Metformin inhibits the radiation-induced invasive phenotype of esophageal squamous cell carcinoma

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Abstract. Esophageal cancer is one of the most aggressive tumor types because of its invasiveness and metastatic potential. Several reports have described an association between increased invasiveness after ionizing radiation (IR) treatment and epithelial-to-mesenchymal transition (EMT). The biguanide metformin is reported to prevent transforming growth factor-β (TGF-β)-induced EMT and proliferation of cancer. This study examined whether IR induces EMT and promotes the invasive potential of TE-9 esophageal squamous cell carcinoma cells and the effect of metformin on IR-induced EMT. After IR exposure, TE-9 cells showed a spindle-shaped morphology and lost cell-cell adhesion. Immunoblotting showed that IR induced expression of mesenchymal markers (vimentin and N-cadherin), transcription factors (Slug, Snail, and Twist), and matrix metalloproteinases. A scratch wound assay and Matrigel invasion assay showed that IR enhanced the invasive potential and migratory capacity of TE-9 cells. Expression of hypoxia-related factor-1α and TGF-β was increased after IR. IR also induced phosphorylation of Smad2 and Smad3. Metformin inhibited radiation-induced EMT-like morphological changes, and enhanced invasion and migration of TE-9 cells. Metformin inhibited IR-induced phosphorylation of Smad2 and Smad3. Although phosphorylation of AMP-activated protein kinase was enhanced by IR and metformin, phosphorylation of mammalian target of rapamycin was enhanced by IR and suppressed by metformin. These results indicated that metformin suppressed IR-induced EMT via suppression of the TGF-β-Smad phosphorylation pathway, and a part of the non-Smad pathway. Metformin might be useful to prevent IR-induced invasion and metastasis of esophageal squamous cell carcinoma.

Introduction

Esophageal cancer is a common malignancy among human gastrointestinal tumors. In 2008, it was estimated that 482,300 new esophageal cancer cases and 406,800 deaths had occurred worldwide (1). In the most high-risk area, which stretches from Northern Iran through the central Asian republics to North-Central China is called the esophageal cancer belt, 90% of the cases are squamous cell carcinoma. Esophageal cancer is one of the most aggressive types of tumor with an average 5-year survival rate of ~17% (2). It has been established that the rates of mortality and incidence of esophageal cancer are quite similar, probably because of the relatively late stage at diagnosis and rapid progression of the tumor. The treatment failure of esophageal cancer can be explained by the extensive local invasion and regional lymph node metastasis, which make it difficult to completely resect the tumors (2).

Chemoradiotherapy has an important role in the treatment of esophageal cancer in both inoperable and pre-operative settings (3). Even after complex multidisciplinary treatment, the rate of local recurrence and distant metastasis remains high. Acquired radioresistance during radiotherapy has been considered as one of the major reasons for treatment failure (4,5).

It has been reported that ionizing radiation (IR) activates multiple signaling pathways and affects the activity or abundance of proteases, growth factors, cytokines, and adhesion proteins that are involved in tissue remodeling (6,7). Recently, several preclinical and clinical studies have shown that IR enhances the invasiveness and metastatic potential of human cancer cells. Tsukamoto *et al* (8) and Wild-Bode *et al* (9)

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Abbreviations: ESCC, esophageal squamous cell carcinoma; EMT, epithelial-to-mesenchymal transition; IR, ionizing radiation; TGF-β, transforming growth factor-β; HIF-1α, hypoxia-related factor-1α

Key words: metformin, esophageal cancer, epithelial-to-mesenchymal transition, radiation, transforming growth factor-β

showed that sub-lethal doses of IR enhance the migration and invasiveness of human endometrial cancer and glioblastoma cells.

Recently, epithelial-to-mesenchymal transition (EMT) has been recognized as a key event in tumor invasion and metastasis (10,11). EMT is a cellular process by which an epithelial cell changes its characteristics in response to both intracellular and extracellular signaling pathways (12). It is a complex process that involves cytoskeletal remodeling, cell-cell and cell-matrix adhesions, and transcriptional regulation, leading to the transition from a polarized epithelial phenotype to an elongated fibroblastoid phenotype.

