After 24 h, the culture medium was replaced with fresh culture medium containing 0, 1, 5 or 10 mM metformin. Cell viability was assayed after 48 h. The results show that 5 and 10 mM metformin significantly inhibited cell proliferation (Student's t-test, $^{**}P<0.01$ n=8, error bars represent the SD). (B) Cell viability was assayed daily from 0 to 72 h. The viability of metformin-treated cells differed significantly in a time- and dose-dependent manner from the control cells ($^{**}P<0.01$, by two-way ANOVA).

5 mM metformin for 48 h. P-RTK array membranes were blocked with 5% BSA/TBS (0.01 M Tris-HCl, pH 7.6) for 1 h. The membranes were then incubated with 2 ml of lysate prepared from cell lines after normalization with equal amounts of protein. After extensive washing with TBS including 0.1% v/v Tween-20 (3 washings of 10 min each) and TBS alone (2 washings of 10 min each) to remove unbound materials, the membranes were incubated with anti-phospho-tyrosine-HRP antibody for 2 h at room temperature. The unbound HRP antibody was washed out with TBS including 0.1% Tween-20. Finally, the membranes were exposed to X-ray film using a chemiluminescence detection system (Perkin-Elmer).

Antibody array for angiogenesis. The RayBio Human Angiogenesis Antibody Array C1 (RayBiotech Inc., Norcross, GA, USA) was used according to the manufacturer's protocol. HuTu80 cells ($1.0x10^6$ cells in a 100-mm dish) were treated with or without 5 mM metformin for 48 h. Each array membrane was exposed to X-ray film using a chemiluminescence detection system (Perkin-Elmer).

Analysis of miRNA microarray. Total RNA was extracted from HuTu80 cells ($1.0x10^6$ cells in a 100-mm dish) treated with or without 10 mM metformin for 48 h using the miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA samples typically showed $_{A260/280}$ ratios between 1.9 and 2.1, using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). After RNA measurement with an RNA 6000 Nano kit (Agilent Technologies), the samples were labeled using a miRCURY Hy3/Hy5 Power labeling kit and were hybridized to a human miRNA Oligo chip (v.21.0; Toray Industries, Inc., Tokyo, Japan). The chips were scanned with the 3D-Gene Scanner 3000 (Toray Industries). 3D-Gene extraction version 1.2 software (Toray Industries) was used to read the raw intensity of the image. The changes in miRNA expression between metformin-treated and control samples were analyzed with GeneSpringGX v 10.0 (Agilent Technologies). Quantile normalization was performed on the raw data that were above the background level. Differentially expressed miRNAs were determined by the Mann-Whitney U test. The false discovery rate was computed with the Benjamini-Hochberg procedure for multiple testing (23). Hierarchical clustering was performed using the furthest neighbor method with the absolute uncentered Pearson's correlation coefficient as a metric. We produced a heat map with the relative expression intensity for each miRNA, in which the base-2 logarithm of the intensity was median-centered for each row.

Statistical analyses. All statistical analyses were performed by GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between the treatment and control groups were performed by two-tailed paired or unpaired Student's t-tests. Two-way ANOVA was also used to compare tumor size in mice after different treatments. In all analyses, a $P<0.05$ was considered significant.

Results

Metformin inhibits the proliferation of human SBAC cells in vitro. To evaluate the effect of metformin on the proliferation of human SBAC cells, HuTu80 cells were grown in 10% FBS and treated with 0, 1, 5 or 10 mM metformin for 72 h. Metformin treatment led to inhibition of cell proliferation in a time- and dose-dependent manner (Fig. 1).

Metformin induced cell cycle arrest in the G0/G1 phase and regulates cell cycle-related proteins in SBAC cells. To examine whether the growth inhibition was due to cell cycle change, we analyzed the cell cycle profiles of HuTu80 cells 24-72 h after treatment with or without 10 mM metformin by flow cytometry. Metformin treatment increased the proportion of cells in the G0/G1 phase in treated cells compared with the control cells (71.8 vs. 49.4% after 24 h, 67.3 vs. 46.1% after 48 h, 69.1 vs. 49.9% after 72 h) (Fig. 2). The proportion of cells in S phase and G2/M phase decreased accordingly.

The effects of metformin on the expression of cell cycle regulatory proteins were evaluated by western blotting. HuTu80 cells were treated with or without 10 mM metformin for 24-72 h. We found notably lower levels of cyclin D1, cyclin E, Cdk4 and pRb in cells treated with metformin (Fig. 3), which suggested that metformin suspended the transition from G0/G1 phase to S phase by modulating cyclin D1, cyclin E, Cdk4 and pRb.

