

Metformin inhibits TGF- β 1-induced epithelial-mesenchymal transition and liver metastasis of pancreatic cancer cells

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Abstract. Epithelial-mesenchymal transition (EMT) is considered a crucial event in the development of cancer metastasis. Metformin is a drug used in the treatment of type 2 diabetes. Recently, increasing evidence has indicated that metformin possesses anti-tumor activities. However, the effects of metformin on EMT and metastases in pancreatic cancer remain unknown. Thus, the present study investigated whether metformin inhibits EMT of human pancreatic cancer cell lines. Pancreatic cancer cells were stimulated with transforming growth factor β 1 (TGF- β 1), an activator of EMT signaling, with or without metformin. After 48 h, the levels of epithelial and mesenchymal markers were evaluated by western blot analysis, immunocytochemistry and RT-qPCR. Cancer cell migration was evaluated by an *in vitro* wound healing assay. The cells stimulated with TGF- β 1 acquired an elongated and fusiform morphology, which was inhibited by metformin. The wound healing assay revealed that metformin significantly suppressed the TGF- β 1-stimulated migration of pancreatic cancer cells. Following treatment with metformin, E-cadherin expression (epithelial marker) was upregulated, and the levels of mesenchymal markers were downregulated, which had been increased by TGF- β 1 in these cells. Exposure of the cells to TGF- β 1 activated the Smad2/3 and Akt/mammalian target of rapamycin (mTOR) pathways, and this effect was inhibited by metformin, suggesting that metformin inhibits TGF- β 1-induced-EMT through the down-regulation of the Smad pathway in PANC-1 cells and the downregulation of the Akt/mTOR pathway in

BxPC-3 cells. In an animal model of surgical orthotopic implantation, metformin inhibited liver metastasis without a significant reduction in the size of the primary pancreatic tumor. On the whole, the findings of the present study suggest that metformin inhibits EMT and cancer metastasis through the Smad or Akt/mTOR pathway.

Introduction

Pancreatic cancer is the fourth most common cause of cancer-related mortality in Japan, and patients with pancreatic cancer have a poor prognosis with an overall 5-year survival rate of <5% (1). Factors contributing to the high mortality rates are late diagnosis due to the lack of early symptoms, a high resistance to treatment and a high invasive potential, which render the tumor surgically incurable. Due to resistance to chemotherapy, radiotherapy and immunotherapy, the radical resection of pancreatic cancer is the only available method that has the potential to cure the disease. Therefore, the inhibition of local recurrence and distant metastasis following surgical resection is crucial.

Longstanding type 2 diabetes is a risk factor for the development of pancreatic cancer due to insulin resistance and associated hyperinsulinemia, hyperglycemia and inflammation are related to the development of cancer (2). Metformin is widely used in the treatment of type 2 diabetes mellitus and has been investigated due to its anti-tumor effects (3). In various types of cancer, metformin has been suggested to exhibit anti-tumor activities and to prevent cancer development (4-6). Previous studies have also suggested that there is a positive association between metformin and overall survival in pancreatic cancer treatment (7,8). Wan *et al* reported in a meta-analysis of data from 36,791 patients with pancreatic cancer, that metformin treatment was significantly associated with a favorable overall survival. Subgroup analyses revealed a marked reduction in the mortality risk of patients with stage I-II disease treated with metformin and in patients receiving surgery following treatment with metformin (7). To date, a number of *in vitro* and *in vivo* studies have been performed to investigate the anti-tumor activity of metformin (3); however, there are limited studies available to date describing the effects

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of metformin on epithelial-mesenchymal transition (EMT) and metastasis.

EMT is one of the earliest and most crucial steps in cancer invasion and metastasis (9-12). The EMT phenotype is characterized by the loss of cell-cell adhesion and apical-basolateral polarity, and a phenotypic change in which cells shift from an epithelial morphology to an elongated fibroblast-like morphology with invasive properties. EMT involves the upregulation of mesenchymal markers, such as vimentin and α -smooth muscle actin (α SMA), and the downregulation of epithelial adhesion molecules, such as E-cadherin and cytokeratins. EMT is triggered by the interplay of extracellular signals (such as collagen) and a number of secreted soluble factors, such as transforming growth factor β 1 (TGF- β 1), fibroblast growth factor, epidermal growth factor and hepatocyte growth factor. Among these, TGF- β has been identified as the main factor involved in EMT in the tumor microenvironment. TGF- β 1 has been shown to activate various downstream pathways, including Smads, Akt/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK), thereby inducing EMT (9).

Recent studies have suggested that metformin inhibits EMT in several types of cancer (13-19). However, the effects of metformin on EMT in pancreatic cancer remain unclear. Hence, the objective of the present study was to clarify whether metformin inhibits the EMT and liver metastasis of pancreatic cancer cells.

Materials and methods

Cell lines and culture. The human pancreatic cell lines, PANC-1 and MIAPaCa-2, were purchased from RIKEN Bioresources Center Cell Bank, BxPC-3 cells were from DS Pharma Biochemical Co., and the mouse pancreatic cancer cell line Panc02 was obtained from the National Cancer Institute. PANC-1, BxPC-3 and Panc02 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine and penicillin (100 U/ml)/streptomycin (100 μ g/ml). MIAPaCa-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with low glucose supplemented with 10% fetal bovine serum (FBS), L-glutamine and penicillin (100 U/ml)/streptomycin (100 μ g/ml). All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

TGF- β 1-induced EMT and metformin treatment. After the PANC-1, MIAPaCa-2 and BxPC-3 cells were grown to >80% confluency, they were transferred to a serum-free culture medium with 10 ng/ml TGF- β 1 in a humidified 5% CO₂ atmosphere at 37°C for the induction of EMT. For metformin treatment, the cells were incubated with 10 mM metformin at 37°C for 48 h (Wako Pure Chemical Industries, Ltd.) prior to stimulation with TGF- β 1.

Evaluation of cell viability by WST-8 assay. The cells were seeded into 96-well plates at a density of 5x10³ cells/well. The following day, the cells were treated with or without metformin and incubated at 37°C up to 48 h. Cell viability was examined by the addition of 10 μ l of 5 mg/ml tetrazolium salt solution to the medium of each well and incubated at 37°C for 4 h, and

the absorbance was read at 450 nm with a microplate reader (SpectraMax M2; Molecular Devices, LLC). The absolute values of the absorbance were converted to surviving fraction data and reported as the percentage of living cells relative to the control.

Evaluation of cell migration by wound healing assay. The wound-healing assay was performed as previously described with minor modifications (20,21). Briefly, cells were grown to 100% confluency in P60 culture dishes, and starved in serum-free culture medium for 24 h before scratching. A circle the cell layer of the monolayer approximately 500 μ m in diameter was then made by scrapping with a 10- μ l extra-long micro-pipette tip. The cells were washed twice with PBS and incubated with serum-free culture medium with or without TGF- β 1 and metformin. Microphotographs were acquired with a digital camera at 0, 12 and 24 h after scratching, and the cell-free area was measured using ImageJ software (version 1.51). The migration area was evaluated as the cell-free area at 12 and 24 h, as a percentage of the scratch at 0 h.