EMT is induced by multiple factors. Numerous studies have revealed that transforming growth factor-β (TGF-β) stimulates EMT in various types of cancer cells. TGF-β signals through a complex of two types of serine/threonine kinase receptors. It binds to the type II receptor and stimulates the type I receptor that activates the intracellular mediators Smad2 and Smad3 by phosphorylation. Smad2 and Smad3 combine with Smad4 to form complexes that translocate into the nucleus to activate gene expression related to EMT (12). IR can cause cancer cell death through apoptosis, senescence, and genomic instability. However, IR increases extra- and intracellular levels of TGF-β and induces acceleration of metastasis (8,13). Recently, radiation was found to enhance cell migration, invasion and metastasis through induction of EMT in various cancer cell lines including esophageal squamous cell carcinoma (ESCC) (11,14).

The biguanide metformin is the most widely used drug to treat type 2 diabetes. It reduces blood glucose levels by suppressing gluconeogenesis in the liver and increasing glucose uptake by skeletal muscle. It improves insulin sensitivity and thus decreases insulin resistance. Activation of AMP-activated protein kinase (AMPK), an enzyme that is the central regulator of metabolic pathways, has been credited with the glucose-lowering effects of metformin (15). Retrospective epidemiological studies have provided evidence that type 2 diabetic patients receiving metformin have substantially lower cancer incidence and mortality than those on other treatments (16-18). Numerous preclinical and clinical studies have demonstrated multiple anticancer effects of metformin (19). Metformin has shown antigrowth effects in various tumor types using xenograft models (20). The main mechanism of tumor growth inhibition by metformin has been attributed to activation of AMPK, leading to various downstream effects that act together to restrain tumor growth (20-22). Metformin suppresses tumor growth by phosphorylation of AMPK, leading to suppression of mammalian target of rapamycin (mTOR) (20,22). Metformin has also been reported to prevent TGF-β-induced conversion of cancer cells into the migratory mesenchymal phenotype (23,24).

In this study, we examined whether IR induced EMT and enhanced the migration and invasiveness of ESCC cells. In addition, we investigated the possibility of metformin to prevent radiation-induced EMT *in vitro*.

Materials and methods

Cell lines and treatments. The human ESCC cell line TE9 was kindly provided by Dr Tetsuro Nishihira (Higashimatsuyama Medical Association Hospital, Saitama, Japan). The cells were cultured in RPMI-1640 (Gibco®, Life Technologies Corp., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan), 100 IU/ml penicillin, 100 μg/ml streptomycin (Life Technologies Corp.), 2 mM glutamine (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) at 37˚C with 5% CO_2.

Metformin (Wako Laboratory Chemicals, Tokyo, Japan) was dissolved in phosphate-buffered saline at a stock concentration of 100 mM and stored at -20˚C. Cultures were irradiated using an MBR-1520R-3 (Hitachi Medicotechnology, Hitachi, Japan) at a dose rate of 0.66 Gy/min. The power output of the X-ray irradiation was 125 kV and 20 mA. Forward-scattered radiation 0.5 mm Al and 0.2 mm Cu filters were used.

Proliferation assay. The proliferation of cells treated with metformin was determined by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. TE9 cells were seeded at a density of $4x10^3$ cells/well in a 96-well culture plate and allowed to attach for 24 h. The cells were treated with metformin at various concentrations (0.1-5 mM) for 48 h. The percentage inhibition was determined by comparing the cell density of the drug-treated cells with that of untreated controls. All experiments were repeated at least three times.