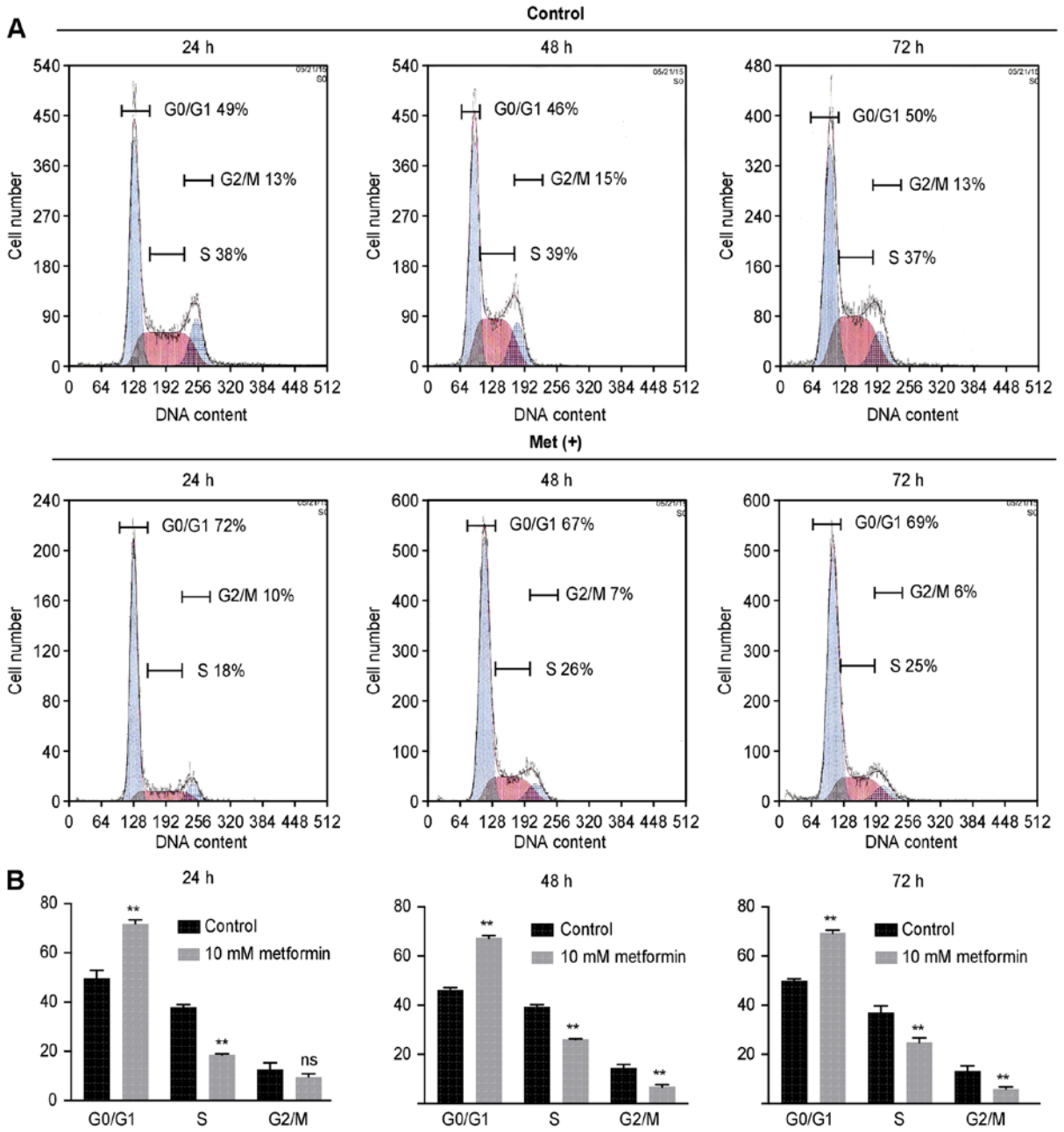

Figure 2. Metformin blocks cell cycle progression at the G0/G1 phase. (A) Flow cytometric analysis of proliferating HuTu80 cells, 24, 48 and 72 h after administration of 10 mM metformin (Met). (B) Metformin increased the proportion of cells in the G0/G1 phase in the treated cells (Student's t-test, $^{**}P<0.01$ n=3, error bars represent the SD). The proportion of cells in S phase and G2/M phase decreased accordingly.

Additionally, p-AMPKα increased after a 24-h treatment with 10 mM metformin compared with the control, revealing the activation of the AMPK pathway. p-mTOR and p-p70s6k, which are downstream of AMPKα, were also decreased in metformin-treated cells without substantial differences in the total protein levels of AMPKα, mTOR or p70s6k (Fig. 3). These results suggest that metformin decreases the expression level of cell cycle-related proteins and the phosphorylation of key cell cycle regulatory proteins via the AMPKα/mTOR pathway leading to G0/G1 arrest in human SBAC cells.

Metformin affected p-RTKs in vitro. We performed a p-RTK array to identify key RTKs associated with the antitumor effect of metformin. The antibody array (Fig. 4A) enabled 49 of the expressed and activated RTKs to be screened in HuTu80 cells. We also performed the array using 10 mM metformin for 48 h; however, most of the phospho-RTKs were strongly inhibited, and we could not conduct an evaluation. Therefore, we performed the array in the presence and absence of 5 mM metformin for 48 h. Metformin reduced the expression of phosphorylated epidermal growth factor receptor (EGFR), ROR2 (Fig. 4B). Densitometry showed that the ratios of the EGFR and ROR2 of metformin-treated to untreated cells were 57 and 75%, respectively (Fig. 4C).