Immunocytochemistry. After the PANC-1, MIAPaCa-2, and BxPC-3 cells were grown to >80% confluency in 35-mm μ -dishes (Ibidi), they were washed with PBS and fixed with 4% paraformaldehyde for 20 min at 25°C. The cells were then incubated with mouse anti-human-vimentin (Santa Cruz Biotechnology, Inc.) for 2 h at 25°C followed by 1 h of incubation with anti-rabbit IgG conjugated with Alexa Fluor 594 (Alexa Fluor 594, Life Technologies; Thermo Fisher Scientific, Inc.) at 25°C. The cells were observed using an epi-illumination on a laser scanning confocal microscope (Olympus Corp.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed to detect the mRNA expression levels of E-cadherin, vimentin and α -SMA. Total RNA was extracted from cells using the acid guanidinium phenol chloroform method with Isogen (Nippon Gene Co. Ltd.) from the cultured pancreatic cancer cells (PANC-1, MIAPaCa-2 and BxPC-3). The isolated RNA was stored at -80°C until use in RT-qPCR. Subsequently, complementary DNA (cDNA) was synthesized from reverse-transcribed 1 μ g extracted RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The expression levels of E-cadherin, vimentin, Snail, ZEB-1 and GAPDH genes were analyzed by the 7300 Real-Time PCR system (Applied Biosystems) using the DNA-binding dye SYBR-Green to detect the PCR products. The relative quantification of gene expression with the RT-qPCR data was performed using the standard curve method, with GAPDH as a reference gene. The PCR conditions were as follows: Denaturation at 95°C for 15 sec, primer annealing and elongation at 60°C for 1 min, followed by melting curve analysis, in which the temperature was increased from 60 to 95°C. The sequences of primers used for RT-qPCR were as follows: E-cadherin sense, 5'-GAAGGT GACAGAGCCTCTGGAT-3' and antisense, 5'-CATTCCCGT TGGATGACACA-3'; vimentin sense, 5'-ACACCCTGCAAT CTTTCAGACA-3' and antisense, 5'-GATTCCACTTTGCGT TCAAGGT-3'; α -SMA sense, 5'-GACCGAATGCAGAAG GAGAT-3' and antisense, 5'-CCACCGATCCAGACAGAG

TA-3'; GAPDH sense, 5'-ACCACAGTCCATGCCATCACT-3' and antisense, 5'-CCATCACGCCACAGTTTCC-3'.

Western blot analysis. Cell debris were removed by washing with ice-cold PBS; the cells were lysed in Lysis Buffer (CellLytic M; Sigma-Aldrich; Merck KGaA), scraped and incubated on ice for 15 min. Tissues were frozen using liquid nitrogen, crushed, lysed in lysis buffer, scraped and incubated on ice for 15 min. The supernatants were collected, and total protein was mixed with an SDS sample buffer. The Bradford method was used to measure the protein content; 20 μ g protein were used per lane. The protein lysates were then separated by electrophoresis on 10% SDS-PAGE and transblotted to a polyvinylidene fluoride membrane (Atto Corporation). The membrane was blocked with 10% EzBlock (Atto Corporation) in TBS-T [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween-20 V/V] for 60 min at room temperature, washed with TBS-T 3 times, and incubated overnight at 4°C with goat anti-mouse- β -actin (ab8229, Abcam), mouse anti-human-E-cadherin (mab1838, R&D Systems, Inc.), mouse anti-mouse- α -SMA (a2547, Sigma-Aldrich; Merck KGaA), mouse anti-human-vimentin (sc32322, Santa Cruz Biotechnology, Inc.), mouse anti-human-Smad2/3 (sc8332, Santa Cruz Biotechnology, Inc.), rabbit anti-human-Snail (3879, Cell Signaling Technology, Inc.), rabbit anti-mouse-phospho-Akt (Ser473) (4058, Cell Signaling Technology, Inc.), rabbit anti-mouse-Akt (4691, Cell Signaling Technology, Inc.), rabbit anti-human-phospho-mTOR (2971, Cell Signaling Technology, Inc.), rabbit anti-human-mTOR (Cell Signaling Technology, Inc. 2983) and rabbit anti-human-phospho-Smad2/3 (8828, Cell Signaling Technology, Inc.) antibodies in TBS-T (diluted 1:500-1:1,000). The membrane was then washed with TBS-T 3 times and incubated with the secondary anti-rabbit (616520, GE Healthcare Japan Corporation), anti-goat (628420, GE Healthcare Japan Corporation) and anti-mouse (054220, GE Healthcare Japan Corporation) IgG antibodies in TBS-T (diluted 1:5,000-1:10,000) for 1 h at room temperature. Immuno-reactive proteins were detected using an ECL-kit (ECL plus, GE Healthcare Bio-Sciences K.K.). The blots were analyzed using ImageJ (version 1.51) software.

Animal models. A total of 33 of C57BL/6 immunocompetent female mice (aged 6-8 weeks; body weight, 15-17 g) were provided by Shimizu. Mice were kept at 18-24°C and 40-70% relative humidity, with a 12-h light/dark cycle. They were provided with free access to water and food (CE-2; CLEA Japan). Cultured Panc02 cells were collected and washed twice with PBS. Panc02 cells (3×10^6) were injected subcutaneously into both flanks of 3 mice within 30 min of collection. After 3 weeks, subcutaneous tumors growing to a maximum diameter of approximately 10 mm were collected as tumor fragments for subsequent surgical orthotopic implantation (SOI). SOI of tumor fragments was performed in 30 of the C57/BL6 mice, as previously described (22). For implantation, a small 6-10 mm transverse incision was created on the left flank of the mouse. The tail of the pancreas and spleen was carefully exposed through this incision, and a single tumor fragment (3 mm³), harvested from a subcutaneous tumor grown on a mouse, was sewn to the tail of the pancreas using

7-0 nylon surgical sutures (ELP). The pancreatic tail with the tumor fragment and spleen were stored in the abdomen, and the incision was sutured using 4-0 nylon surgical sutures (ELP). Mice were anesthetized with 5% isoflurane during SOI. A total of 15 mice each were used in the control and metformin groups. Mice were randomly assigned to receive either daily peritoneal injections of metformin (125 mg/kg) or normal saline for 28 days. The mice were monitored daily for any signs of toxicity or abnormalities, including their appetite and behavior. Body weight for each animal was measured once a week. Humane euthanasia was performed when mice reached an experimental endpoint or if their ascites increased and they gained >20% of their body weight, or if they were very weak and lost >20% of their body weight. Following SOI, it was difficult to monitor internal tumor growth exactly until sacrifice. Therefore, body weight was used as a humane endpoint index for internal tumor development. At the time of sacrifice, mice were sacrificed by an intraperitoneal injection of pentobarbital sodium overdose (120 mg/kg). All animal experiments were approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of medicine under Assurance Number M30-618.

