Metformin in combination with 5-fluorouracil suppresses tumor growth by inhibiting the Warburg effect in human oral squamous cell carcinoma

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Abstract. Cancer cells show enhanced glucose consumption and lactate production even in the presence of abundant oxygen, a phenomenon known as the Warburg effect, which is related to tumor proliferation, progression and drug-resistance in cancers. Hypoxia-inducible factor-1 (HIF-1) and several members of Phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway positively contribute to the Warburg effect, whereas AMP activated protein Kinase (AMPK) acts as a negative regulator. Targeting the regulator molecules of Warburg effect might be a useful strategy to effectively kill cancer cells. Metformin was reported to be effective against various cancers as it inhibits cell proliferation by activating AMPK, and inhibiting mTOR and HIF-1α. Several studies suggested the efficacy of metformin with 5-fluorouracil (5-FU) against esophageal and colon cancer. In this study, we evaluated the efficacy of metformin and 5-FU combined therapy against human oral squamous cell carcinoma (OSCC) *in vitro* and *in vivo*. MTT assay and TUNEL assay revealed that metformin (4 mg/ml) and 5-FU (2.5 μg/ml) combination treatment effectively inhibited cell growth and induced apoptosis in OSCC cell lines (HSC2, HSC3 and HSC4) compared to either agent alone. Lactate colorimetric assay detected decreased level of lactate in the supernatants of metformin and 5-FU treated cells compared to cells treated with metformin or 5-FU. Western blot analysis showed marked downregulation of HIF-1α and mTOR expression, and upregulation of AMPKα in cells treated with metformin and 5-FU combination treatment. Combination therapy with metformin (200 mg/kg, i.p.) and 5-FU (10 mg/kg, i.p.) for 4 weeks (5 days/week) effectively reduced HSC2 tumor growth (77.6%) compared to metformin (59.9%) or 5-FU (52%) alone in nude mice. These findings suggest that metformin and 5-FU combined therapy could exert strong antitumor effect against OSCC through the inhibition of Warburg phenomenon in tumor cells.

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Introduction

Oral squamous cell carcinoma (OSCC) is the most common cancer of the oral cavity; it accounts for more than 90% all oral neoplasms (1). Oral cancers account for 2-4% of all cancer cases worldwide and OSCC is the 8th most common cancer in humans (2,3,4). For several decades increasing trends of oral cancer incidences have been observed in either gender in the general population all over the world (5,6). Current approaches of treatment of OSCC include surgery, radiation therapy and/or chemotherapy. However, despite the progress in research and therapy, survival has not improved considerably in recent years (7). If detected during its early stage, the 5-year survival rate of oral cancer is 60-80% (1,8). However, the overall survival rate of OSCC is approximately 50% in the advanced stage of the disease (9,10). Moreover, these therapeutic approaches usually cause adverse effects that reduce quality of life. Therefore, it is necessary to identify novel, effective and less cytotoxic therapeutic agents for OSCC treatment.

Proliferating tumor cells do not exploit the full capacity of oxidative metabolism of glucose to produce ATP, instead it shows enhanced lactate production during glucose metabolism even in the presence of abundant oxygen (11). This phenomenon is known as the Warburg effect or aerobic glycolysis, which is a common metabolic characteristic of cancer cells and is related to tumor proliferation, progression and drug-resistance in cancer (12,13). The Warburg effect has been directly associated with the upregulation of HIF-1α and lactate dehydrogenase (LDH) as well as downregulation of pyruvate dehydrogenase (PDH) (14,15). Several members of PI3K/AKT/mTOR signaling pathway are recognized as the major control points to support the metabolic autonomy of tumor cells and the Warburg effect (14-16). Herein, Akt acts as a key enzyme of the Warburg effect in tumor cells by favoring the glucose-to-lactate metabolic pathway. PI3K and mTOR are located up- and down-stream of Akt respectively, and also act as active players (16). On the contrary, it was reported that, silencing AMPK in tumor cells results in a metabolic shift towards aerobic glycolysis. HIF-1α has a significant role in this

metabolic shift, therefore AMPK acts as a negative regulator of the Warburg effect (17). Targeting the regulator molecules of Warburg effect might be a useful strategy to overcome drug-resistance and effectively kill cancer cells.

Metformin, a low cost antidiabetic drug has been reported to be effective in the treatment of different types of cancers including oral cancer and head and neck cancer, and it is well tolerated by patients (18-27). Metformin exerts inhibitory effects on multiple pathways involved in the initiation of carcinogenesis, as well as proliferation, survival and metastasis of cancer cells (28). It targets cancer stem cells and several regulatory molecules of Warburg effect, and it also induces apoptosis in cancer cells (29). Merformin inhibits cell proliferation by activating AMPK, and inhibiting mTOR and HIF-1α (30). It can also ameliorate the cytotoxicity of some drugs and several studies demonstrated the combined effect of metformin and 5-fluorouracil (5-FU) against esophageal and colon cancer cells (31,32).

In this study, we evaluated the efficacy of combined therapy with metformin and 5-FU against human OSCC cell lines *in vitro* and *in vivo*.

Materials and methods

Cell lines and cell culture. OSCC cell lines (HSC2, HSC3 and HSC4) were obtained from Cell Bank, RIKEN BioResource Center (Ibaraki, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 μg/ml streptomycin, 100 U/ml penicillin (Thermo Fisher Scientific) in a humidified atmosphere containing 5% CO_2.