Metformin did not affect angiogenesis-related molecules in vitro. We performed an angiogenesis array (Fig. 5A) to identify the key angiogenesis-related molecules associated

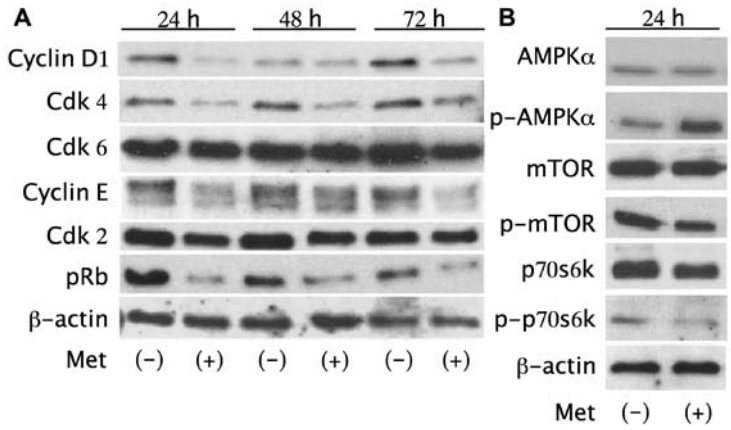

Figure 3. Metformin reduces cell cycle regulatory proteins and activates the AMPKα pathway. (A) Western blot analysis of cyclin D1, cyclin E, Cdk2, Cdk4, Cdk6, Rb, pRb in HuTu80 cells treated with 10 mM metformin for 24-72 h. The expression level of cyclin D1, cyclin E, Cdk4 and pRb decreased in treated cells. (B) Metformin increased p-AMPKα after a 24-h treatment with 10 mM metformin. p-mTOR and p-p70s6k, downstream of AMPKα, decreased accordingly in treated cells. There were no substantial differences in total AMPK, mTOR or p70s6k expression between metformin-treated cells and the control cells.

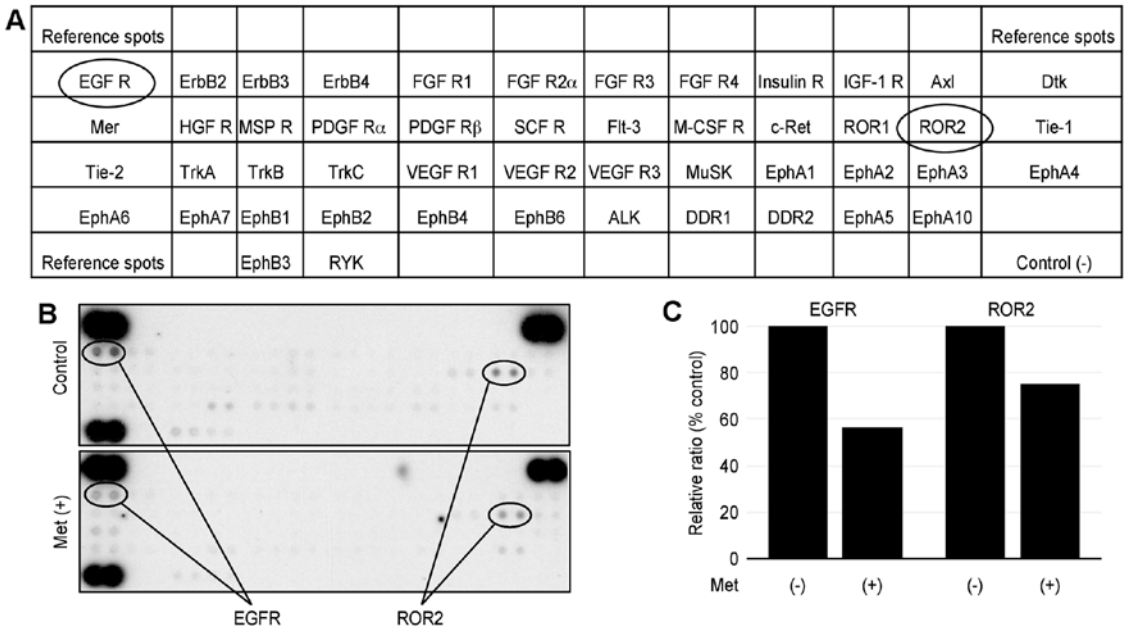

Figure 4. Metformin reduces the expression of phosphorylated EGFR and ROR2. (A) The template indicates locations of tyrosine kinase antibodies spotted onto a human phospho-RTK array. (B) Representative expression of various phosphorylated tyrosine kinase receptors in HuTu80 cells with or without 5 mM metformin for 48 h. (C) The densitometric ratios of the EGFR, and ROR2 spots were 57 and 75%, respectively (the ratio of metformin-treated cells to control cells).

with the antitumor effects of metformin on HuTu80. The 20 screened angiogenesis molecules showed no change after 5-mM metformin treatment for 48 h compared with the control (Fig. 5B).

Metformin inhibits SBAC proliferation in vivo. To evaluate the antitumor effect *in vivo*, each nude mouse was subcutaneously inoculated with HuTu80 cells ($5x10^6$ cells/animal) in the flank region, followed by an i.p. injection. All animals survived the experimental period. Additionally, metformin did not affect body weight (data not shown), and the mice did not exhibit signs of behavioral alterations or suffering. Metformin (1 mg/body/day) significantly reduced tumor growth by 72% relative to the PBS-injected control (Fig. 6).

