# Mechanisms of metformin's anti-tumor activity against gemcitabine-resistant pancreatic adenocarcinoma

KEIICHI SUZUKI[1], OSAMU TAKEUCHI[2], YUKIO SUZUKI[3] and YUKO KITAGAWA[4]

[1]Department of Surgery; [2]Biomedical Laboratory, Kitasato Institute Hospital, Tokyo 108-8642;
[3]Department of Pharmacy, Research and Education Center for Clinical Pharmacy,
Division of Clinical Medicine, Kitasato University School of Pharmacy, Tokyo 108-8641;
[4]Department of Surgery, Keio University School of Medicine, Tokyo 160-8582, Japan

**Abstract.** Metformin (MET) is the first-line treatment for type 2 diabetes mellitus. Several epidemiological studies have suggested the potential anti-cancer effects of MET, including its activity against pancreatic ductal adenocarcinoma (PDAC). Gemcitabine (GEM) has become the standard chemotherapy for PDAC; however, acquired resistance to GEM is a major challenge. In this study, we evaluated the anti-tumor effects of MET against GEM-resistant PDAC in a mouse xenograft model. GEM-resistant BxG30 PDAC cells were implanted into BALB/c nude mice. The mice were divided into 4 groups (control, GEM, MET, and combined treatment with GEM + MET) and treated with the drugs for 4 weeks. Compared with the control mice, the final tumor volumes were significantly decreased in the mice treated with GEM + MET. Treatment to control volume ratios (T/C%) were calculated as 80.2% (GEM), 54.0% (MET) and 47.2% (GEM + MET). The anti-tumor activity of GEM alone against BxG30 tumor xenografts was limited. MET treatment alone exerted satisfactory anti-tumor effects; however, the optimal T/C% was achieved by treatment with GEM + MET, indicating that this combined treatment regimen potently inhibited the growth of GEM-resistant PDAC. The expression of hypoxia-inducible factor $1\alpha$ (HIF-$1\alpha$) and the phosphorylation of ribosomal protein S6 (S6), an important downstream effector of the mammalian target of rapamycin (mTOR) signaling pathway, were also assessed by western blot analysis. The phosphorylation of S6 was inhibited by incubation with MET, but not with GEM, and the expression of HIF-$1\alpha$ under hypoxic conditions was significantly inhibited by MET treatment, but not by GEM treatment. The production of vascular endothelial growth factor was also suppressed by MET treatment, but not by GEM treatment, as determined by ELISA. Taken together, the data of this study demonstrate that the anti-tumor activity of MET is mediated via the suppression of mTOR-HIF-1 signaling, reflecting a different underlying mechanism of action than that of GEM. These results may prove to be clinically significant and reveal the potential of MET as an effective therapeutic drug for PDAC.

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive tumor with a very poor prognosis (1). The 5-year survival rate of patients with PDAC is approximately 6% with a mean survival of 6 months (2). The curative potential is optimal when surgical resection is involved; however, only 15-20% of patients are eligible for surgery at the time of diagnosis, as the majority of patients with PDAC present with either locally advanced disease (which is rarely resectable) or metastatic disease (3).

Gemcitabine (GEM) is a difluorinated analog of the naturally-occurring nucleoside, deoxycytidine (4). GEM has been widely used in the treatment of diverse carcinomas, including non-small cell lung cancer, bladder cancer and breast cancer, and has become the standard chemotherapy for PDAC. Although recently some modern chemotherapeutics, such as FOLFIRINOX (5) and nab-paclitaxel (PTX) plus GEM (6) have been used as first-line chemotherapy, GEM remains a key drug for use in the treatment of PDAC, particularly for second-line treatment. However, acquired resistance of PDAC to GEM has become a major issue. When resistance to GEM develops, clinicians usually have to switch treatment to a third-line drug, and there are no effective therapies available for patients that fail third-line therapy.

Metformin (MET) is the first-line treatment for type 2 diabetes mellitus and is the most widely prescribed drug worldwide. Recently, several epidemiological studies have indicated the potential anti-tumor effects of metformin against various types of cancer, including PDAC (7-10). Furthermore,

*Correspondence to:* Dr Keiichi Suzuki, Department of Surgery, Kitasato Institute Hospital, 5-9-1 Shirogane, Minato-ku, Tokyo 108-8642, Japan
E-mail: k1suzuki@me.com

basic research has revealed the mechanisms underlying the anticancer effects of MET, which mainly involve the induction of AMP-activated protein kinase (AMPK) (11). Tumor growth inhibition by MET is mediated primarily by the inhibition of mammalian target of rapamycin (mTOR) signaling (12). The mTOR signaling cascade serves as a master regulator of metabolism, cell growth and proliferation. According to a previous study, 15-20% of patients with PDAC have very high levels of active phosphorylated mTOR[S2448], and patients with such tumors have a significantly reduced survival (13). Therefore, targeted anti-mTOR therapies may offer a clinical benefit for patients with PDAC.

A previous study also reported that mTOR-dependent signals stimulated hypoxia-inducible factor 1α (HIF-1α) accumulation and HIF-1-mediated transcription in cells exposed to hypoxic conditions (14). Vascular endothelial growth factor (VEGF) is primarily induced during hypoxia and is regulated by HIF-1 (15). The majority of PDACs are characterized by a hypoxic tumor microenvironment (16). The oxygen pressure in a solid tumor is generally lower than in the surrounding interstitial tissue, and tumors exhibiting extensive hypoxia have been shown to be more aggressive than corresponding tumors that are better oxygenized (17,18). Based on these data, in this study, we evaluated the activities of HIF-1α, and its downstream target, VEGF, following treatment with MET.

Recently, we reported a method for cloning GEM-resistant cell lines and established multiple GEM-resistant clones derived from the human pancreatic cancer cell line, BxPC-3 (19). In this study, we used a moderately GEM-resistant cell line, BxG30. This study was designed to evaluate the anti-tumor effects of MET against GEM-resistant PDAC in a mouse xenograft model. Our results demonstrated that the anti-tumor effect of MET was mediated by the suppression of the function of mTOR. Furthermore, we provide evidence that MET inhibits HIF-1α and VEGF activation in hypoxic cells. These findings are of significant clinical interest and reveal the potential use of MET as an anti-tumor agent in the treatment of PDAC.

## Materials and methods

*Chemotherapeutic drugs*. MET was provided free of charge by Sumitomo Dainippon Pharma Co., Ltd. (Osaka, Japan). GEM was purchased from Eli Lilly Japan (Hyogo, Japan).