In vitro cell growth assay. Cells ($5x10^3$ per well) were seeded on 96-well plates (Becton Dickinson Labware, Franklin lakes, NJ, USA) in DMEM supplemented with 10% FBS. Twenty-four hours later, the cells were treated with 5-FU (0.5-10 μg/ml) (Kyowa Hakko Kirin Co., Ltd, Tokyo, Japan), Metformin hydrochloride (metformin; 1-10 mg/ml) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or both for 24, 48 or 72 h. Then, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well (25 μl/well) and incubated for 4 h. The blue dye taken up by cells was dissolved in dimethyl sulfoxide (100 μl/well), and the absorbance was measured with a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) at 490 nm. All assays were run in triplicate.

TUNEL (terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling) assay. To detect apoptotic cells in cell lines and in mouse tumor tissues, TUNEL assay was performed by labeling 3'-OH DNA ends generated by DNA fragmentation. Cells ($5x10^3$ cells per well) were seeded on cover glass (Matsunami Glass Ind. Ltd., Osaka, Japan) in DMEM containing 10% FBS. After incubation for 24 h, cells were treated with 5-FU (2.5 μg/ml) and/or metformin (4 mg/ml) and incubated for 48 h. Then, the cells on the cover glass were washed twice with phosphate buffered saline (PBS), air dried, and fixed in 4% paraformaldehyde at room temperature for 30 min. The TUNEL assay was performed using a DeadEnd™ Colorimetric TUNEL System according to the manufacturer's instructions (Promega Corp., Madison, WI, USA). Briefly, the cells on the cover glass were incubated in 20 μg/ml proteinase K for 15 min. Endogenous peroxidase of cells on the cover glass was blocked by incubating in a 3% hydrogen peroxide solution for 5 min after cells were rinsed in distilled water. After being washed with PBS, the cells were incubated with equilibration buffer (0.05 M phosphate buffer containing 0.145 M sodium chloride, pH 7.4) and then Tdt enzyme in a humidified chamber at 37˚C for 60 min. They were subsequently put into pre-warmed working strength stop wash buffer for 10 min. After being rinsed in PBS, the cells were incubated with anti-digoxigenin-peroxidase conjugate for 30 min. Peroxidase activity in each cell was demonstrated by the application of diaminobenzidine. Hematoxylin was used as a counterstain. At least 1000 cells were counted under a microscope in three random fields of each cover glass. The number of apoptotic cells was calculated by dividing the number of TUNEL positive cells by the total number of counted cells and the result was expressed as a percentage.

In the same manner, TUNEL assay was performed in 4 μm paraffin sections of mouse tumor tissues using a DeadEnd Colorimetric TUNEL System according to the manufacturer's instructions (Promega Corp.).

Lactate colorimetric assay. To detect the lactate production from 5-FU (2.5 μg/ml) and/or metformin (4 mg/ml) treated cells, lactate colorimetric assay was carried out to measure the total lactate content in the cell culture supernatant of the untreated control, 5-FU (2.5 μg/ml) and/or metformin (4 mg/ml) treated cells for 48 h using a Lactate Assay kit according to the manufacturer's instructions (BioVision Inc., Milpitas, CA, USA).

Western blot analysis. After the cells were treated with 5-FU (2.5 μg/ml) and/or metformin (4 mg/ml) for 48 h, they were collected and lysed. Whole cell lysates were subjected to electrophoresis on 10% SDS-polyacrylamide gels, and then transferred to a PVDF membrane. The membranes were incubated with the anti-HIF-1α mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-mTOR rabbit monoclonal antibody (Cell Signaling Technology Inc., Danvers, MA, USA), anti-Akt1 mouse monoclonal antibody (Santa Cruz), and anti-AMPKα rabbit monoclonal antibody (Cell Signaling Technology Inc.) followed by Novex® alkaline-phosphatase conjugated (goat) anti-rabbit or (goat) anti-mouse immunoglobulin G (IgG) secondary antibody (Thermo Fisher Scientific). The antibody was detected using a chromogenic immunodetection system, WesternBreeze (Thermo Fisher Scientific) according to the manufacturer's instructions. Moreover, anti-α-tubulin monoclonal antibody (Santa Cruz Biotechnology, Inc.) was used for normalization of western blot analysis.

Nude mice and tumor inoculations. Female athymic nude mice with CAnN.Cg-Foxnlnu/CrlCrlj genetic background (CLEA Japan, Inc. Tokyo, Japan) were purchased at 4 weeks of age and kept under sterile conditions in a pathogen-free environment. The mice were provided with sterile water and food. In addition, all manipulations were carried out aseptically inside a laminar flow hood. Cells were used as a xenograft model in

the nude mice. Briefly, cells ($1x10^6$) were suspended in 0.1 ml of serum-free medium and injected into the subcutaneous tissue of 5-week-old nude mice (average weight 20.0 g) using a 27-gauge needle. Tumors were allowed to grow for 14 days before treatment. The mice were then divided into 4 groups, 5 mice in each group with similar mean tumor volumes (600-800 mm^3). All *in vivo* experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University.

In vivo treatment protocol. For this experiment, suitable doses for 5-FU and metformin were selected as 10 and 200 mg/kg, respectively, as these doses were reported to be effective in tumor xenograft models of other cancer types (33,34). The treatment protocol of the four experimental groups of mice is shown in Fig. 5A. After tumor formation, these mice were treated with sterile saline (100 μl), 5-FU (10 mg/kg) and/or metformin (200 mg/kg) by intraperitoneal (i.p.) injection for 4 weeks (5 days/week).

The size of the tumors was measured every three days and tumor volumes were calculated as 0.5 x length x $width^2$. At 28 days, mice were sacrificed by an overdose of Somnopentyl (200 mg/kg; Merck & Co., Inc., Whitehouse Station, NJ, USA), and the tumors were dissected, fixed in neutral-buffered formalin and embedded in paraffin for further study.