Immunofluorescence. Cells were cultured on Lab-Tec chamber slides (Nalge Nunc International, New York, NY, USA). The medium was changed to serum-free medium after 24 h. The cells were treated with 0.5 mM metformin for 48 h and then irradiated (2 Gy). They were fixed in a mixture of methanol and acetone (1:1) for 5 min. The cells were blocked with 1.5% bovine serum albumin and incubated with a mouse monoclonal antibody against vimentin (1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or a rabbit polyclonal antibody against E-cadherin (1:100 dilution; Santa Cruz Biotechnology, Inc.) and mouse monoclonal antibody against β-catenin (1:100 dilution; Santa Cruz Biotechnology Inc.) at 4˚C overnight. The immunoreactivity was visualized by incubating the cells with anti-rabbit IgG conjugated with Alexa Fluor 488 or 594 (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. The cells were counterstained with bisbenzimide (Hoechst 33258, 100 ng/ml; Sigma-Aldrich Co., LCC) to visualize the nuclei. Finally, the cells were examined under an immunofluorescence microscope (BX50/BX-FLA, Olympus, Tokyo, Japan).

Scratch wound assay. TE9 cells were seeded at a density of $1x10^5$ in a 6-well plate and grown overnight to confluency. The monolayers of cells were scratched with a 1000-μl pipette tip to create a wound and then washed twice with serum-free RPMI-1640 to remove detached cells. The medium was then replaced with serum-free medium with or without 0.25 mM metformin, and the cells were irradiated (2 Gy). The rate of wound closure was assessed and photographed after 48 h. Each value was derived from three randomly selected fields.

Matrigel invasion assay. TE9 cells were treated with or without 0.25 mM metformin after incubation in medium without FBS for 24 h. Then, the cells were irradiated (2 Gy) at 48 h after metformin treatment. The cells were collected by trypsiniza-

Table I. Primary antibodies used for immunoblot analysis.

Primary antibody		Isotype	Dilution
Anti-E-cadherin[a]	H-108	Rabbit polyclonal IgG	1:2,000
Anti-vimentin[a]	V9	Mouse monoclonal IgG	1:2,000
Anti-N-cadherin[a]	H-63	Rabbit polyclonal IgG	1:1,000
Anti-β-catenin[a]	E-5	Mouse monoclonal IgG	1:1,000
Anti-Slug[a]	H-140	Rabbit polyclonal IgG	1:400
Anti-twist[a]	H-81	Rabbit polyclonal IgG	1:500
Anti-TGF-β1[a]	V	Rabbit polyclonal IgG	1:500
Anti-phospho-Smad2/3[a]	Ser 423/425	Goat polyclonal IgG	1:1,000
Anti-Smad2/3[a]	E-20	Goat polyclonal IgG	1:1,000
Anti-Snail[b]	C15D3	Rabbit monoclonal IgG	1:1,000
Anti-phospho-AMPKα (Thr172)[b]	40H9	Rabbit monoclonal IgG	1:2,000
Anti-AMPKα[b]	D63G4	Rabbit monoclonal IgG	1:2,000
Anti-phospho-mTOR (Ser2448)[b]	D9C2	Rabbit monoclonal IgG	1:2,000
Anti-mTOR[b]	7C10	Rabbit monoclonal IgG	1:2,000
Anti-Raptor[b]	24C12	Rabbit monoclonal IgG	1:2,000
Anti-Rictor[b]	53A2	Rabbit monoclonal IgG	1:2,000
Anti-β-actin[c]		Mouse monoclonal IgG	1:10,000
Anti-HIF1α[d]	H1α67	Mouse monoclonal IgG	1:500

Purchased from: [a]Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); [b]Cell Signaling Technology Inc. (Beverly, MA, USA); [c]Sigma-Aldrich Co. (Oakville, ON, Canada); [d]Thermo Scientific Inc. (Wilmington, DE, USA).

tion, resuspended in serum-free medium at $1x10^5$ cells/ml, and added to the upper chamber of a 24-well transwell insert (Corning Life Sciences, Corning, NY, USA) that was coated with 50 μl Matrigel (1:10 dilution in serum-free medium; BD Biosciences, Franklin Lakes, NJ, USA). Medium supplemented with 10% serum with or without 0.25 mM metformin was added to the bottom chamber. After 24 h, cells that had migrated through the Matrigel and 8-μm pore size membrane were fixed, stained and counted under a light microscope. All experiments were performed in triplicate.