*Cells and cell culture*. The human pancreatic cancer cell line, BxPC-3, was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) in a humidified 5% CO2 incubator at 37˚C. BxG30, a moderately GEM-resistant cell line, was established as previously described (18). Briefly, the BxPC-3 cells were allowed to acclimatize by stepwise exposure to increasing concentrations of GEM beginning at 1.5 ng/ml and increasing by 1-10 ng/ml increments at every passage for 6 months. A BxPC-3-derived cell line cultured in the presence of a final concentration of 30 ng/ml GEM was named BxG30.

*Xenograft models*. Animals were maintained according to institutional regulations in facilities approved by the Animal Care Committee of Kitasato University (Tokyo, Japan) and in accordance with Japanese government guidelines for animal experiments. The protocol of the animal experiments was reviewed and approved by The Laboratory Animal care and use Committees of Kitasato University on April 1st, 2013 (approval no. 13023). Animal experiments were performed in accordance with the ethical guidelines of the Kitasato Institute. Isoflurane was used as an inhaled anesthetic. Humanitarian endpoints, such as performing euthanasia treatment at an appropriate time, when unbearable pain was involved, were considered. The Institutional Animal Care and Use Committee Guidebook (20) was referred to as regards the humanitarian endpoints. In this study, no mice had to be euthanized due to poor conditions before the end of the experiment.

During a preliminary toxicity test of MET monotherapy, certain adverse events, such as appetite loss and body weight loss, occurred at doses of 800 mg/kg and increased significantly from the dose of 1,500 mg/kg. Finally, we decided on a 600 mg/kg dose of MET, as this induced a consistent anti-tumor effect without any adverse events. For the purposes of this study, 96 male BALB/c Slc-nu/nu mice (6 weeks old) were obtained from CLEA Japan Inc. (Tokyo, Japan). The body weights of the mice ranged from 15 to 19 g on arrival. The mice were maintained in a specific-pathogen-free condition (temperature, 23±2˚C; humidity, 55±10%) and were provided with autoclaved food and water. After being allowed to acclimatize for 1 week, $2x10^6$ BxPC-3 and BxG30 cells in 0.1 ml of 1% phosphate-buffered saline (PBS) were injected into both the flanks of the mice. The mice were randomly divided into 4 groups as follows: i) The control group [no treatment, final tumor number (n)=10]; ii) the GEM-treated group (80 mg/kg, n=11); iii) the MET-treated group (600 mg/kg, n=11); and iv) the combination treatment group (GEM + MET, n=12). The BxG30 cells were injected into the other mice, and the mice were divided into 4 groups according to the treatments (control group, n=14; GEM group, n=13; MET group, n=10; GEM + MET group, n=14). Treatment was initiated 2 weeks following implantation. MET was diluted in PBS to 600 mg/kg and administered orally every day for 4 weeks. GEM was dissolved in PBS and injected intraperitoneally at a dose of 80 mg/kg every week (on days 15, 22, 29 and 36). Estimated tumor volumes and body weights were measured each week. The estimated tumor volume was calculated using the Battelle Columbus Laboratories Protocol according to the following formula (21): $TV = (AxB^2)/2$, where TV = tumor volume, A = major axis and B = minor axis.

Following sacrifice on day 42, the tumors were dissected and weighed. The estimated tumor volumes of the control group (C) and treatment groups (T) were calculated as described above. The relative tumor volumes (TRW and CRW) were calculated at defined time points by evaluating the ratio of (T) and (C) to the estimated tumor volumes at the beginning of treatment, and the treatment to the control ratio (T/C%) was calculated as TRW divided by CRW. The minimal T/C% over the treatment period was assessed and used to calculate the therapeutic effect. A value of T/C% <50% was required for the treatment to be considered effective.

Although some tumor ulceration was observed, all mice exhibiting ulceration, bleeding or necrosis also exhibited good general conditions without any signs of infection, anemia or abnormal behavior. Thus, no treatment was administered for the ulcerations as it was not deemed necessary.

*Cytotoxicity assays.* The BxG30 cells were seeded at a density of $2.2 \times 10^3$ cells per well in 96-well plates containing culture medium supplemented with 10% FBS. After 24 h, the cultures were washed and incubated in medium alone (control) or medium containing MET or GEM at various concentrations (final concentrations of MET: 0, 1.65, 2.475, 3.3 and 4.125 $\mu$g/ml; final concentrations of GEM: 0, 2, 3, 4, 5 and 10 ng/ml) in quadruplicate. The number of viable cells was counted after 72 h using a Cell Counting Kit-8 (Nacalai Tesque, Inc., Kyoto, Japan) according to the manufacturer's instructions. The assay reagent was a tetrazolium compound (WST-8) that is reduced by live cells to produce a colored formazan product whose absorbance can be measured at 450 nm. The quantity of the formazan product is directly proportional to the number of live cells in the culture. The inhibition index (I.I., %) was calculated using the following formula: I.I. (%) = (b-c)/(b-a) x100, where a = the optical density (OD) of the cells, b = the OD of the cells + WST-8 reagent, and c = the OD of the cells + WST-8 reagent + anti-tumor agent) and $IC_{50}$ values were calculated. A classical isobologram was used to evaluate the synergistic effects of GEM and MET. All experiments were repeated at least 3 times.

*Determination of protein concentrations in culture supernatants.* The BxG30 cells were pre-cultured at a density of $1.5 \times 10^5$ cells per well in RPMI-1640 medium containing 10% FBS for 24 h. After removing the culture medium and washing with PBS, the cells were treated with MET and GEM (final concentrations of MET: 0, 0.825, 1.65 and 3.3 $\mu$g/ml; final concentrations of GEM: 0, 1, 2, 4 and 10 ng/ml). The cells were cultured for 24 h and lysed in lysis buffer [Cell Signaling Technology (CST) Japan, Tokyo, Japan] containing Protease Inhibitor Cocktail Set III (Wako Pure Chemical Industries, Osaka, Japan) and Phosphatase Inhibitor Cocktail Solution I (Wako Pure Chemical Industries). The lysates were centrifuged at 23,000 x g for 20 min at 4°C and the supernatants were collected. The samples were stored at -80°C and used for western blot analysis.