Immunohistochemistry. The avidin-biotin complex immunohistochemical technique was used to detect Warburg effect related factors (HIF-1-α, mTOR, Akt1 and AMPKα) in mouse tissue specimens, using the EnVision™ kit (Dako, Glostrup, Denmark). Paraffin-embedded 4 μm tissue sections were deparaffinized in xylene and rehydrated through graded alcohols. Endogenous peroxidase was quenched with a 0.3% hydrogen peroxide/methanol mixture for 30 min. Sections were rinsed and pre-incubated with 2% blocking serum for 30 min, followed by incubation with the anti-HIF-1α mouse monoclonal antibody (Santa Cruz Biotechnology, Inc.), anti-mTOR rabbit monoclonal antibody (Cell Signaling Technology Inc.), anti-Akt1 mouse monoclonal antibody (Santa Cruz Biotechnology, Inc.), and anti-AMPKα rabbit monoclonal antibody (Cell Signaling Technology Inc.) for 8 h at 4˚C. After rinsing the tissue sections in phosphate buffered saline (PBS) for 10 min, the antibody was detected using the EnVision kit according to the manufacturer's instructions. Tissues were finally rinsed in PBS for 5 min followed by tap water for 5 min, and then counterstained with hematoxylin for 1 min. The tissue sections were subsequently dehydrated in graded ethanol followed by xylene and mounted with glass coverslips using DPX.

Statistical analysis. All statistical significance was set at P<0.05. Statistical analyses were performed using the StatView software (version 5.0J, SAS Institute Inc., Cary, NC, USA).

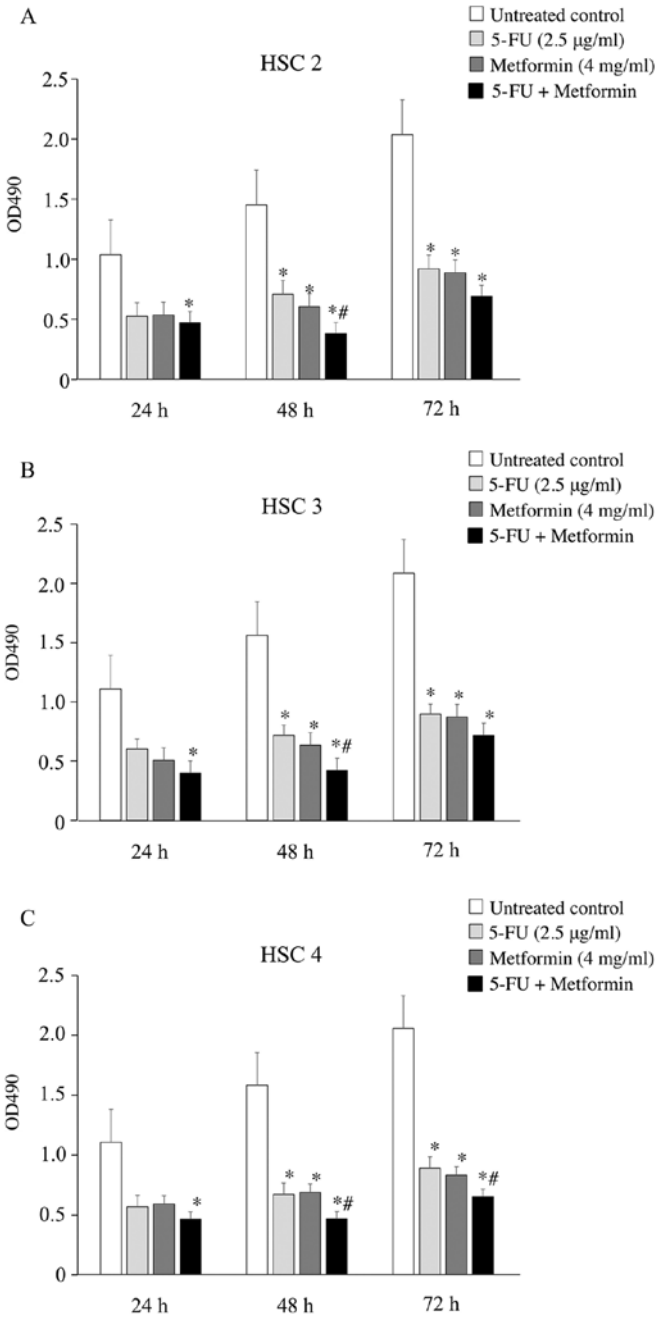

Figure 1. Effect of 5-FU and/or metformin treatment on OSCC cell proliferation *in vitro*. Inhibition of cell growth was evaluated by MTT assay. (A) 5-FU and metformin combined treatment significantly inhibited the growth of HSC2 cells compared to 5-FU or metformin alone, or the untreated control after 48 h of treatment. (B) In case of HSC3 cells, similar result was observed as HSC2. (C) 5-FU and metformin combined treatment significantly inhibited the growth of HSC4 cells compared to either agent alone, or the untreated control after 48 and 72 h of treatment. Error bars represent the standard deviation of the mean of six independent experiments. *P<0.01 when compared to that of control (Mann-Whitney U test). #P<0.01 when compared to that of each agent alone (Mann-Whitney U test).