Tumor growth and metastatic pattern analysis. The animals were sacrificed 28 days following SOI. Tumor length, width, height and weight were measured and tumor volume was calculated as follows: Tumor volume = (length x width x height)/2. For the evaluation of metastasis, liver and lung surfaces were observed, and were further pathologically sectioned and metastases were observed under a microscope. The paraffin-embedded liver and lung tissues were sectioned at 4- μ m thickness, then hematoxylin and eosin staining was performed. Briefly, the sections were deparaffinized and rehydrated at 37°C with xylene 3 times for 5 min; then with 100% ethanol 2 times for 10 min each; and finally, in a series of 95, 80 and 70% ethanol for 5 min each. The sections were washed with deionized water for 5 min, and stained with hematoxylin for 5 min at room temperature. The sections were then rinsed with deionized water for 20 min and stained with eosin for 5 min at room temperature. Gradient dehydration was performed with 70, 90, 95 and 100% ethanol for 2, 2, 2 and 10 min, respectively, then with xylene 3 times for 5 min each at room temperature. The sections were sealed with neutral gum and placed in a ventilated room at room temperature overnight. Furthermore, western blot analysis, RT-qPCR and immunohistochemistry were performed for EMT-related markers of the primary tumors in the pancreas.

Immunohistochemistry of vimentin in the primary pancreatic tumors. The paraffin-embedded tumor tissues were sectioned at 4- μ m thickness using a microtome cryostat and mounted on MAS-coated slides. Hemo-De was used to clear the sections 3 times for 5 min. The sections were incubated with 100% ethanol for 5 min 3 times, 95% ethanol for 5 min, 90% ethanol for 5 min and 70% ethanol for 5 min to block endogenous peroxidase activity, followed by rinsing with distilled water for 5 min. The slides were submerged in DAKO REAL Target Retrieval solution and then heated in a water bath for 20 min at 95°C for antigen retrieval. After cooling down to room temperature, they were rinsed with distilled

water for 5 min; Dako Cytomation protein block (Dako, Tokyo, Japan) was used for 30 min at room temperature to block nonspecific background. The sections were then incubated overnight at 4°C with specific primary antibody against vimentin (sc32322, Santa Cruz Biotechnology, Inc.) diluted at 1:200 with antibody dilution (Dako; Agilent Technologies, Inc.). After washing the sections 3 times in PBS Tween-20 for 7 min, they were incubated with secondary antibody [Histofine Simple Stain mouse MAX PO (rabbit); Nichirei Biosciences, Inc.] for 30 min at room temperature. After removing unbound antibodies by washing 3 times in PBS for 7 min, diaminobenzidine, a chromogen substrate reagent, was used to visualize the bound antibodies. After counterstaining with Mayer's hematoxylin, the sections were washed for 30 min, dehydrated in graded ethanol (70% ethanol for 5 min, 90% ethanol for 5 min, 95% ethanol for 5 min and 100% ethanol for 5 min 3 times), cleared in Hemo-De (3 times for 5 min each) and cover-slipped.

Statistical analysis. All analyses were performed using the JMP ver13.0 (SAS Institute, Inc.). The differences between 2 groups were analyzed with the unpaired Student's t-test and a Chi-square test. Differences between >2 groups were determined by one-way ANOVA followed by Tukey's multiple comparison test to compare the mean values. All data are presented as the means \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Metformin inhibits morphological changes consistent with EMT in the presence of TGF- β 1. Cell was examined using the WST-8 assay. Cell viability was not significantly altered by treatment with 10 mM metformin compared to that in the untreated group (data not shown). Therefore, the concentration of 10 mM metformin was used for the subsequent experiments. Following the addition of 10 ng/ml TGF- β 1 for 48 h, the PANC-1 and BxPC-3 pancreatic cancer cells acquired an elongated and fusiform morphology, suggesting that 10 ng/ml TGF- β 1 was sufficient to induce EMT. Metformin treatment at 1 h prior to TGF- β 1 exposure inhibited these morphological changes (Fig. 1). The MIAPaCa-2 cell line also acquired an elongated morphology after TGF- β 1 exposure, and metformin treatment suppressed these morphological changes. However, the morphological changes of this cell line were smaller than other cell lines (data not shown).

Metformin suppresses the migratory potential of PANC-1 and MIAPaCa-2 cells. To examine the effect of metformin treatment on the migratory capability of PANC-1 and MIAPaCa-2 cells, wound healing assays were performed; at 24 h after scratching, cell migration into the wound was visualized using a light microscope and images were captured. The PANC-1 cells treated with TGF- β 1 migrated significantly more rapidly than the control cells after 24 h. Metformin reduced wound healing in cells treated with TGF- β 1 during the same period (Fig. 2A). Similar results were observed in the MIAPaCa-2 cells. However, the TGF- β 1-induced migration of MIAPaCa-2 cells was weaker than in that of the PANC-1 cells. As a result, the difference in the migrated area between the

TGF- β 1-treated group and the metformin + TGF- β 1-treated group in the MIAPaCa-2 cells was smaller than that for the PANC-1 cells; thus, the cell images for the MIAPaCa-2 are not shown (Fig. 2B). Wounds could not be made accurately in the BxPC-3 cells due to their strong adhesion to the plate, and therefore the wound healing assay in these cells could not be evaluated.

Effect of metformin on molecular markers of EMT. Western blot analysis and RT-qPCR for E-cadherin were performed to determine the effects of metformin on EMT. The results of western blot analysis revealed no marked changes in the expression of E-cadherin following exposure to TGF- β 1, and an increase in E-cadherin expression was observed following metformin treatment in the BxPC3 cells (Fig. 3A). However, E-cadherin protein expression was not observed even in the absence of TGF- β 1 in either the PANC-1 or MIAPaCa-2 cells (data not shown). RT-qPCR analysis revealed a decrease in the mRNA expression of E-cadherin following TGF- β 1 exposure, which was blocked by metformin treatment in the PANC-1 cells (Fig. 3B). The mRNA expression of E-cadherin was not downregulated by TGF- β 1 exposure in the BxPC3 cells, but was upregulated with a combination of TGF- β 1 and metformin treatment (Fig. 3B).

Subsequently, immunocytochemistry, western blot analysis and RT-qPCR of mesenchymal markers (i.e., vimentin and α -SMA) and the EMT transcription factor, Snail, were performed. Immunofluorescence staining for vimentin revealed a strong expression following exposure to TGF- β 1 for 48 h, and a stronger expression was observed at the tip of spindle-shaped cells, which was reversed by metformin treatment in the PANC-1 cells (Fig. 4A). Vimentin protein expression was observed only in PANC-1 cells using immunocytochemistry. As shown by western blot analysis, the expression of vimentin and Snail was significantly upregulated by TGF- β 1 treatment, which was blocked by metformin treatment in the PANC-1 cells (Fig. 4B). In the BxPC-3 cells, vimentin expression was not observed (data not shown); however, the expression of α -SMA was upregulated by TGF- β 1 treatment, which was blocked by metformin treatment. Furthermore, Snail expression was downregulated by the combination of TGF- β 1 and metformin treatment (Fig. 4B). The mRNA expression of vimentin was significantly upregulated by TGF- β 1 treatment, which was blocked by metformin treatment (Fig. 4C). Exposure to TGF- β 1 slightly increased the expression of α -SMA, although the change was not statistically significant, and this slight increase in expression was blocked by metformin treatment in BxPC-3 cells (Fig. 4C).