*Western blotting of pS6 and HIF-1α.* For protein extraction, cells were plated into 6-well cell culture plates (Corning, Inc. Corning, NY, USA) at a density of $1.5 \times 10^5$ cells in serum-containing medium and then incubated for 24 h at 37°C. The cells were rinsed twice with PBS and scraped into Cell Lysis Buffer (CST) and Phosphatase Inhibitor Cocktail Solution I (Wako, Osaka, Japan) was added. Following incubation for 20 min on ice, cell lysates were cleared by 20 min of centrifugation at 15,000 x g at 4°C. Protein concentration was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA), and conditioned to 15 $\mu$g per lane. Samples were boiled for 3 min at 100°C and 4X LDS Sample Buffer (Life Technologies Japan Ltd., Tokyo, Japan), and 5% 2-mercaptoethanol (Nacalai Tesque Inc.). Equal amounts of total protein were separated on a 12.5% SDS polyacrylamide gel at a constant current of 20 mA. Separated proteins were transferred to PVDF membranes (Life Technologies Japan Ltd.) at 30V by using iBlot® Gel Transfer Device (Life Technologies Japan Ltd.). The membrane was blocked with 5% dry-milk with 0.1% Tris Buffered Saline-Tween-20 (TBS-T) for 60 min. The membrane was then incubated overnight at 4°C and with primary antibody, and then probed with secondary antibodies for 1 h at room temperature. Chemiluminescence was developed using the ECL Select Western Blotting Detection System and the band intensities were then detected using Image Quant LAS 500 (both from GE Healthcare Japan, Tokyo, Japan) and analyzed using NIH Image J software. The BxG30 cells were treated with several concentrations of MET and GEM (final concentrations of MET: 0, 0.825, 1.65 and 3.3 $\mu$g/ml; final concentrations of GEM: 0, 1, 2, 4 and 10 ng/ml).

The following antibodies were used for western blotting: pS6 rabbit antibody, S6 mouse antibody, HIF-1α rabbit antibody (all from CST; #2317S, #3716S) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit polyclonal IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; #sc-25778) as primary antibodies, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (CST; #7074P2) or HRP-conjugated anti-mouse IgG antibody (GE Healthcare Japan, Tokyo, Japan; NA931-100UL) as secondary antibodies. Each sample was analyzed 3 times independently.

*Exposure to hypoxia.* On the day of the experiment, the culture medium was replaced with fresh RPMI-1640 medium containing 10% FBS. Culture dishes were placed in a modular incubator chamber (Billups-Rothenberg Inc., San Diego, CA, USA), a humidified airtight chamber with air flow valves. To simulate hypoxic conditions, the cells were incubated with 1% $O_2$, 5% $CO_2$ and 94% $N_2$ for 48 to 78 h.

*ELISA for the detection of VEGF.* ELISA was performed to quantify secreted VEGF from BxG30 cells using a Novex ELISA kit (Life Technologies Japan Ltd.) according to the manufacturer's instructions. The BxG30 cells were incubated under hypoxic conditions as described above for 72 h with various concentrations of MET (final concentrations: 0.825, 1.65 and 3.3 $\mu$g/ml) and GEM (final concentrations: 1, 2 and 4 ng/ml). At the end of the ELISA procedure, stop solution was added and the absorbance of each well was measured at 450 nm using a Multiskan FC instrument (Thermo Fisher Scientific). Each sample was analyzed 3 times independently.

*Statistical analysis.* Dunnett's test was used to compare the results from the treatment groups with the control group. One-way factorial ANOVA followed by the Dunnett's test as a post hoc test was used to analyze multiple comparisons between the control group and each of the treatment groups. Differences were considered statistically significant at P<0.05.

## Results

*Anti-tumor activity of MET in the BxPC-3 xenograft model.* The estimated tumor volumes and body weights of the mice were measured each week following implantation of the BxPC-3 xenografts. The final tumor weights were 0.59±0.05 g (mean ± SEM) in the control group (n=10), 0.32±0.07 g in the

GEM-treated mice (n=11), 0.42±0.08 g in the MET-treated mice (n=11) and 0.23±0.06 g in the mice treated with GEM and MET (n=12) (Fig. 1A and B). Both the GEM-treated groups exhibited significantly reduced tumor weights compared with the control group. However, there were no differences in tumor weight between the mice treated with MET alone and the control animals. The minimal T/C% on day 42 was 55.6% in the GEM-treated mice, 88.7% in the MET-treated mice and 48.8% in the mice treated with GEM and MET (Fig. 1C).

*Anti-tumor activity of MET in the BxG30 xenograft model*. The anti-tumor activity of MET against BxG30, a GEM-resistant PDAC cell line, was evaluated using a BxG30 xenograft model. The final tumor weights were 0.26±0.05 g in the control mice (n=14), 0.21±0.05 g in the GEM-treated mice (n=13), 0.15±0.06 g in the MET-treated mice (n=10) and 0.11±0.03 g in the mice treated with GEM and MET (n=14). Compared with the control animals, the final tumor volumes were significantly decreased only in mice treated with both GEM and MET (Fig. 2A and B). The T/C% was 80.2% in the GEM-treated mice, 54.0% in the MET-treated mice and 47.2% in the mice treated with both GEM and MET. The anti-tumor effect of GEM against BxG30 cells was clearly limited. The MET-treated animals exhibited a satisfactory inhibition of tumor growth, although the minimal T/C% was <50% on day 42 only in the group of mice treated with both GEM and MET (Fig. 2C). These data indicated that combination therapy with GEM and MET exerted marked anti-tumor activity against GEM-resistant PDAC.

It was decided that the specimens were inadequate for use in further experiments, as some specimens harvested from both the BxPC-3 and BxG30 xenograft model were necrotic in some places with ulceration of the skin and/or bleeding from tumors.

*Cytotoxicity assay*. We initially evaluated the cytotoxic effects of MET and GEM in BxG30 cells using an *in vitro* WST cytotoxicity assay. Both agents inhibited cell growth in a concentration- and time-dependent manner (data not shown). The $IC_{50}$ values (means ± SEM) of MET and GEM were 3.36±0.13 $\mu$g/ml and 3.75±0.22 ng/ml, respectively. The $IC_{50}$ values of MET and GEM were connected by a dotted line in a classical isobologram to evaluate potential synergistic effects (Fig. 3), but none were observed.