Results

Effects of 5-FU and/or metformin on the growth of OSCC cells in vitro. The growth inhibitory effect of 5-FU and metformin on HSC2, HSC3 and HSC4 cells was analyzed by the MTT assay. Cells were treated with 5-FU (0.5-10 μg/ml) and/or metformin (1-10 mg/ml) for 24, 48 and 72 h. 5-FU or metformin inhibited cell growth in a dose-dependent manner, and single treatment with 2.5 μg/ml 5-FU or 4 mg/ml metformin inhibited ≥50% cell growth in all three cell lines (data not shown). Therefore, these concentrations of 5-FU and metformin were chosen for all *in vitro* experiments as single or combination treatments. As shown in Fig. 1, 5-FU and metformin combination significantly inhibited the growth of HSC2, HSC3 and HSC4 cells compared

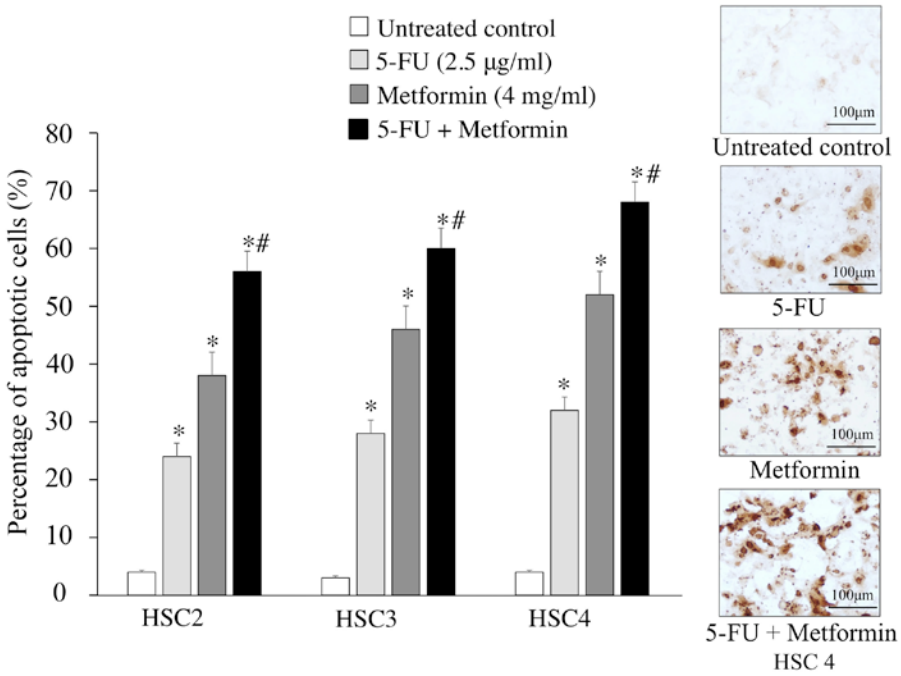

Figure 2. Effect of 5-FU and/or metformin treatment on OSCC cell apoptosis *in vitro*. TUNEL assay was performed to confirm the apoptotic inducing activity of 5-FU and/or metformin treatment. The numbers of apoptotic cells were significantly increased after 5-FU and metformin combined treatment than treatment with either agent alone. Error bars represent the standard deviation of the mean of TUNEL positive cells in three random fields of each cover glass. TUNEL positive cells (brown color) in HSC4 cells are shown on the right. *P<0.01 when compared to that of control (Mann-Whitney U test). #P<0.01 when compared to that of each agent alone (Mann-Whitney U test).

to 5-FU or metformin alone, or the untreated control. In addition, 48 h treatment was the most effective (growth inhibition ratio: 70-73%) for all three cell lines.

Effects of 5-FU and/or metformin on induction of apoptosis in vitro. To understand whether the enhanced cell growth inhibitory effect of 5-FU and metformin combined treatment was due to apoptosis, we performed TUNEL assay to detect DNA fragmentation and chromatin condensation in treated cells. TUNEL assay showed that 5-FU (2.5 μg/ml) and metformin (4 mg/ml) combination treatment for 48 h induced apoptosis (56-68%) more strongly in cells compared to single agent chemotherapy. Briefly, the numbers of apoptotic cells were significantly increased after 5-FU and metformin combined treatment than treatment with either agent alone (Fig. 2).

Effects of 5-FU and/or metformin on the production of lactate in vitro. To clarify the mechanisms of the antitumor activity of 5-FU and metformin combined treatment, we examined the production of lactate in cells that was relevant to the Warburg effect. Lactate colorimetric assay detected a decreased level (≤1.7 ng/mol) of lactate in the supernatants of 5-FU (2.5 μg/ml) and metformin (4 mg/ml) treated cells compared to untreated control cells (≤7 ng/mol), or cells treated with 5-FU (≤5.7 ng/mol) or metformin (≤3.9 ng/mol) alone. Briefly, metformin reduced the production of lactate in the three cell groups compared to 5-FU, whereas 5-FU and metformin combined treatment markedly reduced the production of lactate in the three cell groups compared to either agent alone (Fig. 3).

Effects of 5-FU and/or metformin on the expression levels of Warburg effect related factors in vitro. In order to clarify

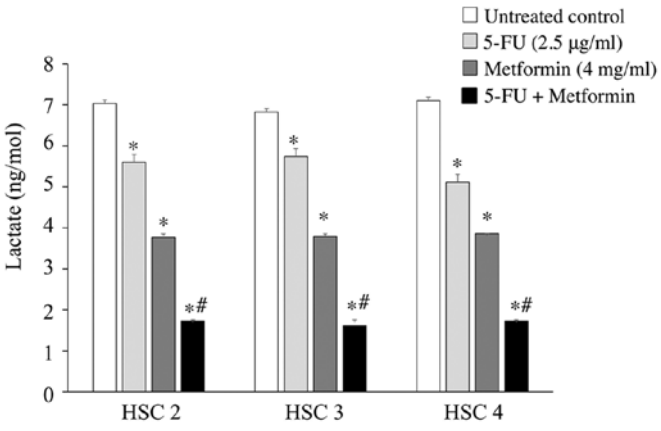

Figure 3. Effect of 5-FU and/or metformin treatment on lactate production *in vitro*. Lactate colorimetric assay detected a decreased level of lactate in the supernatants of 5-FU and metformin treated cells compared to untreated control cells, or cells treated with 5-FU or metformin alone. *P<0.01 when compared to that of control (Mann-Whitney U test). #P<0.01 when compared to that of each agent alone (Mann-Whitney U test).