Effect of metformin on the Smad and Akt/mTOR pathways. To elucidate the mechanisms through which metformin inhibits TGF- β 1-induced EMT, the levels of phosphorylation of Smad2/3, Akt, and mTOR were examined in the pancreatic cell lines. First, the role of metformin in the regulation of Smad2/3 expression and phosphorylation, which is involved in the downstream signaling pathway of TGF- β 1, was investigated by western blot analysis. In the PANC-1 cells, exposure to TGF- β 1 resulted in the phosphorylation of Smad2/3, which was blocked by metformin treatment (Fig. 5A). However, the exposure of BxPC-3 cells, which have a naturally low expression of Smad2/3, to TGF- β 1 and metformin did not alter the

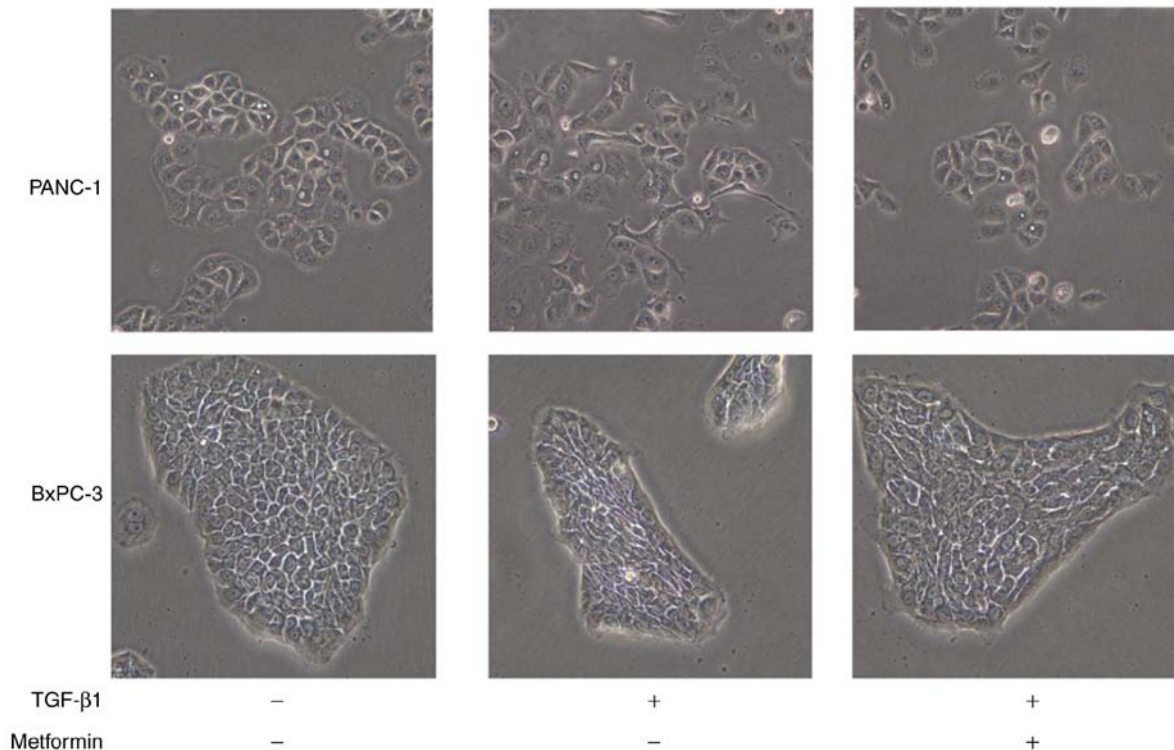


Figure 1. Morphological changes in pancreatic cancer cells. PANC-1 and BxPC-3 cells were incubated with 10 ng/ml TGF- β 1 in a humidified 5% CO₂ atmosphere at 37°C for 48 h. Cells were treated with 10 mM metformin 1 h prior to the exposure of the cancer cells to TGF- β 1. Following treatment with TGF- β 1, the pancreatic cancer cells acquired an elongated and fusiform morphology. Metformin treatment for 1 h prior to TGF- β 1 exposure inhibited the morphological changes.

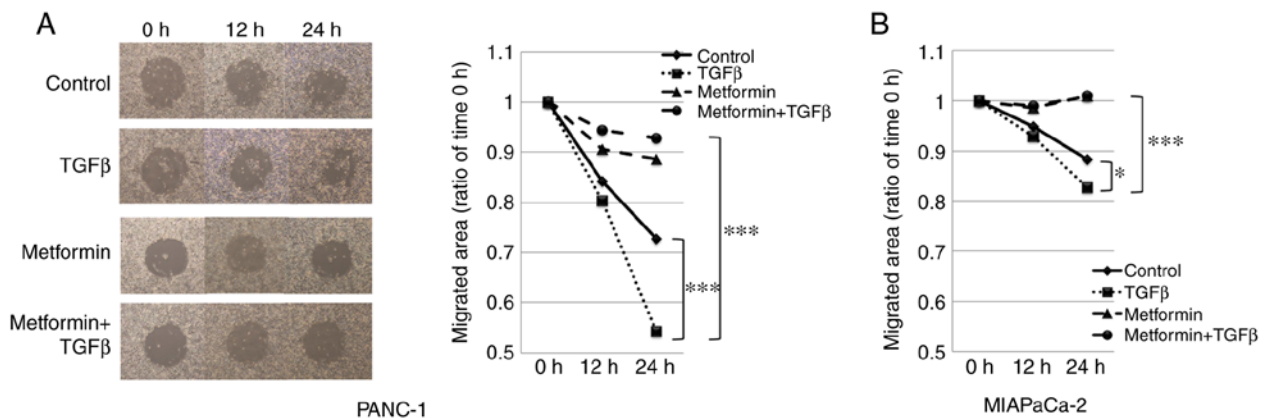


Figure 2. Wound healing assay of pancreatic cancer cells. (A) Significant wound healing was observed after 24 h in cells treated with TGF- β 1 compared with the control cells, and metformin inhibited wound closure in cells treated with TGF- β 1 in PANC-1. (B) Significant wound healing was observed after 24 h in cells treated with TGF- β 1 compared with the control cells, and metformin inhibited wound closure in MIPaCa-2 cells cells treated with TGF- β 1 as well. Cell migration was quantified in 8 experiments, and each line represents the ratio of time 0. *P<0.05, ***P<0.001.

phosphorylation of Smad2/3 (Fig. 5A). Subsequently, other potential pathways of TGF- β 1-induced EMT were investigated. The role of metformin in regulating Akt and mTOR expression and phosphorylation is involved in the downstream signaling pathway of TGF- β 1. The exposure of the PANC-1 cells to TGF- β 1 did not result in the phosphorylation of Akt and mTOR, suggesting that this pathway is not involved in TGF- β 1-induced EMT in these cells. On the other hand, in the BxPC-3 cells, exposure to TGF- β 1 resulted in the phosphorylation of Akt and mTOR. Metformin treatment decreased the expression of Akt. Moreover, in the BxPC cells, metformin

treatment decreased the phosphorylation of Akt primarily due to a reduction in total AKT protein levels. Metformin treatment also inhibited mTOR phosphorylation (Fig. 5B).