Effects of metformin on miRNA expression. We analyzed the levels of expression of 2555 miRNA probes in HuTu80 cells with or without metformin treatment with a custom microarray platform. Unsupervised hierarchical clustering analysis showed that the treated cells clustered separately from the control cells (Fig. 7). We identified 17 miRNAs that were significantly upregulated and 33 downregulated miRNAs in treated cells, compared with the control (Table I).

Discussion

The present study revealed that metformin inhibited cell proliferation and tumor growth of human SBAC *in vitro* and *in vivo*. Previous studies have shown that metformin induces

A

POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	bFGF
GRO	IFN-gamma	IGF-1	IL-6	IL-8	Leptin	MCP-1	PDGF-BB
PLGF	RANTES	TGF beta1	TIMP-1	TIMP-2	TPO	VEGF-A	VEGF-D
BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS

B

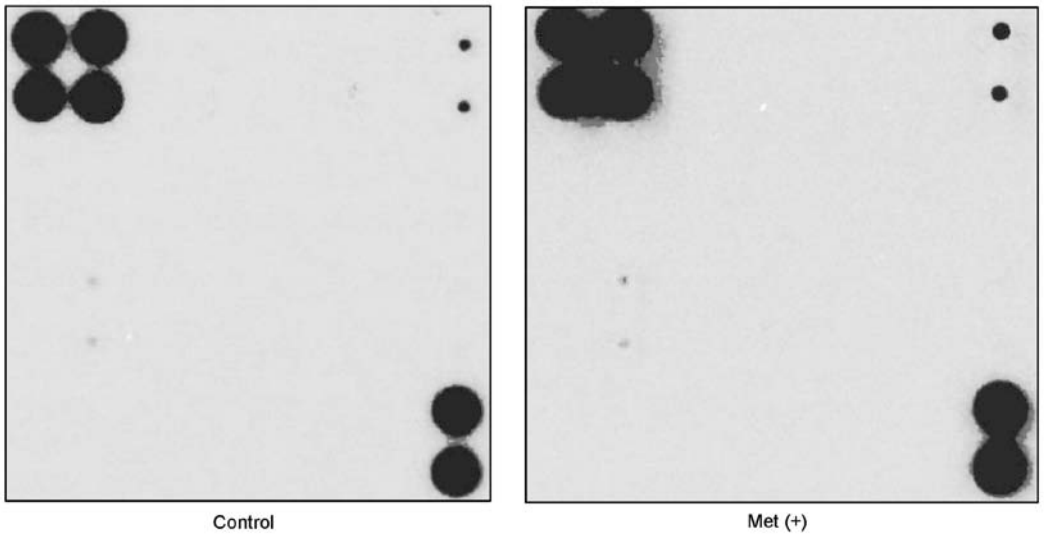

Figure 5. Metformin did not affect angiogenesis-related molecules *in vitro*. (A) The template indicates locations of angiogenesis-related proteins spotted onto a human angiogenesis array. (B) The 20 screened angiogenesis molecules showed no changes after 5-mM metformin treatment for 48 h compared with the control.

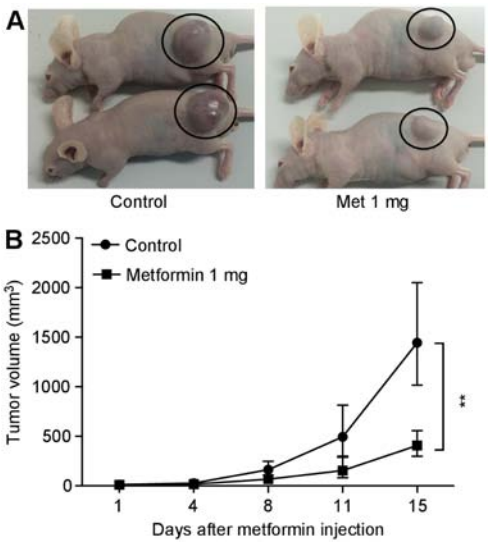

Figure 6. Metformin inhibits tumor growth *in vivo*. When tumors became palpable, 0 or 1 mg of metformin was injected intraperitoneally 5 times per week for 2 weeks. (A) Representative images of the gross HuTu80 tumors treated with metformin, or of the control. (B) The tumor volume was calculated as [tumor length (mm) x tumor width $(mm)^2$]/2. The tumors were significantly smaller in the metformin-treated group than in the control group. Each point represents the mean ± standard deviation of 8 mice ($^{**}P<0.01$, by two-way ANOVA).