whether or not 5-FU and/or metformin treatment modulates the Warburg effect related factors in tumor cells, we examined the expression levels of HIF-1-α, mTOR, Akt1 and AMPKα in 5-FU and/or metformin treated (48 h) cells by western blotting. Metformin markedly reduced the expression of HIF-1-α and mTOR, and slightly reduced the expression of Akt1 in all three cell lines and induced the expression of AMPKα in HSC3 and HSC4. In addition, 5-FU and metformin combined treatment reduced the expression of HIF-1-α, mTOR and Akt1, and induced the expression of AMPKα markedly in all three cell lines (Fig. 4).

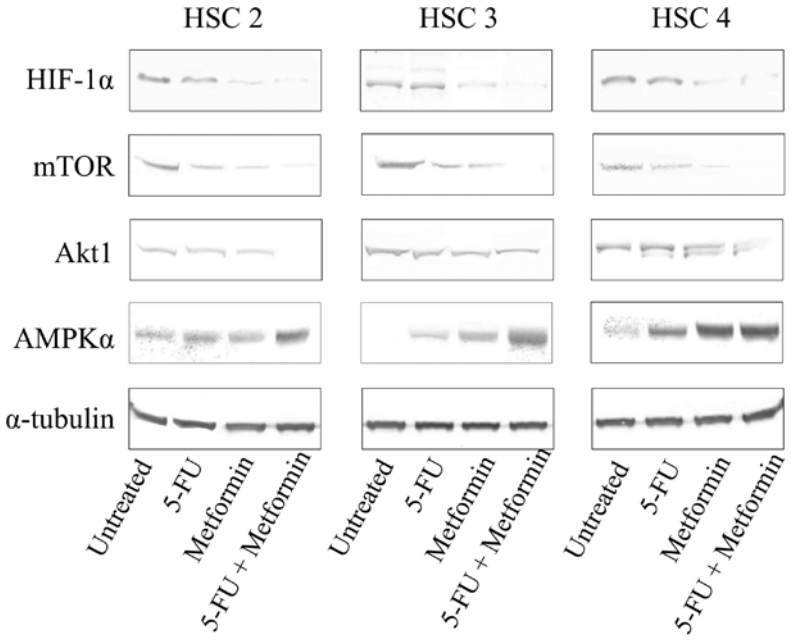

Figure 4. Expression of HIF-1α, mTOR, Akt1 and AMPKα protein in 5-FU and/or metformin treated OSCC cells. Western blot analysis demonstrated marked downregulation of HIF-1α, mTOR and Akt1 expression, and upregulation of AMPKα in cells treated with 5-FU and metformin combination treatment.

Effects of 5-FU and/or metformin on tumor growth inhibition in vivo. Nude mice with HSC2 tumor xenografts were used to examine the antitumor activity of 5-FU and metformin single/combination treatment. Control group received saline 200 μl only, while treatment groups were treated with either 5-FU (10 mg/kg/day, 5 times/week) or metformin (200 mg/kg/day, 5 times/week) alone, or in combination for 4 weeks (Fig. 5A). Fig. 5B shows the result of the *in vivo* experiment. All the treatment groups significantly inhibited tumor growth compared to the untreated control. Antitumor effect of 5-FU alone (52%) was in the same range to metformin alone (59.9%). However, the maximum reduction (77.6%) of tumor growth was observed with 5-FU and metformin combination therapy, which is significantly different than treatment with either agent alone. Compared to the control, mice in all treatment groups showed no toxicity or significant weight loss during the treatment (Fig. 5C).

Effects of 5-FU and/or metformin on the expression of Warburg effect related factors in vivo. We examined the expression levels of Warburg effect related factors (HIF-1-α, mTOR, Akt1 and AMPKα) in mouse tumors by immunohistochemistry. The expression of HIF-1-α was detected in the nucleus of untreated HSC2 tumor cells and 5-FU treated HSC2 tumor cells. However, the expression of HIF-1-α was not detected in the nucleus of metformin treated tumor cells and 5-FU plus metformin treated tumor cells. Similar result was observed in case of mTOR, except mTOR expression was detected in the cytoplasm of tumor cells. The expression of Akt1 was detected strongly in both the nucleus and the cytoplasm of untreated HSC2 tumor cells. However, the expression of Akt1 was detected weakly in 5-FU treated tumor cells, but it was not detected in metformin treated tumor cells or 5-FU plus metformin treated tumor cells. The expression of AMPKα was not detected in cytoplasm of untreated HSC2 tumor cells, but it was detected weakly in 5-FU treated tumor cells, moderately in metformin treated tumor cells, and strongly in 5-FU plus metformin treated tumor cells (Fig. 6).

Effects of 5-FU and/or metformin on induction of apoptosis in vivo. To detect the degree of apoptosis induced by 5-FU and/or metformin *in vivo*, the number of apoptotic cells in mouse tumor tissue sections was quantified by the TUNEL assay. Although treatment with 5-FU or metformin alone moderately induced apoptosis in mouse tumors compared to the untreated control, 5-FU and metformin combined treatment significantly upregulated the expression levels of TUNEL-positive cells in mouse tumors than all other treatment groups or control (Fig. 7).