Metformin suppresses tumor growth and metastasis in vivo. Pancreatic tumors survived and grew in the pancreatic tails of all sacrificed mice in the present study. A total of 2 mice in the metformin group and 1 mouse in the control group were euthanized due to a >20% weight gain due to ascite retention. A total of 2 mice in the metformin group died within 3 days following SOI, presumably due to suture failure. Consequently, 14 mice in the

Table I. Tumor growth and metastatic pattern in mice administered metformin and control mice.

	Control (n=14)	Metformin (n=11)	P-value
Body weight at SOI (g)	19.9±1.2	20.0±0.9	0.835
Body weight at sacrifice (g)	21.0±1.7	20.9±1.5	0.861
Tumor growth			
yes	14	11	1.000
no	0	0	
Tumor volume (mm ³)	601.4±179.2	492.1±262.3	0.249
Tumor weight (mg)	456.5±182.9	368.1±212.2	0.295
Liver metastasis			
yes	11	4	0.049
no	3	7	
Lung metastasis			
yes	4	1	0.341
no	10	10	

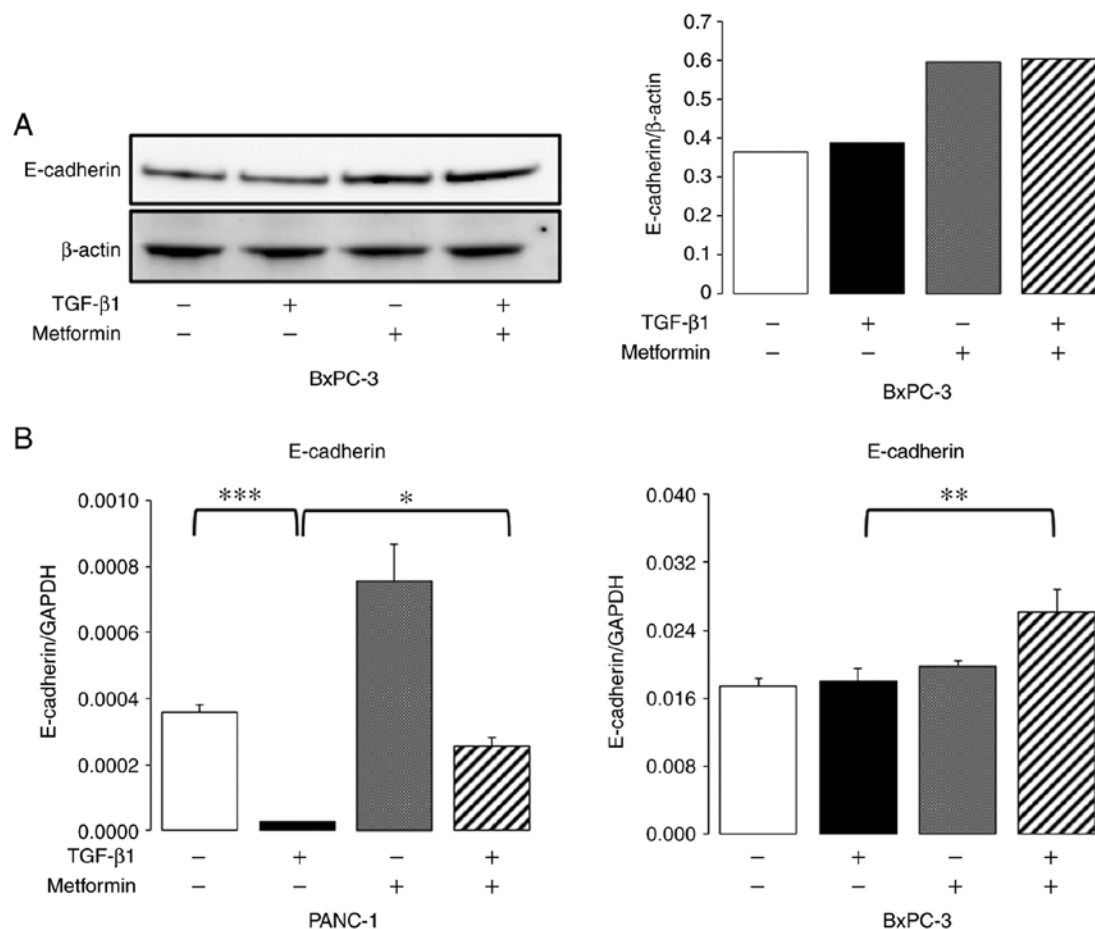


Figure 3. Effect of metformin on epithelial markers. PANC-1 and BxPC-3 cells were incubated with 10 ng/ml TGF-β1 in a humidified 5% CO₂ atmosphere at 37°C for 48 h. Cells were treated with 10 mM metformin 1 h prior to exposure of cancer cells to TGF-β1. (A) The BxPC3 cells were lysed and the lysates were subjected to western blot analysis. The expression of E-cadherin did not change markedly following exposure to TGF-β1, and metformin treatment increased its expression only in BxPC3 cells, as shown by western blot analysis. The graph represents single values as the experiment could not be repeated. (B) E-cadherin mRNA expression in the PANC1 cells and BxPC3 cells. RNA was reverse-transcribed and subjected to RT-qPCR to determine the levels of each mRNA in the PANC1 and BxPC3 cells. Each value represents the means ± SEM of 6 experiments. *P<0.05, **P<0.01, ***P<0.001.

control group and 11 mice in the metformin group were evaluated for metastasis. Primary pancreatic tumors tended to be smaller in

both size and weight in the metformin group than in the control group, but this was not statistically significant (Table I) (Fig. 6A).