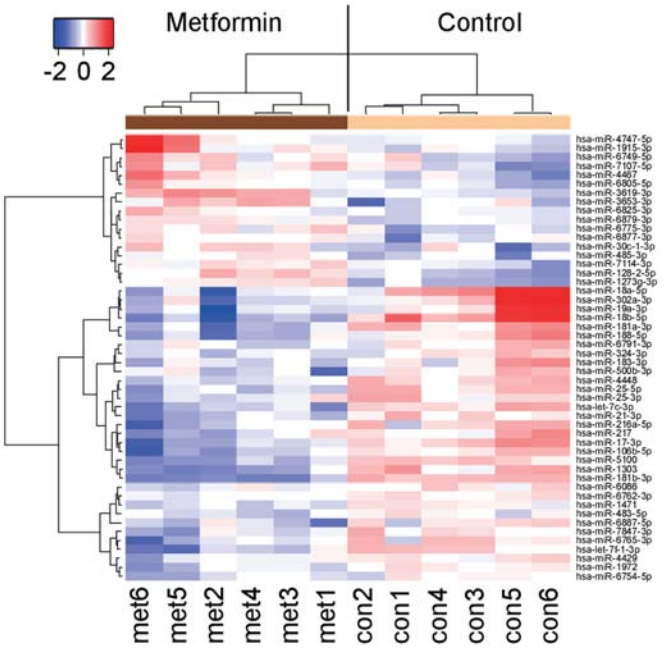

Figure 7. Hierarchical clustering of HuTu80 cells with or without metformin. HuTu80 cells were clustered according to the expression profiles of 50 miRNAs differentially expressed by HuTu80 cells with or without metformin. The analyzed samples are shown in the columns, and the miRNAs are shown in the rows. The miRNA clustering color scale shown at the top indicates the relative expression levels of miRNAs; with red and blue representing high and low expression levels, respectively.

cell cycle arrest via modulating cell cycle regulatory proteins in various cancer cell lines (10-14,17). Cell cycle dysregulation is a feature of tumor cells, and cell cycle arrest is a major indicator of antitumor activity (24). Specific cyclin/Cdk complexes are activated at different intervals during the cell cycle. Complexes of Cdk4/6 and cyclin D1 are required for G1 phase progression. Complexes of Cdk2 and cyclin E are required for G1 to S phase transition (25). In the present study, flow cytometry showed that metformin induced cell cycle

Table I. Statistical results and chromosomal location of miRNAs in HuTu80 cells treated with or without metformin.

miRNA	Fold change (treated/ non-treated)	P-value	Chromosomal localization
Upregulated			
hsa-miR-1915-3p	2.12	0.0411	10
hsa-miR-4745-5p	1.98	0.0411	19
hsa-miR-3619-3p	1.77	0.0411	22
hsa-miR-128-2-5p	1.77	0.0022	3
hsa-miR-4467	1.76	0.0087	7
hsa-miR-12733g g-3p	1.73	0.0061	1
hsa-miR-3653-3p	1.67	0.0260	22
hsa-miR-7107-5p	1.58	0.0450	12
hsa-miR-30c-1-3p	1.51	0.0260	1
hsa-miR-6749-5p	1.49	0.0411	11
hsa-miR-6805-5p	1.41	0.0303	19
hsa-miR-6775-3p	1.37	0.0260	16
hsa-miR-485-3p	1.34	0.0260	14
hsa-miR-7114-3p	1.32	0.0152	9
hsa-miR-6879-3p	1.27	0.0152	11
hsa-miR-6877-3p	1.25	0.0367	9
hsa-miR-6825-3p	1.24	0.0411	3
Downregulated			
hsa-miR-18b-5p	0.23	0.0022	X
hsa-miR-18a-5p	0.30	0.0200	13
hsa-miR-302a-3p	0.34	0.0152	4
hsa-miR-181b-3p	0.36	0.0048	1
hsa-miR-19a-3p	0.37	0.0152	13
hsa-miR-1303	0.43	0.0043	5
hsa-miR-17-3p	0.45	0.0050	13
hsa-miR-181a-3p	0.47	0.0043	1
hsa-miR-5100	0.49	0.0049	10
hsa-miR-188-5p	0.49	0.0087	X
hsa-let-7c-3p	0.50	0.0022	21
hsa-miR-106b-5p	0.53	0.0063	7
hsa-let-7f-1-3p	0.55	0.0087	9
hsa-miR-25-5p	0.55	0.0022	7
hsa-miR-216a-5p	0.59	0.0152	2
hsa-miR-21-3p	0.59	0.0022	17
hsa-miR-217	0.61	0.0411	2
hsa-miR-183-3p	0.62	0.0411	7
hsa-miR-6765-3p	0.63	0.0303	14
hsa-miR-4448	0.64	0.0043	3
hsa-miR-25-3p	0.66	0.0129	7
hsa-miR-6887-5p	0.67	0.0152	19
hsa-miR-500b-3p	0.67	0.0411	X
hsa-miR-7847-3p	0.68	0.0152	11
hsa-miR-324-3p	0.73	0.0411	17
hsa-miR-6791-3p	0.74	0.0446	19
hsa-miR-1972	0.74	0.0129	16
hsa-miR-1471	0.76	0.0087	2
hsa-miR-6754-5p	0.78	0.0303	11
hsa-miR-4429	0.80	0.0303	2
hsa-miR-483-5p	0.80	0.0260	11
hsa-miR-6086	0.81	0.0411	X
hsa-miR-6762-3p	0.83	0.0087	12

arrest at the G0/G1 phase in SBAC. Additionally, cyclin D1, Cdk4, cyclin E and pRb, which are major cell cycle regulatory proteins, are markedly attenuated in treated cells. Therefore, this finding suggests that these proteins could be intracellular targets of the metformin-mediated anti-proliferative effect by inducing G0/G1 cycle arrest in SBAC cells.