Discussion

The efficacy of 5-FU in combination with metformin on OSCC both *in vitro* and *in vivo* was shown. In addition, this study suggests that metformin may enhance the effect of 5-FU on OSCC through the inhibition of the Warburg effect.

Metformin is an oral hypoglycemic drug that has been used to treat type 2 diabetes mellitus, and belongs to the biguanide class. Because of its low cost compared to insulin, metformin is used worldwide. Epidemiologic studies and meta-analyses have suggested that type 2 diabetes mellitus patients have a higher incidence of malignancies in recent years (35-37). However, a Taiwanese study demonstrated that the increasing trends of oral cancer may not be ascribed to the increasing incidence of diabetes over the same period (5). The type 2 diabetes mellitus patients who received metformin have not only showed lowered cancer-associated mortality but also demonstrated decreased tumor incidence (38-41). Several studies indicated the preventive effects of metformin against thyroid, bladder, colon, prostate, breast, endometrial,

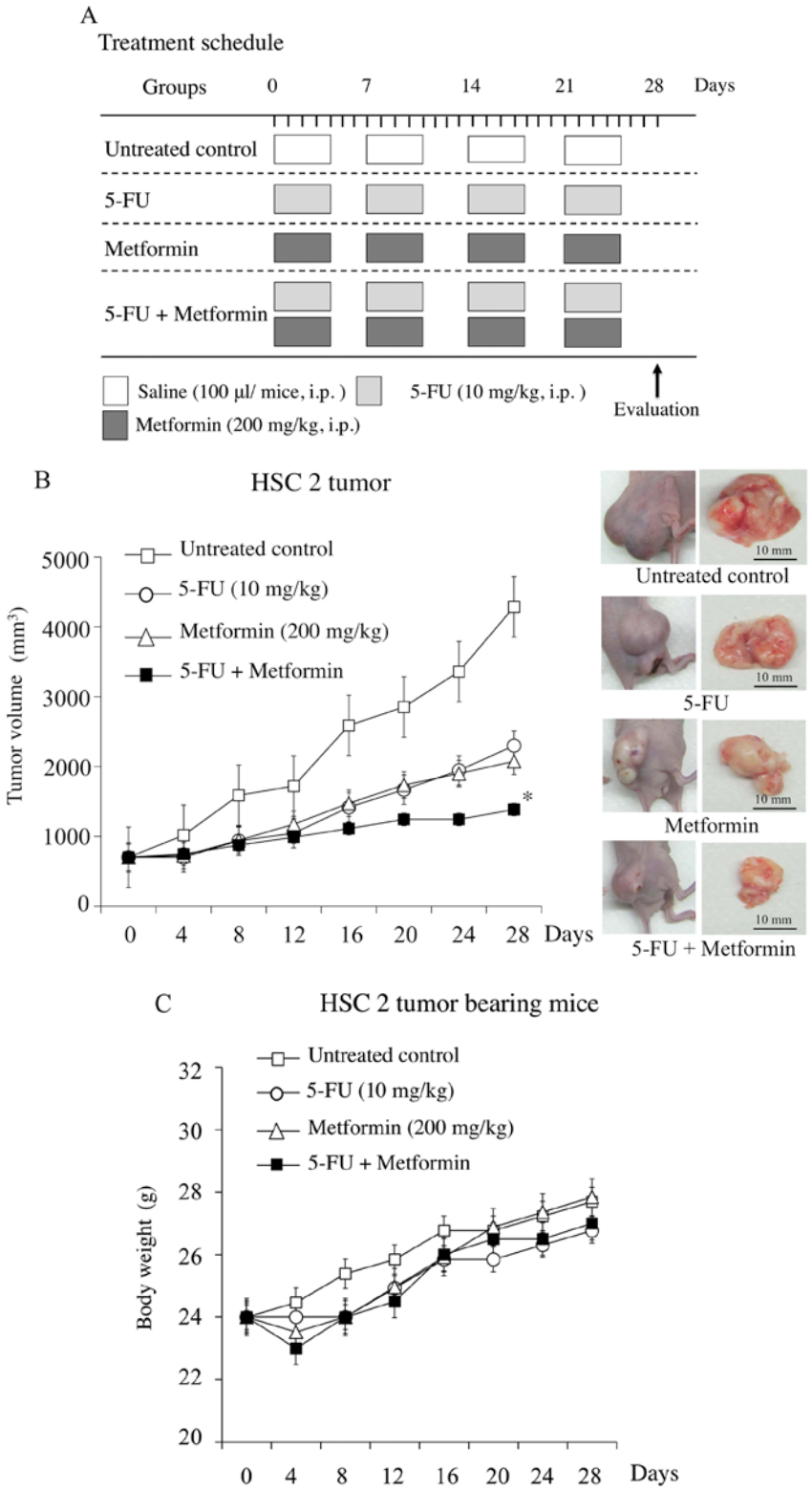

Figure 5. Effect of 5-FU and/or metformin treatment on HSC2 tumor growth and mouse body weight. (A) Treatment schedule. (B) Change of HSC2 tumor volume. (C) Change of mouse body weight. Cells ($1x10^6$) were inoculated into the backs of nude mice. When the tumors reached 600-800 mm^3 in volume, they were treated with either 5-FU (10 mg/kg, 5 times/week) or metformin (200 mg/kg, 5 times/week) alone, or in combination for 4 weeks. Tumor volume and body weight was measured every three days. 5-FU and metformin combination treatment most effectively reduced tumor growth compared to 5-FU or metformin single treatment, or untreated control (saline alone). Mice in all treatment groups showed no significant weight loss during the treatment when compared to control mice. Error bars represent the standard error of the mean results from five mice (n=5). *P<0.01 when compared to that of each agent alone or control (one-way ANOVA).