*Western blot analysis of pS6 and HIF-1α*. The phosphorylation of ribosomal protein S6 (pS6), one of the most important downstream effectors of the mTOR signaling pathway, was assessed by western blot analysis. The relative expression of pS6 and total S6 (pS6/S6) was markedly decreased in a dose-dependent manner in the cells treated with MET. S6 phosphorylation was inhibited by incubation with MET, but not with GEM (Fig. 4A and B). The pS6/S6 ratios of each MET concentration were 84.08±29.66% (0.825 $\mu$g/ml), 78.48±6.74% (1.65 $\mu$g/ml) and 42.39±2.74% (3.3 $\mu$g/ml) relative to the control, respectively. Treatment with 3.3 $\mu$g/ml of MET significantly inhibited the activation of S6 compared with the control cells (P<0.05). HIF-1α is a well-known downstream target of mTOR and its expression level was also evaluated by western blot analysis. Hypoxic conditions clearly induced the overexpression
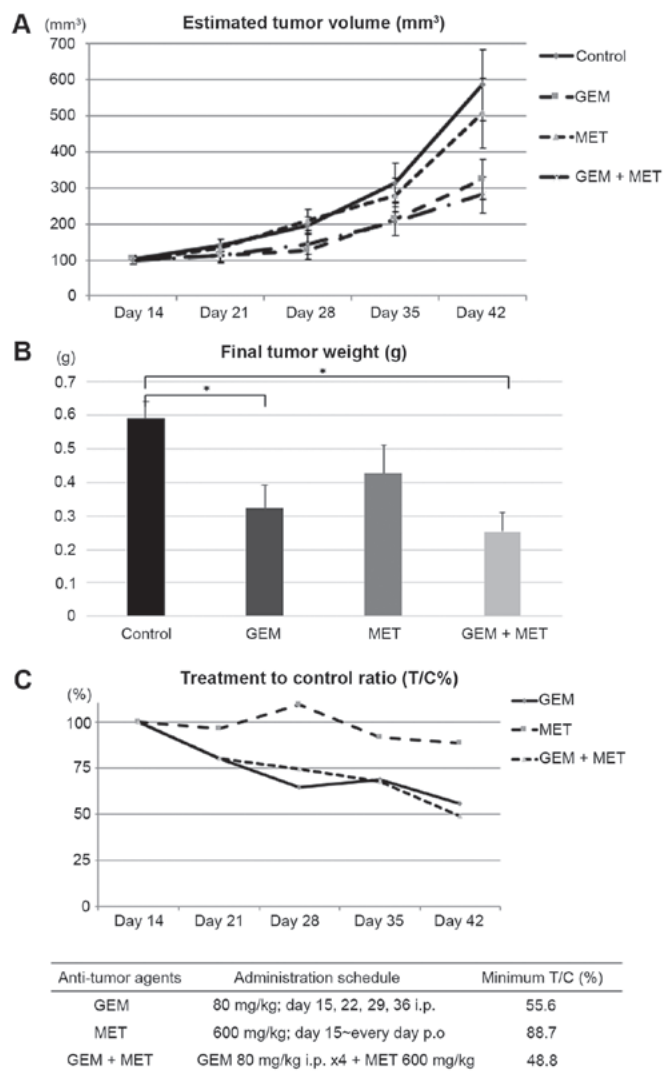


Figure 1. Anti-tumor effects of metformin (MET), gemcitabine (GEM), and a combination of the two in a BxPC-3 pancreatic ductal adenocarcinoma (PDAC) mouse xenograft model. Treatments were initiated 14 days following the implantation of tumor cells. (A) The estimated tumor volumes were calculated each week. (B) Following sacrifice on day 42, the tumors were dissected. One-way factorial ANOVA followed by post-hoc Dunnett's test was conducted to detect the significant differences between control group and each of treatment groups. The final tumor weights were significantly reduced in both GEM-treated groups compared with the control group (*P<0.05). MET treatment did not provide a significant reduction in tumor weight. (C) The treatment-control ratios (T/C%) for the BxPC-3 PDAC mouse xenograft model. Only the combination treatment group (GEM + MET) exhibited a minimal T/C% of <50.

of HIF-1α. When the BxG30 cells were treated with various concentrations of MET, the expression of HIF-1α was suppressed in a dose-dependent manner. Treatment with >1.65 $\mu$g/ml of MET significantly suppressed HIF-1α expression, even under hypoxic conditions (Fig. 5).

*Detection of VEGF production by ELISA*. The production of VEGF by the BxG30 cells was evaluated by ELISA under hypoxic conditions. The results revealed that VEGF production was suppressed by MET treatment in comparison with the untreated cells (P<0.01). However, no significant difference was observed in the secretion of VEGF between the GEM-treated and untreated cells (Fig. 6).
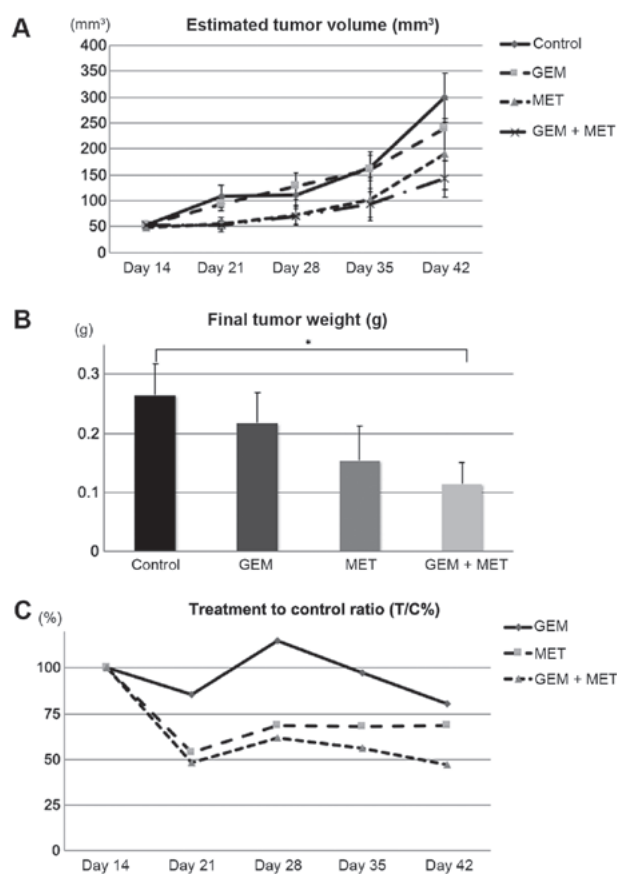
Figure 2. The anti-tumor effects of metformin (MET), gemcitabine (GEM), and a combination of the two in gemcitabine-resistant BxG30 pancreatic ductal adenocarcinoma (PDAC) mouse xenograft model. (A) Estimated tumor volumes were calculated each week. GEM treatment did not show anti-tumor activity against BxG30 cells. (B) The final tumor weights were significantly reduced in mice treated with GEM and MET compared with control mice ($^*P<0.05$). One-way factorial ANOVA followed by post-hoc Dunnett's test was conducted to detect the significant differences between control group and each of treatment groups. (C) The treatment to control ratios (T/C%) for the gemcitabine-resistant BxG30 PDAC mouse xenograft model. The T/C% was <50% only in mice treated with GEM and MET, indicating that combination therapy exhibited satisfactory anti-tumor activity even against GEM-resistant PDAC cells.