AMPK is a well-known downstream target of metformin, and metformin inhibits cancer cell proliferation by activating AMPK (26). The antitumor effect of metformin is related to the activation of AMPK (12,26-30), apoptosis (16,31) and angiogenesis (32) in various cancer cells. Previous studies demonstrated that metformin displays AMPK-dependent or -independent effects, at least partially, on cancer cell proliferation (27-30,33). More recently, metformin was shown to induce cyclin D1 degradation via an AMPK/GSK3β signaling axis that involved the ubiquitin/proteasome pathway in ovarian cancer cells (29). Our results showed that treatment with metformin increased the expression of p-AMPK (Thr172) and reduced the expression of p-p70s6k (Thr389), which confirms the ability of metformin to activate AMPK and inactivate mTOR. These results indicate that metformin may reduce the expression of cell cycle-related proteins and induce cell cycle arrest via an AMPKα/mTOR/p70s6k axis.

In addition to its effect on the cell cycle, metformin can interfere with some receptors because metformin decreases the levels of EGFR oncoprotein in various cancer cells (10,11,13,31). EGFR plays a key role in cancer cell proliferation and cell cycle progression (34-36). In the present study, we found that metformin reduced phosphorylated EGFR and ROR2 in SBAC cells. This is the first report to show that metformin inhibited ROR2 activation. ROR2 correlates with a poor prognosis in various cancer types (37-39), cell migration/invasion and metastasis (40-42); therefore, it has potential as a novel drug target in cancer therapy.

Using an angiogenesis antibody array, metformin was previously shown to inhibit angiogenesis by reducing angiogenesis-related molecules (11,12). On the other hand, the 20 screened angiogenesis molecules did not exhibit differences in metformin-treated or non-treated SBAC cells. These data suggest that metformin may not affect tumor angiogenesis by reducing various angiogenesis-related molecules in SBAC.

miRNAs are small, endogenous, non-coding siRNAs that are 21-30 nucleotides in length and that modulate the expression of various target genes at the post-transcriptional and translational levels (43). We assessed the miRNAs associated with the antitumor effects of metformin in SBAC cells using miRNA expression arrays. The cluster analysis indicated that the metformin treatment affected the expression of numerous miRNAs. We identified 50 miRNAs differentially expressed (17 upregulated and 33 downregulated) in the cluster. These changes in miRNA expression may provide clues to the molecular basis of the antitumor effect of metformin.

Our data revealed that miR-1915 exhibited the highest upregulation in HuTu80 cells treated with metformin. miR-1915 targets the anti-apoptotic protein Bcl-2 and modulates multidrug resistance in colorectal cancer cells (44). miR-1915 is a p53-responsive miRNA, and it negatively regulates Bcl-2 expression at the post-translational level (45). Additionally, miR-1915 was shown to inhibit Bcl-2 expression and sensitize cancerous cells to anticancer drugs (44) we

investigated the possible role of microRNAs in the development of multidrug resistance (MDR). Metformin has also been shown to significantly downregulate miR-21 in HuTu80 cells, and downregulation of miR-21 has been shown to restore the PTEN/Akt axis in colon cancer cells and inhibit their growth (46). Therefore, these changes in miRNA expression may contribute to suppressed cancer proliferation.

There are several limitations in the present study. First, we conducted the *in vitro* study with higher doses of metformin than those that are used in human treatment (6-30 μmol/l); however, these doses are similar to those used in previous studies on prostate (14), colon cancer (17) and melanoma (16). The cell culture was conducted in hyperglycemic conditions with 10% FBS. The high concentration of nutrition may lead to strong growth stimulation and high-dose requirements for metformin. However, we confirmed the antitumor effect in the *in vivo* study, in which the use of metformin, 1 mg (40-50 mg/kg) was almost equivalent to the dose used in human treatments. Second, we used only a single cell line in this study because no other human SBAC cell lines were available. Instead, we obtained the human ileocecal adenocarcinoma cell line HCT-8 and performed the cell proliferation assay with this cell line. The cells were treated with the same concentrations of metformin (0, 1, 5 or 10 mM) as the HuTu80 cells and were cultured for 48 h. The proliferation of HCT-8 cells was also inhibited with metformin in a time- and dose-dependent manner (data not shown).

In conclusion, our results indicate that metformin inhibits SBAC cell proliferation by suppressing cell cycle-related molecules, activating AMPK and inhibiting mTOR and p70s6k signaling, and altering the production of miRNAs. Therefore, metformin may become a novel therapeutic agent for SBAC treatment, providing synergistic effects based on different mechanisms of action.