Immunoblot analysis. Cell lysates were prepared in denaturing sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. Proteins (20 μg) from each sample were loaded onto a 10% polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA). The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) and blocked with commercial gradient buffer (PVDF Blocking Reagent for Can Get Signal, Toyobo, Tokyo, Japan) at room temperature for 30 min. The membranes were incubated with the primary antibody overnight at 4˚C. After washing, the membranes were incubated with horseradish peroxidase-labeled anti-mouse or anti-rabbit IgG (GE Healthcare UK, Ltd., Buckinghamshire, UK) or anti-goat IgG (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Antibody-antigen complexes were detected using an ECL Plus kit (GE Healthcare Japan Co. Ltd.) and the Light Capture system (Atto Co. Ltd., Tokyo, Japan). Quantification was performed with the CS analyzer program (Atto Co. Ltd.). Primary antibodies used are described in Table I.

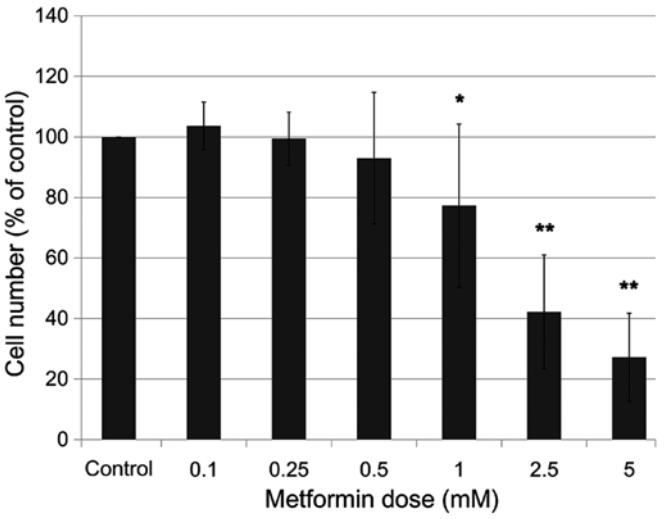

Figure 1. Effect of metformin on cell proliferation. Effects of metformin on cell proliferation were assessed by an MTT assay. Data are expressed as the means ± SD of triplicate experiments. Statistically significant inhibition of cell proliferation after metformin exposure was observed at concentrations of 1 mM (77.3%, P=0.014), 2.5 mM (42.2%, P<0.001), and 5 mM (27.2%, P<0.001), but the inhibition did not reach statistical significance at 0.5 mM (93.0%, P=0.30).

Results

Metformin inhibits the proliferation of TE9 cells. First, we examined the effects of metformin on cell proliferation using the MTT assay. Dose-dependent inhibition of proliferation was observed at 1-5 mM metformin (Fig. 1). Because 0.5 mM