## Discussion

The results of this study revealed that MET exerted anti-tumor activity against PDAC. Combination therapy with GEM and MET exerted potent anti-tumor activity not only against wild-type PDAC xenografts, but also against GEM-resistant PDAC xenografts. The data of this study also demonstrated that MET suppressed the expression of HIF-1α and VEGF in tumor cells through the inhibition of the mTOR signaling pathway. These results suggested that the anti-tumor activity of MET was mediated through the inhibition of angiogenesis, which is a very different mechanism of action from that of GEM.

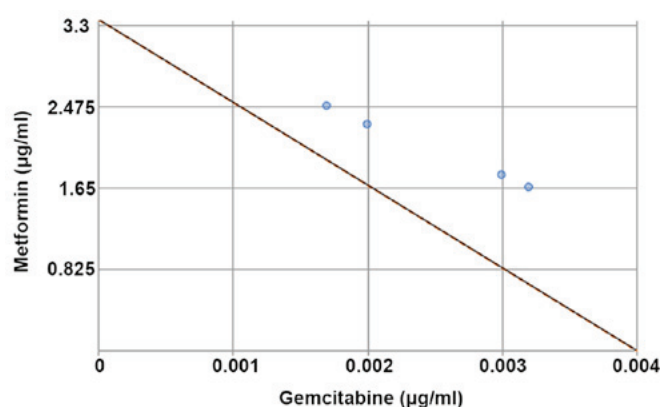Pancreatic cancer is the third-leading cause of cancer-related mortality in the United States, with a 5-year



Figure 3. Evaluation of inhibition of tumor cell proliferation using in vitro WST assay. The $IC_{50}$ values of metformin (MET) and gemcitabine (GEM) were connected by a dotted line in a classical isobologram to evaluate potential synergistic effects, but no synergism was detected.

survival rate of approximately 7-8% (22). Although novel modern chemotherapeutics have been developed, such as FOLFIRINOX and nab-PTX + GEM, the prognosis of patients with PDAC remain dismal. Contemporary drug therapy for PDAC centers on combining multiple cytotoxic agents with overlapping dose-limiting toxicities, and thus there is an urgent need for the development of novel drugs with anti-tumor mechanisms different from those of the cytotoxic drugs currently in use.

Up to 85% of patients with PDAC have diabetes or hyperglycemia, which frequently manifest as early as 2 to 3 years prior to PDAC diagnosis (23). Additionally, pancreatic surgery is becoming more common in the developed world, with an increasing number of patients developing diabetes subsequent to either partial or total pancreatectomy. Secondary diabetes following pancreatectomy has been reported in 5-10% of patients in western countries (24,25). MET, an anti-hyperglycemic drug, is the first-line treatment for type II diabetes and is a widely prescribed anti-diabetic drug; hence, one might expect that MET is used in a large number of patients with PDAC. Numerous epidemiological studies have indicated that the administration of MET in patients with type II diabetes is associated with a reduced cancer incidence and cancer-related mortality (7,8,26). Therefore, MET has been regarded as a potential therapeutic agent, particularly for diabetic patients with PDAC. GEM is still one of the key agents used for the chemotherapy of PDAC; however, acquired resistance to GEM has become a major concern. Thus, in this study, we assessed the anti-tumor effects of MET against PDAC, which we expected should involve a mechanism of action different from that of GEM. This assumption was based on observations from intractable PDAC cases in patients with diabetes and from experiments using a GEM-resistant PDAC cell line.

We previously reported the cloning of a GEM-resistant cell line derived from the wild-type PDAC cell line, BxPC-3 (19). GEM-resistant clones overexpressed ribonucleotide reductase subunit M1 (RRM1), an enzyme involved in metabolism of GEM. When the expression of RRM1 was high, resistance to GEM was high.