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2016. CA Cancer J Clin 66: 7-30, 2016.
2. Howe JR, Karnell LH, Menck HR and Scott-Conner C: The American College of Surgeons Commission on Cancer and the American Cancer Society. Adenocarcinoma of the small bowel: Review of the National Cancer Data Base, 1985-1995. Cancer 86: 2693-2706, 1999.
3. Witters LA: The blooming of the French lilac. J Clin Invest 108: 1105-1107, 2001.
4. Libby G, Donnelly LA, Donnan PT, Alessi DR, Morris AD and Evans JM: New users of metformin are at low risk of incident cancer: A cohort study among people with type 2 diabetes. Diabetes Care 32: 1620-1625, 2009.
5. Landman GW, Kleefstra N, van Hateren KJ, Groenier KH, Gans RO and Bilo HJ: Metformin associated with lower cancer mortality in type 2 diabetes: ZODIAC-16. Diabetes Care 33: 322-326, 2010.
6. Franciosi M, Lucisano G, Lapice E, Strippoli GF, Pellegrini F and Nicolucci A: Metformin therapy and risk of cancer in patients with type 2 diabetes: Systematic review. PLoS One 8: e71583, 2013.
7. Noto H, Goto A, Tsujimoto T and Noda M: Cancer risk in diabetic patients treated with metformin: A systematic review and meta-analysis. PLoS One 7: e33411, 2012.
8. Soranna D, Scotti L, Zambon A, Bosetti C, Grassi G, Catapano A, La Vecchia C, Mancia G and Corrao G: Cancer risk associated with use of metformin and sulfonylurea in type 2 diabetes: A meta-analysis. Oncologist 17: 813-822, 2012.
9. Fujita K, Iwama H, Miyoshi H, Tani J, Oura K, Tadokoro T, Sakamoto T, Nomura T, Morishita A, Yoneyama H, *et al*: Diabetes mellitus and metformin in hepatocellular carcinoma. World J Gastroenterol 22: 6100-6113, 2016.
10. Miyoshi H, Kato K, Iwama H, Maeda E, Sakamoto T, Fujita K, Toyota Y, Tani J, Nomura T, Mimura S, *et al*: Effect of the anti-diabetic drug metformin in hepatocellular carcinoma *in vitro* and *in vivo*. Int J Oncol 45: 322-332, 2014.
11. Kato K, Gong J, Iwama H, Kitanaka A, Tani J, Miyoshi H, Nomura K, Mimura S, Kobayashi M, Aritomo Y, *et al*: The anti-diabetic drug metformin inhibits gastric cancer cell proliferation in vitro and in vivo. Mol Cancer Ther 11: 549-560, 2012.
12. Cai X, Hu X, Tan X, Cheng W, Wang Q, Chen X, Guan Y, Chen C and Jing X: Metformin induced AMPK activation, G0/G1 phase cell cycle arrest and the inhibition of growth of esophageal squamous cell carcinomas in vitro and in vivo. PLoS One 10: e0133349, 2015.
13. Fujihara S, Kato K, Morishita A, Iwama H, Nishioka T, Chiyo T, Nishiyama N, Miyoshi H, Kobayashi M, Kobara H, *et al*: Antidiabetic drug metformin inhibits esophageal adenocarcinoma cell proliferation *in vitro* and *in vivo*. Int J Oncol 46: 2172-2180, 2015.
14. Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, Tanti JF, Le Marchand-Brustel Y and Bost F: The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene 27: 3576-3586, 2008.
15. Nair V, Sreevalsan S, Basha R, Abdelrahim M, Abudayyeh A, Rodrigues Hoffman A and Safe S: Mechanism of metformin-dependent inhibition of mammalian target of rapamycin (mTOR) and Ras activity in pancreatic cancer: Role of specificity protein (Sp) transcription factors. J Biol Chem 289: 27692-27701, 2014.
16. Janjetovic K, Harhaji-Trajkovic L, Misirkic-Marjanovic M, Vucicevic L, Stevanovic D, Zogovic N, Sumarac-Dumanovic M, Micic D and Trajkovic V: In vitro and in vivo anti-melanoma action of metformin. Eur J Pharmacol 668: 373-382, 2011.
17. Zhou XZ, Xue YM, Zhu B and Sha JP: Effects of metformin on proliferation of human colon carcinoma cell line SW-480. Nan Fang Yi Ke Da Xue Xue Bao 30: 1935-1938, 1942, 2010 (In Chinese).
18. Kourelis TV and Siegel RD: Metformin and cancer: New applications for an old drug. Med Oncol 29: 1314-1327, 2012.
19. Zhou JY, Xu B and Li L: A new role for an old drug: Metformin targets microRNAs in treating diabetes and cancer. Drug Dev Res 76: 263-269, 2015.
20. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
21. Towbin H, Staehelin T and Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354, 1979.
22. D'Incalci M, Colombo T, Ubezio P, Nicoletti I, Giavazzi R, Erba E, Ferrarese L, Meco D, Riccardi R, Sessa C, *et al*: The combination of yondelis and cisplatin is synergistic against human tumor xenografts. Eur J Cancer 39: 1920-1926, 2003.
23. Benjamini Y and Hochberg Y: Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc B 57: 289-300, 1995.
24. Abbas T and Dutta A: p21 in cancer: Intricate networks and multiple activities. Nat Rev Cancer 9: 400-414, 2009.
25. Masaki T, Shiratori Y, Rengifo W, Igarashi K, Yamagata M, Kurokohchi K, Uchida N, Miyauchi Y, Yoshiji H, Watanabe S, *et al*: Cyclins and cyclin-dependent kinases: Comparative study of hepatocellular carcinoma versus cirrhosis. Hepatology 37: 534-543, 2003.
26. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, *et al*: Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 108: 1167-1174, 2001.
27. Zakikhani M, Dowling RJ, Sonenberg N and Pollak MN: The effects of adiponectin and metformin on prostate and colon neoplasia involve activation of AMP-activated protein kinase. Cancer Prev Res (Phila) 1: 369-375, 2008.
28. Zakikhani M, Dowling R, Fantus IG, Sonenberg N and Pollak M: Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res 66: 10269-10273, 2006.
29. Gwak H, Kim Y, An H, Dhanasekaran DN and Song YS: Metformin induces degradation of cyclin D1 via AMPK/GSK3β axis in ovarian cancer. Mol Carcinog 56: 349-358, 2017.