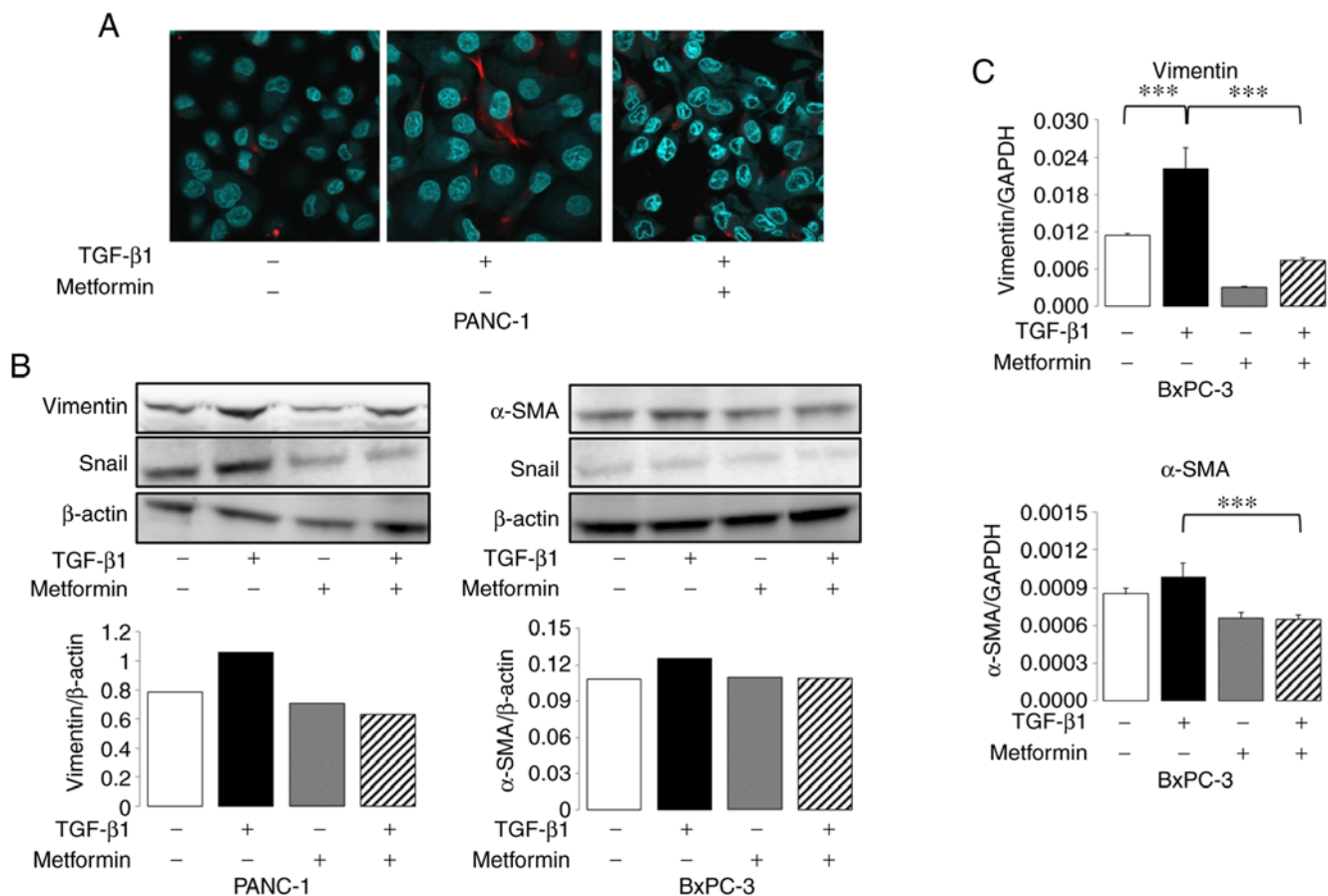


Figure 4. Effect of metformin on mesenchymal markers and transcription factors. PANC-1 and BxPC-3 cells were incubated with 10 ng/ml TGF- β 1 in a humidified 5% CO₂ atmosphere at 37°C for 48 h for 1 h, cells were treated 10 mM metformin 1 h before exposing the cancer cells to TGF- β 1. (A) Immunofluorescence staining for vimentin (red) and nucleus (green). (B) Representative western blots showing EMT transcription factors in PANC-1 and BxPC-3 cells treated with TGF- β 1 with and without metformin treatment. (C) mRNA expression of mesenchymal markers in BxPC-3 cells. Each value represents the mean \pm SEM of 6 experiments. ***P<0.001.

For evaluation of metastasis, we observed liver and lung surfaces, and further sectioned to evaluate the metastasis microscopically (Fig. 6B). Liver metastasis was observed in 15 out of 25 mice. All mice with liver metastases had multiple tumors (range 3-20). In the control group, liver metastasis was observed in 78.6% (11/14) of the mice, whereas in the metformin group, metastasis was observed in only 36.4% (4/11). This suppression of metastasis by metformin treatment was statistically significant (P=0.049, Table I). Lung metastasis was observed in 5 out of 25 mice, and although the frequency of lung metastasis was slightly higher in the control group (28.6% vs. 9.1%), it did not reach statistical significance (Table I). Subsequently, the levels of EMT-related markers in the primary pancreatic tumors in 12 mice were measured. RT-qPCR analysis revealed that E-cadherin expression tended to increase and vimentin expression tended to decrease with metformin treatment (Fig. 6C), although neither reached statistical significance (P=0.07 and P=0.07, respectively). As shown by western blot analysis and immunohistochemical analysis, the expression of vimentin in the metformin group was significantly lower than that in the control group (Fig. 6D and E).

Discussion

EMT is a crucial biological process in cancer invasion and metastasis. However, the details of the mechanisms

of EMT in pancreatic cancer and how this process can be suppressed remain to be explored. The present study focused on whether metformin, widely used in the treatment of type 2 diabetes mellitus, can inhibit the EMT of pancreatic cancer cells. The results indicated that metformin suppressed the TGF- β 1-induced EMT of pancreatic cells and inhibited liver metastasis *in vivo*. Moreover, the results strongly suggested that metformin inhibited EMT by inhibiting the TGF- β signaling pathways (i.e., the Smad2/3 and Akt/mTOR pathways). To the best of our knowledge, this is the first study to demonstrate that metformin inhibits EMT and metastasis formation by suppressing the intracellular TGF- β signaling pathways in pancreatic cancer.

Recently, metformin has attracted attention due to its anti-tumor effects (3). Strong associations between metformin use and the reduced risk of several cancer types, including pancreatic cancer have been found in diabetic patients (4,23). Preclinical studies have suggested that metformin can inhibit the growth of pancreatic cancer both *in vitro* and *in vivo* (24,25). Metformin systemically ameliorates hyperinsulinemia, hyperglycemia and hyperlipidemia, which are involved in both cancer initiation and progression. An anti-tumor effect of metformin is triggered when several signals, such as phosphoinositide 3-kinase (PI3K) and MAPK are suppressed (26-28). Metformin also inhibits the mTOR pathway