In mice implanted with wild-type BxPC-3 xenografts, MET treatment alone exerted limited inhibitory effects on tumor
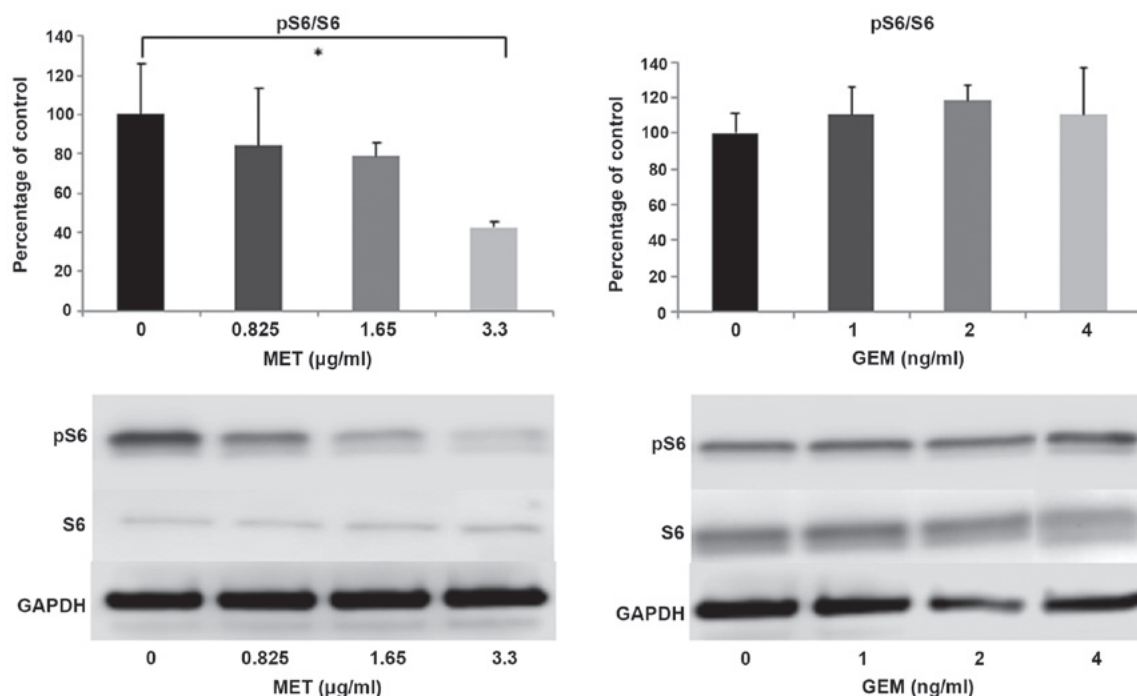
Figure 4. Evaluation of phosphorylation of S6 (pS6) by western blot analysis. One-way factorial ANOVA followed by post-hoc Dunnett's test was conducted to detect the significant differences between control group and each of treatment groups. Relative expression of pS6 to total S6 (pS6/S6) was markedly decreased in a dose-dependent manner in cells treated with metformin (MET). S6 phosphorylation was significantly inhibited by incubation with (A) MET ($^*$P<0.05), but not with (B) gemcitabine (GEM).
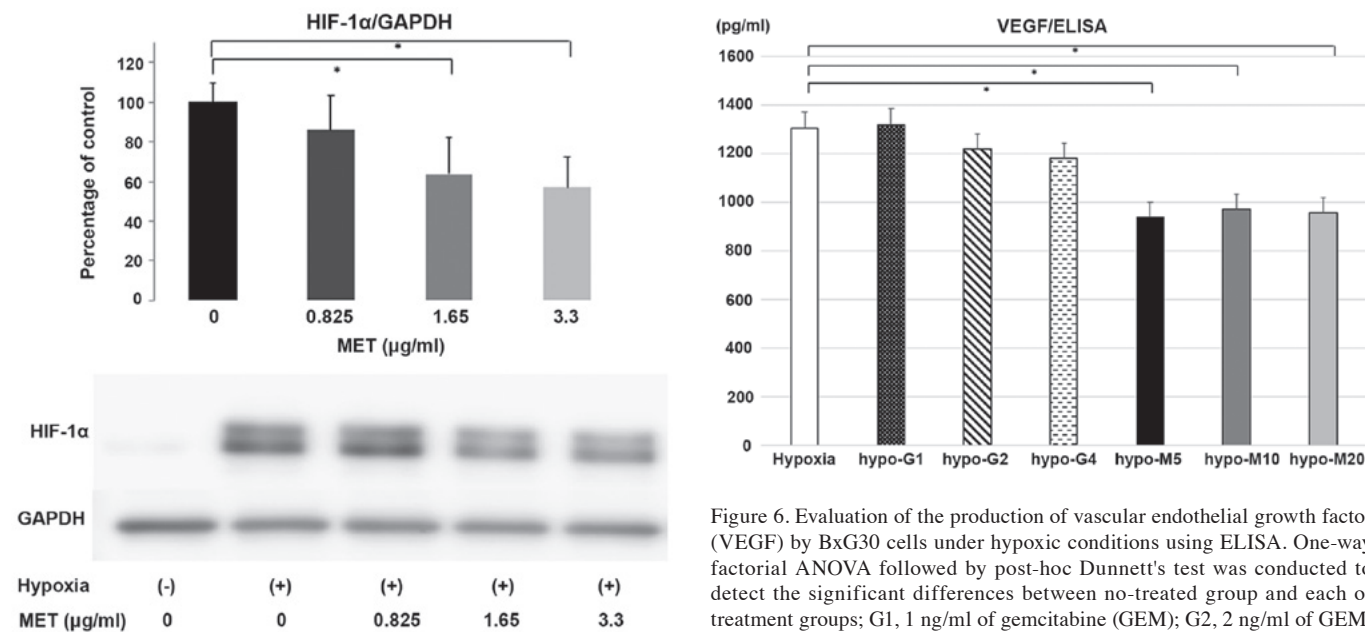


Figure 5. Evaluation of the expression of hypoxia-inducible factor 1$\alpha$ (HIF-1$\alpha$) under hypoxic conditions by western blot analysis. One-way factorial ANOVA followed by post-hoc Dunnett's test was conducted to detect the significant differences between the untreated group and each of the treatment groups. Hypoxic conditions (1% $O_2$, 5% $CO_2$ and 94% $N_2$) induced the overexpression of HIF-1$\alpha$. Metformin (MET) treatment reduced the expression level of HIF-1$\alpha$ in a dose-dependent manner. Incubation with >1.65 $\mu$g/ml of MET significantly suppressed the expression of HIF-1$\alpha$, even under hypoxic conditions ($^*$P<0.05).



Figure 6. Evaluation of the production of vascular endothelial growth factor (VEGF) by BxG30 cells under hypoxic conditions using ELISA. One-way factorial ANOVA followed by post-hoc Dunnett's test was conducted to detect the significant differences between no-treated group and each of treatment groups; G1, 1 ng/ml of gemcitabine (GEM); G2, 2 ng/ml of GEM; G4, 4 ng/ml of GEM; M5, 5 mM of metformin (MET); M10, 10 mM of MET; and M20, 20 nM of MET. Treatment of BxG30 cells with MET significantly inhibited VEGF production compared with control cells ($^*$P<0.01). There were no significant differences in theproduction of VEGF between GEM-treated cells and control cells.

growth compared with GEM treatment alone. GEM treatment exerted more favorable anti-tumor effects on wild-type BxPC-3 xenografts; however, only combination therapy with MET and GEM resulted in effective anti-tumor activity as measured using the T/C%, the minimal treated-control ratio, which revealed that inhibition was <50% on day 42. According to these data, combination therapy with MET and GEM was regarded as the promising treatment.

We also evaluated the anti-tumor effect of MET in mice implanted with GEM-resistant xenografts. The results indicated that the anti-tumor effects of GEM on BxG30 xenografts were limited, while MET treatment markedly inhibited tumor growth. Combination therapy with MET and GEM resulted in <50% of minimal T/C% on day 42, confirming that combination therapy exerted potent anti-tumor effects even against GEM-resistant tumors.