30. Sesen J, Dahan P, Scotland SJ, Saland E, Dang VT, Lemarié A, Tyler BM, Brem H, Toulas C, Cohen-Jonathan Moyal E, *et al*: Metformin inhibits growth of human glioblastoma cells and enhances therapeutic response. PLoS One 10: e0123721, 2015.
31. Wang LW, Li ZS, Zou DW, Jin ZD, Gao J and Xu GM: Metformin induces apoptosis of pancreatic cancer cells. World J Gastroenterol 14: 7192-7198, 2008.
32. Rattan R, Graham RP, Maguire JL, Giri S and Shridhar V: Metformin suppresses ovarian cancer growth and metastasis with enhancement of cisplatin cytotoxicity in vivo. Neoplasia 13: 483-491, 2011.
33. Ben Sahra I, Regazzetti C, Robert G, Laurent K, Le Marchand-Brustel Y, Auberger P, Tanti JF, Giorgetti-Peraldi S and Bost F: Metformin, independent of AMPK, induces mTOR inhibition and cell-cycle arrest through REDD1. Cancer Res 71: 4366-4372, 2011.
34. Wang L, Xiao J, Gu W and Chen H: Sex difference of Egfr expression and molecular pathway in the liver: Impact on drug design and cancer treatments? J Cancer 7: 671-680, 2016.
35. Engel BJ, Bowser JL, Broaddus RR and Carson DD: MUC1 stimulates EGFR expression and function in endometrial cancer. Oncotarget 7: 32796-32809, 2016.
36. Perry JE, Grossmann ME and Tindall DJ: Epidermal growth factor induces cyclin D1 in a human prostate cancer cell line. Prostate 35: 117-124, 1998.
37. Huang J, Fan X, Wang X, Lu Y, Zhu H, Wang W, Zhang S and Wang Z: High ROR2 expression in tumor cells and stroma is correlated with poor prognosis in pancreatic ductal adenocarcinoma. Sci Rep 5: 12991, 2015.
38. Lu C, Wang X, Zhu H, Feng J, Ni S and Huang J: Over-expression of ROR2 and Wnt5a cooperatively correlates with unfavorable prognosis in patients with non-small cell lung cancer. Oncotarget 6: 24912-24921, 2015.
39. Sun B, Ye X, Lin L, Shen M and Jiang T: Up-regulation of ROR2 is associated with unfavorable prognosis and tumor progression in cervical cancer. Int J Clin Exp Pathol 8: 856-861, 2015.
40. O'Connell MP, Fiori JL, Xu M, Carter AD, Frank BP, Camilli TC, French AD, Dissanayake SK, Indig FE, Bernier M, *et al*: The orphan tyrosine kinase receptor, ROR2, mediates Wnt5A signaling in metastatic melanoma. Oncogene 29: 34-44, 2010.
41. Henry CE, Llamosas E, Djordjevic A, Hacker NF and Ford CE: Migration and invasion is inhibited by silencing ROR1 and ROR2 in chemoresistant ovarian cancer. Oncogenesis 5: e226, 2016.
42. Lyros O, Nie L, Moore T, Medda R, Otterson M, Behmaram B, Mackinnon A, Gockel I, Shaker R and Rafiee P: Dysregulation of WNT5A/ROR2 signaling characterizes the progression of Barrett's-associated esophageal adenocarcinoma. Mol Cancer Res 14: 647-659, 2016.
43. Morishita A and Masaki T: miRNA in hepatocellular carcinoma. Hepatol Res 45: 128-141, 2015.
44. Xu K, Liang X, Cui D, Wu Y, Shi W and Liu J: miR-1915 inhibits Bcl-2 to modulate multidrug resistance by increasing drug-sensitivity in human colorectal carcinoma cells. Mol Carcinog 52: 70-78, 2013.
45. Nakazawa K, Dashzeveg N and Yoshida K: Tumor suppressor p53 induces miR-1915 processing to inhibit Bcl-2 in the apoptotic response to DNA damage. FEBS J 281: 2937-2944, 2014.
46. Roy S, Yu Y, Padhye SB, Sarkar FH and Majumdar APN: Difluorinated-curcumin (CDF) restores PTEN expression in colon cancer cells by down-regulating miR-21. PLoS One 8: e6854, 2013.