ovarian and oral cancer in patients with type 2 diabetes mellitus (20-27). Therefore, effectiveness of metformin against oral cancer should be evaluated. Available *in vitro* and *in vivo* studies demonstrated that, metformin could exert

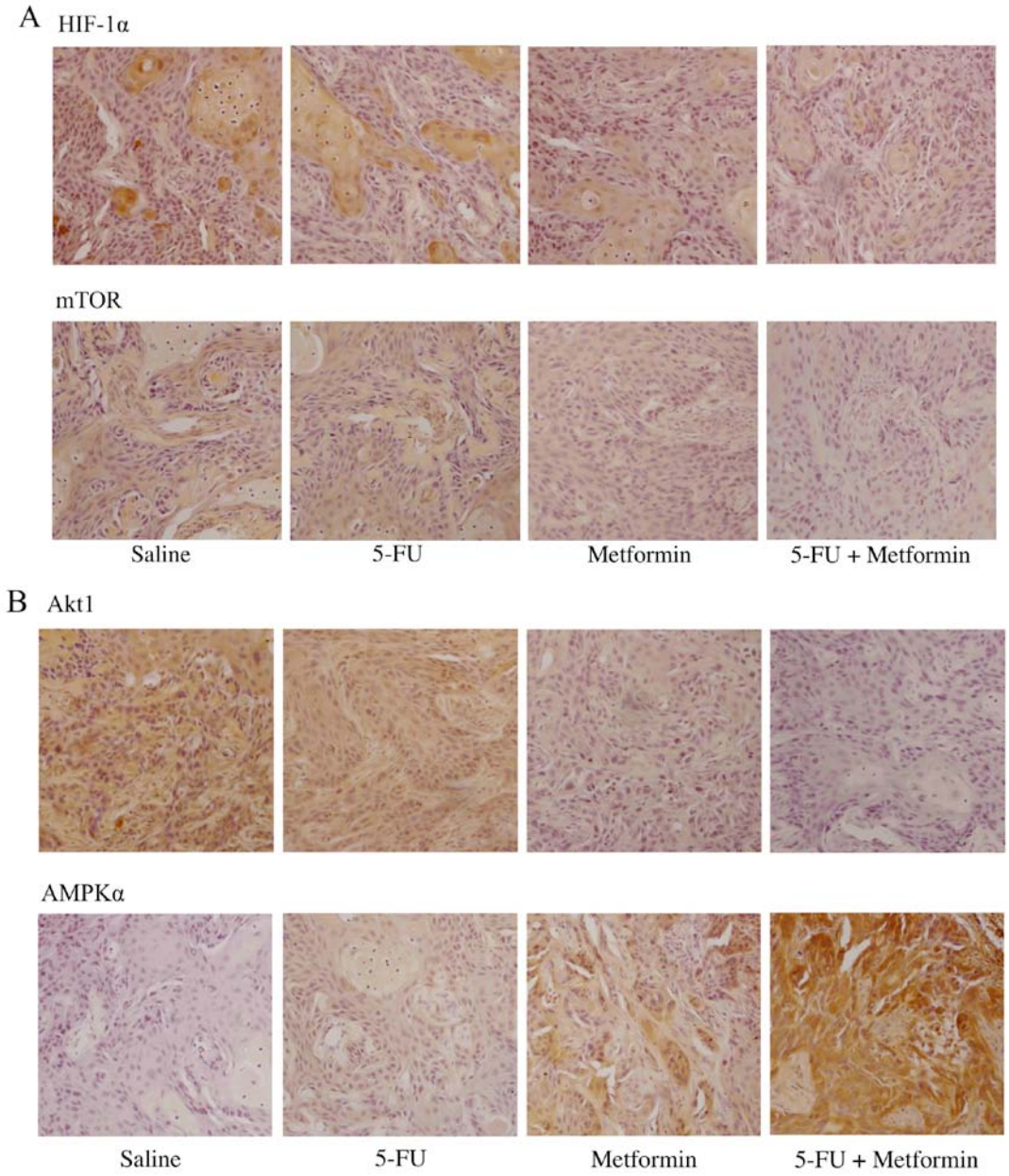

Figure 6. Effects of 5-FU and/or metformin on the expression of Warburg effect related factors *in vivo*. Immunohistchemical staining was performed to investigate the expression of Warburg effect related factors (HIF-1-α, mTOR, Akt1 and AMPKα) in mouse tumors by immunohistochemistry. (A) HIF-1-α and mTOR. (B) Akt1 and AMPKα. Downregulation of HIF-1α, mTOR and Akt1 expression, and upregulation of AMPKα was seen in HSC2 tumor treated with 5-FU and metformin combination.

growth inhibitory effects on various human cancer cell types, such as head and neck, pancreas, prostate, breast, stomach and liver (12,42-46). It was reported that carcinogenesis can be directly promoted by insulin resistance and resultant hyperinsulinemia in diabetic patients and metformin could reduce risk of cancer by maintaining insulin resistance, blood glucose and insulin levels (47,48). However, other antidiabetic drug, e.g. pioglitazone improves insulin resistance in diabetic patients but has a neutral effect against oral cancer. Therefore, metformin might have other additional mechanisms of actions against cancer and unlike other antidiabetic drug it might be useful in the treatment of cancer (49).