We subsequently evaluated the cytotoxic effects of MET and GEM on BxG30 cells using an *in vitro* WST assay. The results of the classical isobologram revealed that combination treatment with MET and GEM did not exert a synergistic effect against BxG30 cells. This discrepancy between the results of the *in vivo* and *in vitro* experiments suggested that the anti-tumor effect of MET may not result from cytotoxicity, but through indirect effects on the tumor microenvironment *in vivo.*

It is well known that MET activates AMPKα, resulting in the inhibition of the mTOR signaling pathway and its downstream effectors (27-30). Indeed, MET has been shown to inhibit the constitutive and induced activation of mTOR in several pancreatic cancer cell lines (31,32). mTOR is a highly evolutionarily conserved protein kinase that plays a key role in the integration of signals from growth factors, nutrients and the energy status of cells (33). mTOR signaling plays a pivotal role in the proliferation and survival of PDAC cells and is activated in pancreatic cancer tissues (34-36). One of the downstream effectors of mTOR is ribosomal S6 kinase (S6K1), which enhances and phosphorylates pS6, leading to the translation of mRNA (37). A previous study indicated that the S6K1 pathway is the major mTOR-dependent downstream mediator of mTOR-regulated G1-phase progression (38). This pathway also mediates the mTOR-dependent control of cell growth and cell size (39). Consequently, mTOR has emerged as an attractive therapeutic target in PDAC.

In this study, the results of western blot analysis revealed a marked decrease in the pS6/S6 ratio in MET-treated cells, indicating the significant suppression of mTOR activity by MET. By contrast, we observed no suppressive effect of GEM on mTOR activity. This alternative anti-tumor effect of MET, which differs from other cytotoxic anti-cancer drugs, may have resulted in the discrepant anti-tumor effects of combination treatment observed in the *in vivo* and *in vitro* experiments.

Hypoxic conditions have been detected in several human malignancies, including PDAC (17). Tumor hypoxia occurs when the consumption of oxygen exceeds its delivery by the vascular system. In experimental studies, hypoxia predicts aggressive growth and spontaneous metastasis formation in a PDAC xenograft (40). Hypoxic conditions contribute to the induction of HIF-1, a key regulator of the cellular response to oxygen deprivation (41). HIF-1 is a heterodimeric transcription factor containing an inducibly-expressed HIF-1α subunit and a constitutively-expressed HIF-1β subunit. HIF-1α is well known as one of the most important downstream effectors of the mTOR signaling pathway, and plays a major role in tumor progression. Thus, the expression of HIF-1α was evaluated by western blot analysis. The results revealed the significant inhibition of HIF-1α expression by MET, but not by GEM, under hypoxic conditions.

The inhibition of HIF-1α activity leads to the suppression of VEGF expression. In this study, ELISA experiments revealed the suppression of VEGF production by MET treatment compared with the untreated cells. By contrast, GEM treatment did not suppress VEGF production in BxG30 cells. PDAC is often considered as a hypoxic cancer. Hence, the majority of pancreatic cancer cells induce a high expression of HIF-1α via the activation of the mTOR signaling cascade. Additionally, the overexpression of HIF-1α leads to the induction of VEGF expression. The findings of this study suggest that MET may inhibit tumor progression through the suppression of the mTOR/HIF-1α/VEGF signaling cascade; this mechanism is in marked contrast with GEM, which does not achieve anti-tumor activity by suppressing this signaling cascade.

To summarize the above-mentioned results, the findings of this study demonstrated that combination therapy exerted excellent anti-tumor effects even for GEM-resistant PDAC in the animal model. Our findings also demonstrated that MET downregulated mTOR activity, through the evaluation of the phosphorylation of ribosomal protein S6. Furthermore, we demonstrated that HIF-1α expression was inhibited by MET treatment, leading to the suppression of VEGF production under hypoxic conditions. Moreover, the cytotoxic effect of MET was not proven by cytotoxic assay, suggesting that the anti-tumor effects of MET are not produced through cytotoxicity, but rather through the environment surrounding the tumor, particularly the inhibition of angiogenesis via the inhibition of VEGF activity. Similarly, a previous study reported that mTOR may play an important role as an upstream activator of HIF-1 function in cancer cells and may carry out its anti-tumor activity through mTOR/HIF1α/VEGF cascade (40).

In conclusion, the data of the present study demonstrated that MET restricts PDAC tumor growth by suppressing mTOR/HIF-1 signaling, which is a different anti-tumor mechanism than that of GEM. These results are of clinical interest and reveal the potential use of MET in the treatment of PDAC.

## Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

## Authors' contributions

KS, OT, YS and YK participated in the conception and design of the study. KS and OT conducted the experiments. KS and

OT performed data analysis. KS wrote or contributed to the writing of the manuscript. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