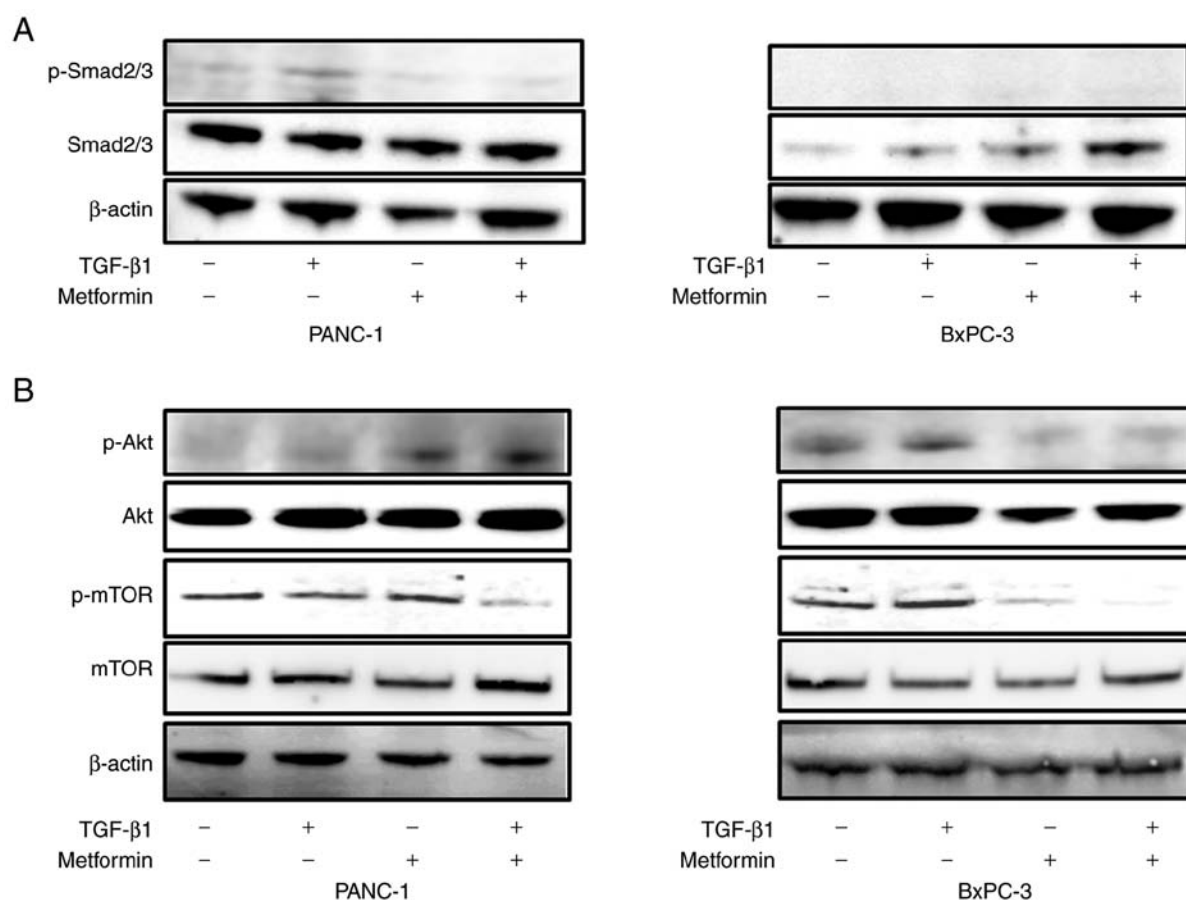


Figure 5. Effect of metformin on Smad and Akt/mTOR pathways of EMT. (A) Cells were incubated with 10 ng/ml TGF- β 1 in a humidified 5% CO₂ atmosphere at 37°C for 1 h. Cells were treated 10 mM metformin 48 h before exposing the cancer cells to TGF- β 1. Cell lysates were extracted and subjected to western blot analysis. (B) Cells were incubated with 10 ng/ml TGF- β 1 in a humidified 5% CO₂ atmosphere at 37°C for 48 h. At 1 h before exposing the cancer cells to TGF- β 1, cells were treated with 10 mM metformin. Cell lysates were extracted and subjected to western blot analysis.

and its downstream substrates (29,30), reduces cyclin D1 expression and cell cycle arrest in a concentration-dependent manner (31), suppresses angiogenesis through the reduction of vascular endothelial growth factor (VEGF) expression (32), and induces apoptosis by p53 activation (3,33).

Recent studies have suggested that metformin inhibits EMT in several types of cancer (13-19). The results of these studies suggest that there are several mechanisms through which metformin inhibits EMT. Zhao *et al* reported that metformin inhibited interleukin (IL)-6-induced EMT in colorectal cancer by blocking signal transducer and activator of transcription 3 (STAT3) phosphorylation (17). Metformin has also been reported to inhibit EMT in prostate cancer through the COX2/PGE2/STAT3 axis (15). TGF- β 1 acts as a potent driver of cancer progression through the induction of EMT in various types of cancer. In TGF- β -induced EMT, both the Smad and non-Smad pathways (i.e., Akt/mTOR and MAP kinase pathways) are activated, thereby increasing EMT-related gene expression (10,34-38). Previous studies have demonstrated that metformin blocks the Smad signaling pathway and inhibits TGF- β -induced EMT (19,39,40). On the other hand, metformin has also been reported to block TGF- β 1-induced EMT through non-Smad pathways, such as the Akt/mTOR/Zeb1 pathway (16). Thus, there are several mechanisms responsible for the inhibition of EMT by metformin, depending on the type of cell used, the EMT inducer, and others.

To the best of our knowledge, until recently, there were no studies available on the effect of metformin on EMT in pancreatic cancer. However, Duan *et al* demonstrated that metformin reduced TGF- β 1 production in pancreatic cells, resulting in the inhibition of autocrine TGF- β 1 signaling, thereby inhibiting EMT (41). However, the effect of metformin on TGF- β 1 signaling pathways was not investigated in this previous study. In pancreatic cancer, there have been no studies published to date on the effect of metformin on the TGF- β -induced Smad and non-Smad signaling pathways involved in EMT, at least to the best of our knowledge. The results of the present study demonstrated that metformin inhibited TGF- β 1-induced-EMT through the downregulation of the Smad pathway in PANC-1 cells and the downregulation of the Akt/mTOR pathway in BxPC-3 cells. These findings suggest that metformin inhibits TGF- β 1-induced-EMT through the downregulation of different pathways, depending on the cell line, and to the best of our knowledge, this is the first report to demonstrate an inhibition of TGF- β 1-induced-EMT by metformin through the downregulation of intracellular TGF- β 1 signaling pathways in pancreatic cancer cells.