The anticancer effects of metformin are its direct pleiotropic inhibitory effects on several pathways involved in survival and metastasis of cancer cells (48). At the cellular level, main mechanisms of metformin against cancer cells are activation of AMPK, mediating the PI3K/Akt signaling pathway, inducing G1-phase arrest with induction of cyclin-dependent kinase inhibitor 1B (p27) and inhibition of mTOR and HIF-1α (19,50,51). Metformin can reduce the anti-senescence effects of EMT program in cancer cells and can inhibit proliferation of CSCs (28). Furthermore, metformin can potentiate the effect of chemotherapeutic agents or reverse drug resistance in cancer cells (13,38,52). Therefore, we examined the possibility of metformin combination therapy with chemotherapeutic agents available for OSCC in Japan pre-experimentally. In the preliminary experiment, we observed that the growth inhibitory effect of 5-FU in combination with metformin against OSCC cells was significantly higher when compared to cisplatin, docetaxel or paclitaxel. However, the

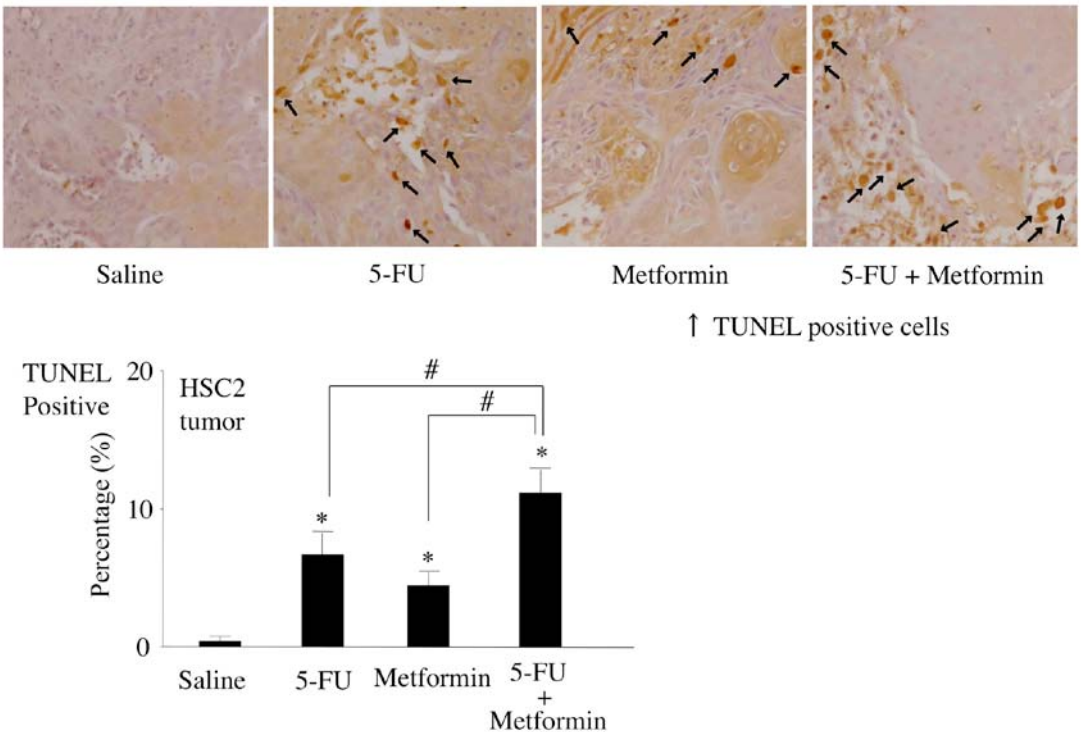

Figure 7. Effects of 5-FU and/or metformin on induction of apoptosis *in vivo*. A TUNEL assay was performed to quantify the number of apoptotic cells in treated HSC2 tumors. Cells stained brown after TUNEL staining, which indicates that apoptosis occurred in tumors treated with 5-FU and/or metformin. The TUNEL assay showed significantly increased number of apoptotic cells in 5-FU and metformin combined treatment tumors. Error bars represent the standard error of the mean results from five mice (n=5). *P<0.01 when compared to that of Control (Mann-Whitney U test). $^{\#}$P<0.01 when compared to that of each agent alone (Mann-Whitney U test).

mechanism of the growth inhibitory effect of 5-FU in combination with metformin is still unclear.

In this study, we focused our attention on inhibition of the Warburg effect by 5-FU in combination with metformin. In accordance with our expectation, metformin alone exerted marked inhibitory effect on Warburg effect in OSCC cells, and in combination with 5-FU it showed more prominent inhibitory effect on Warburg effect. Briefly, 5-FU in combination with metformin significantly suppressed the production of lactate (Fig. 3), and it could reduce the expression of HIF-1-α, mTOR and Akt1 and induce the expression of AMPKα than either agent alone (Figs. 4 and 6). Moreover, the level of inhibition of Warburg effect by 5-FU in combination with metformin seemed to be relative to the growth inhibitory effect (Fig. 1), apoptosis inducing effect (Figs. 2 and 7), and antitumor effect (Fig. 5B). Furthermore, it has been reported that metformin is well tolerated by patients, it is absorbed into the body within 1-3 h after oral administration and 90% of it is eliminated by the renal system (28). The combined treatment with 5-FU and metformin may be an attractive option compared to other cytotoxic therapeutic agents because in our study, mice treated with 5-FU in combination with metformin showed no toxicity or significant weight loss during the treatment when compared to the untreated control mice (Fig. 5C).

As an OSCC treatment strategy, recently we tend to select the heavy use of costly molecularly targeted drugs for OSCC treatment. Instead, a more desirable strategy could be selecting low cost, effective and less cytotoxic drugs against OSCC. In this experiment, we showed that FU and metformin combination therapy effectively suppressed the growth of OSCC cells/tumors and could modulate the regulator molecules of the Warburg effect in cancer cells more than our expectations. These findings suggest that 5-FU and metformin combination treatment might be regarded as a potential treatment strategy for human OSCC. Future studies should aim at defining the most appropriate dose and schedule of administration of this combination treatment.

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