The protocol of the animal experiments was reviewed and approved by The Laboratory Animal Care and Use Committees of Kitasato University on April 1st, 2013 (approval no. 13023). Animal experiments were performed in accordance with the ethical guidelines of the Kitasato Institute.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. Yadav D and Lowenfels AB: The epidemiology of pancreatitis and pancreatic cancer. Gastroenterology 144: 1252-1261, 2013.
2. DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, Alteri R, Robbins AS and Jemal A: Cancer treatment and survivorship statistics, 2014. CA Cancer J Clin 64: 252-271, 2014.
3. Heinemann V: Present and future treatment of pancreatic cancer. Semin Oncol 29 (Suppl 9): 23-31, 2002.
4. Hertel LW, Boder GB, Kroin JS, Rinzel SM, Poore GA, Todd GC and Grindey GB: Evaluation of the antitumor activity of gemcitabine (2',2'-difluoro-2'-deoxycytidine). Cancer Res 50: 4417-4422, 1990.
5. Conroy T, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardière C, *et al*; Groupe Tumeurs Digestives of Unicancer; PRODIGE Intergroup: FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 364: 1817-1825, 2011.
6. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, *et al*: Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 369: 1691-1703, 2013.
7. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR and Morris AD: Metformin and reduced risk of cancer in diabetic patients. BMJ 330: 1304-1305, 2005.
8. 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.
9. Decensi A, Puntoni M, Goodwin P, Cazzaniga M, Gennari A, Bonanni B and Gandini S: Metformin and cancer risk in diabetic patients: A systematic review and meta-analysis. Cancer Prev Res (Phila) 3: 1451-1461, 2010.
10. Singh S, Singh PP, Singh AG, Murad MH, McWilliams RR and Chari ST: Anti-diabetic medications and risk of pancreatic cancer in patients with diabetes mellitus: A systematic review and meta-analysis. Am J Gastroenterol 108: 510-519, quiz 520, 2013.
11. Jalving M, Gietema JA, Lefrandt JD, de Jong S, Reyners AK, Gans RO and de Vries EG: Metformin: Taking away the candy for cancer? Eur J Cancer 46: 2369-2380, 2010.
12. 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.
13. Morran DC, Wu J, Jamieson NB, Mrowinska A, Kalna G, Karim SA, Au AY, Scarlett CJ, Chang DK, Pajak MZ, *et al*; Australian Pancreatic Cancer Genome Initiative (APGI): Targeting mTOR dependency in pancreatic cancer. Gut 63: 1481-1489, 2014.
14. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW and Semenza GL: Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: Implications for tumor angiogenesis and therapeutics. Cancer Res 60: 1541-1545, 2000.
15. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD and Semenza GL: Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16: 4604-4613, 1996.
16. Büchler P, Reber HA, Lavey RS, Tomlinson J, Büchler MW, Friess H and Hines OJ: Tumor hypoxia correlates with metastatic tumor growth of pancreatic cancer in an orthotopic murine model. J Surg Res 120: 295-303, 2004.
17. Vaupel P: The role of hypoxia-induced factors in tumor progression. Oncologist 9 (Suppl 5): 10-17, 2004.
18. Semenza GL: Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 29: 625-634, 2010.
19. Yoneyama H, Takizawa-Hashimoto A, Takeuchi O, Watanabe Y, Atsuda K, Asanuma F, Yamada Y and Suzuki Y: Acquired resistance to gemcitabine and cross-resistance in human pancreatic cancer clones. Anticancer Drugs 26: 90-100, 2015.
20. Office of Laboratory Animal Welfare (OLAW): Institutional Animal Care and Use Committee Guidebook. OLAW National Institutes of Health, Bethesda, MD, 2002.
21. Ovejera AA, Houchens DP and Barker AD: Chemotherapy of human tumor xenografts in genetically athymic mice. Ann Clin Lab Sci 8: 50-56, 1978.
22. Siegel RL, Miller KD and Jemal A: Cancer Statistics, 2017. CA Cancer J Clin 67: 7-30, 2017.
23. Sah RP, Nagpal SJ, Mukhopadhyay D and Chari ST: New insights into pancreatic cancer-induced paraneoplastic diabetes. Nat Rev Gastroenterol Hepatol 10: 423-433, 2013.
24. Cui Y and Andersen DK: Pancreatogenic diabetes: Special considerations for management. Pancreatology 11: 279-294, 2011.
25. Ewald N, Kaufmann C, Raspe A, Kloer HU, Bretzel RG and Hardt PD: Prevalence of diabetes mellitus secondary to pancreatic diseases (type 3c). Diabetes Metab Res Rev 28: 338-342, 2012.
26. Zhang ZJ, Zheng ZJ, Kan H, Song Y, Cui W, Zhao G and Kip KE: Reduced risk of colorectal cancer with metformin therapy in patients with type 2 diabetes: A meta-analysis. Diabetes Care 34: 2323-2328, 2011.
27. Cerezo M, Tichet M, Abbe P, Ohanna M, Lehraiki A, Rouaud F, Allegra M, Giacchero D, Bahadoran P, Bertolotto C, *et al*: Metformin blocks melanoma invasion and metastasis development in AMPK/p53-dependent manner. Mol Cancer Ther 12: 1605-1615, 2013.
28. Kim HG, Hien TT, Han EH, Hwang YP, Choi JH, Kang KW, Kwon KI, Kim BH, Kim SK, Song GY, *et al*: Metformin inhibits P-glycoprotein expression via the NF-κB pathway and CRE transcriptional activity through AMPK activation. Br J Pharmacol 162: 1096-1108, 2011.
29. Rozengurt E, Sinnett-Smith J and Kisfalvi K: Crosstalk between insulin/insulin-like growth factor-1 receptors and G protein-coupled receptor signaling systems: A novel target for the antidiabetic drug metformin in pancreatic cancer. Clin Cancer Res 16: 2505-2511, 2010.
30. Sinnett-Smith J, Kisfalvi K, Kui R and Rozengurt E: Metformin inhibition of mTORC1 activation, DNA synthesis and proliferation in pancreatic cancer cells: Dependence on glucose concentration and role of AMPK. Biochem Biophys Res Commun 430: 352-357, 2013.
31. Karnevi E, Said K, Andersson R and Rosendahl AH: Metformin-mediated growth inhibition involves suppression of the IGF-I receptor signalling pathway in human pancreatic cancer cells. BMC Cancer 13: 235, 2013.
32. Soares HP, Ni Y, Kisfalvi K, Sinnett-Smith J and Rozengurt E: Different patterns of Akt and ERK feedback activation in response to rapamycin, active-site mTOR inhibitors and metformin in pancreatic cancer cells. PLoS One 8: e57289, 2013.
33. Laplante M and Sabatini DM: mTOR signaling in growth control and disease. Cell 149: 274-293, 2012.
34. Asano T, Yao Y, Zhu J, Li D, Abbruzzese JL and Reddy SA: The rapamycin analog CCI-779 is a potent inhibitor of pancreatic cancer cell proliferation. Biochem Biophys Res Commun 331: 295-302, 2005.
35. Pham NA, Schwock J, Iakovlev V, Pond G, Hedley DW and Tsao MS: Immunohistochemical analysis of changes in signaling pathway activation downstream of growth factor receptors in pancreatic duct cell carcinogenesis. BMC Cancer 8: 43, 2008.

36. Ito D, Fujimoto K, Mori T, Kami K, Koizumi M, Toyoda E, Kawaguchi Y and Doi R: In vivo antitumor effect of the mTOR inhibitor CCI-779 and gemcitabine in xenograft models of human pancreatic cancer. Int J Cancer 118: 2337-2343, 2006.
37. Gingras AC, Raught B and Sonenberg N: Regulation of translation initiation by FRAP/mTOR. Genes Dev 15: 807-826, 2001.
38. Fingar DC, Richardson CJ, Tee AR, Cheatham L, Tsou C and Blenis J: mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. Mol Cell Biol 24: 200-216, 2004.
39. Fingar DC, Salama S, Tsou C, Harlow E and Blenis J: Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev 16: 1472-1487, 2002.
40. Chang Q, Jurisica I, Do T and Hedley DW: Hypoxia predicts aggressive growth and spontaneous metastasis formation from orthotopically grown primary xenografts of human pancreatic cancer. Cancer Res 71: 3110-3120, 2011.
41. Semenza GL, Agani F, Feldser D, Iyer N, Kotch L, Laughner E and Yu A: Hypoxia, HIF-1, and the pathophysiology of common human diseases. Adv Exp Med Biol 475: 123-130, 2000.