Of note, it was found that metformin inhibited EMT *in vitro* and liver metastasis *in vivo* at clinical doses. In the present study, metformin was administered to mice daily by intraperitoneal injection at 125 mg/kg, which is equivalent to the human dose of 600 mg/average size individual of

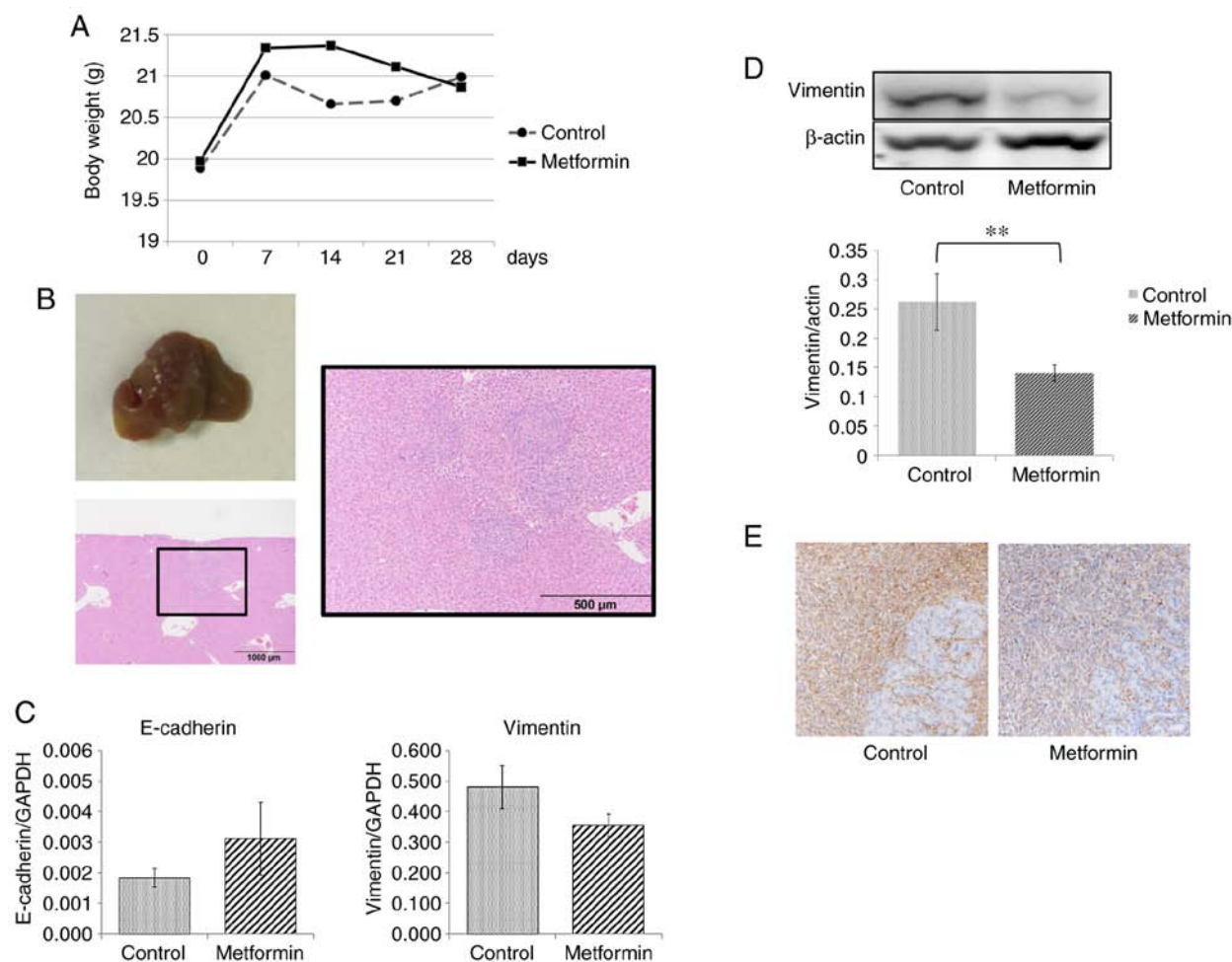


Figure 6. Effect of metformin treatment in animal model. (A) Changes in body weight of the mice are shown. (B) In some mice, multiple liver masses were observed. Metastatic liver tumors could be observed as white nodules from the surface of the liver in some mice. The livers were sectioned as much as possible, and histological analysis was used to evaluate the metastasis. In some mice, metastases were not observed on the liver surface, but were confirmed with thin sections. (C) E-cadherin and vimentin mRNA expression in the primary pancreatic tumor. RNA was reverse-transcribed and subjected to RT-qPCR to determine the levels of each mRNA in the primary pancreatic tumor tissue of 4 control mice and 8 metformin-treated mice. Each value represents the means \pm SEM. (D) The primary pancreatic tumor tissue lysates of 4 control mice and 8 metformin-treated mice were extracted and subjected to western blot analysis. The figure shows western blotting protein expression profiles and statistical analysis using ImageJ (version 1.51). Each value represents the mean \pm SEM. ** $P < 0.01$ (E) Immunohistochemical staining for vimentin in the primary pancreatic tumor tissues with or without metformin. The lower right quarter of the picture shows the normal pancreatic tissue, and the others show tumor tissues.

60 kg (42). Since the dose of metformin for patients with type 2 diabetes mellitus is up to 2,250 mg/individual in Japan (43), it is noteworthy that metformin has the potential to suppress cancer metastasis at relatively low doses used clinically. In the present study, an orthotopic tumor implantation model was used instead of a spontaneous metastasis model. Focusing on the process of metastasis formation, it was considered that this model would be more suitable for the purposes of the present study to assess the effect of metformin, which inhibits cancer cell proliferation, on cancer metastasis. Furthermore, it was confirmed that metformin suppressed liver metastasis without a significant decrease in the primary pancreatic lesion.

There are a few limitations to the present study. Although the assessment of motility is crucial for the assessment of EMT, for the BxPC cells, this assay was not technically feasible due to the potent adhesive force. Alternatively, other assays, such as invasion assays, could overcome this limitation. For proteins with a very low protein content, such as the phosphorylation of Smad, mTOR and Akt, multiple western

blot analyses could not be performed as each assay required too many cells. As a result, statistical analysis could not be performed. In *in vivo* experiments, there were euthanized and dead mice in both groups prior to the analysis for metastasis. Although the present study demonstrated that metformin suppressed liver metastases, whether the long-term use of metformin in tumor-bearing mice improves survival needs to be confirmed in future studies. In addition, in the present study, the number of metastases could not be accurately compared due to the presence of a number of small metastatic foci. The use of Pan02 cells engineered to express luciferase may contribute to the accurate assessment of metastases.

In conclusion, the present study demonstrates that metformin suppresses TGF- β 1-induced EMT in pancreatic cells and inhibits liver metastasis *in vivo*. Moreover, the results demonstrated that the process of inhibiting EMT is mediated through the Smad2/3 or Akt/mTOR pathway. Based on these findings, metformin is expected to be clinically useful for the suppression of cancer metastasis in patients with pancreatic

cancer. To verify the inhibitory effects of metformin on cancer metastasis, prospective clinical studies, such as those in adjuvant settings are necessary.

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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

TI, YN and YI conceived and designed the study and modified the manuscript. JY collected and analyzed the data, designed and performed the experiments, and drafted the manuscript. JY, TI, TeO, NS, KI, KK, KU, and TT performed the cell culture and wound healing assay. JY, TI, YE, SM, TaO, KM and KO performed western blot analysis, RT-qPCR and *in vivo* experiments. JY and YH performed the immunocytochemistry and immunohistochemistry experiments. All authors participated in revising the manuscript, read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of medicine under Assurance Number M30-618.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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