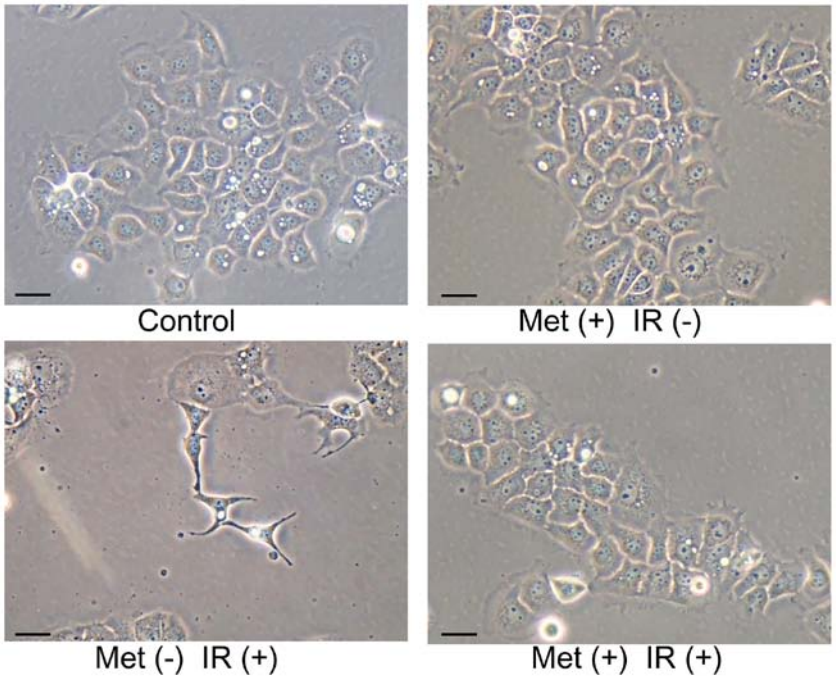

Figure 2. Morphological changes after ionizing radiation (IR) and metformin (Met) treatments. TE-9 cells were treated with 0.5 mM metformin for 48 h and then 2 Gy IR, followed by observation under a phase contrast microscope. Scale bar, 10 μm.

metformin did not significantly inhibit the growth of the cells, the majority of subsequent experiments were performed with 0.25-0.5 mM metformin.

Metformin inhibits mesenchymal-like changes in esophageal cancer cells induced by IR. We examined morphological changes in TE-9 cells after 2 Gy IR. TE-9 cells showed loss of cell polarity, causing a spindle cell morphology, and enlargement between the cell-to-cell spaces at 48 h after IR. Pre-treatment with 0.5 mM metformin inhibited these morphological changes. Metformin did not induce any morphological changes when administered without IR compared with the control (Fig. 2).

Immunofluorescence analysis. Immunofluorescence analysis showed that IR induced vimentin (mesenchymal marker) expression and suppressed E-cadherin (epithelial marker) expression on the cell membrane of TE-9 cells. Pre-treatment with 0.5 mM metformin suppressed the vimentin expression induced after IR. Pretreatment with metformin also recovered E-cadherin expression to a level similar to that in the control (Fig. 3A). Expression of β-catenin (a cadherin-associated protein) on the cell membrane was suppressed by IR. IR induced translocation of β-catenin to the cell nucleus. Metformin inhibited nuclear translocation of β-catenin induced by IR (Fig. 3B).

Metformin inhibits cell migration and invasion promoted by IR. We analyzed the migratory and invasive phenotypes of TE-9 cells using scratch wound and Matrigel invasion assays. As shown in Fig. 4A, the scratch wound assay indicated that the migration of TE-9 cells was significantly stimulated by IR. Metformin itself did not affect the migratory phenotype of the cells. Pretreatment with 0.25 mM metformin inhibited IR-induced migration in TE-9 cells (Fig. 4B). Similarly, the data obtained from the Matrigel invasion assay showed that IR stimulated cell invasion. Pretreatment with 0.25 mM metformin inhibited the IR-induced invasive phenotype of the cells (Fig. 5). Next, we examined the expression of matrix metalloproteinase (MMP)-associated proteins, which are related to the invasion and metastasis of esophageal cancer (25). In the immunoblotting analysis, MMP-2/7/9 expression was increased by IR. Pretreatment with metformin reduced the IR-induced enhancement of MMP expression (Fig. 6A).

Effect of metformin on EMT-associated signaling pathways induced by IR. Expression of mesenchymal marker proteins (vimentin and N-cadherin) was increased by IR in comparison with the control. Metformin counteracted the effects of IR on mesenchymal marker protein expression. There were no apparent differences in the expression of epithelial markers E-cadherin and β-catenin between the metformin-treated and IR groups (Fig. 6B). Snail, Slug, and Twist are EMT-associated transcription factors. The expression of these proteins was elevated in the IR group compared with the control group. After the addition of metformin, protein expression of these transcription factors was suppressed following IR exposure (Fig. 6B). Increased expression of hypoxia-related factor (HIF)-1α was found at 48 h after IR treatment. Metformin had no effect on the increase in HIF-1α expression after IR exposure. TGF-β1 expression was also increased in the IR group, but the increased expression of TGF-β1 was not suppressed by metformin. Metformin suppressed radiation-induced phosphorylation of Smad2/3 (Fig. 6C). Phosphorylation of AMPK was found after metformin treatment, irrespective of IR exposure.

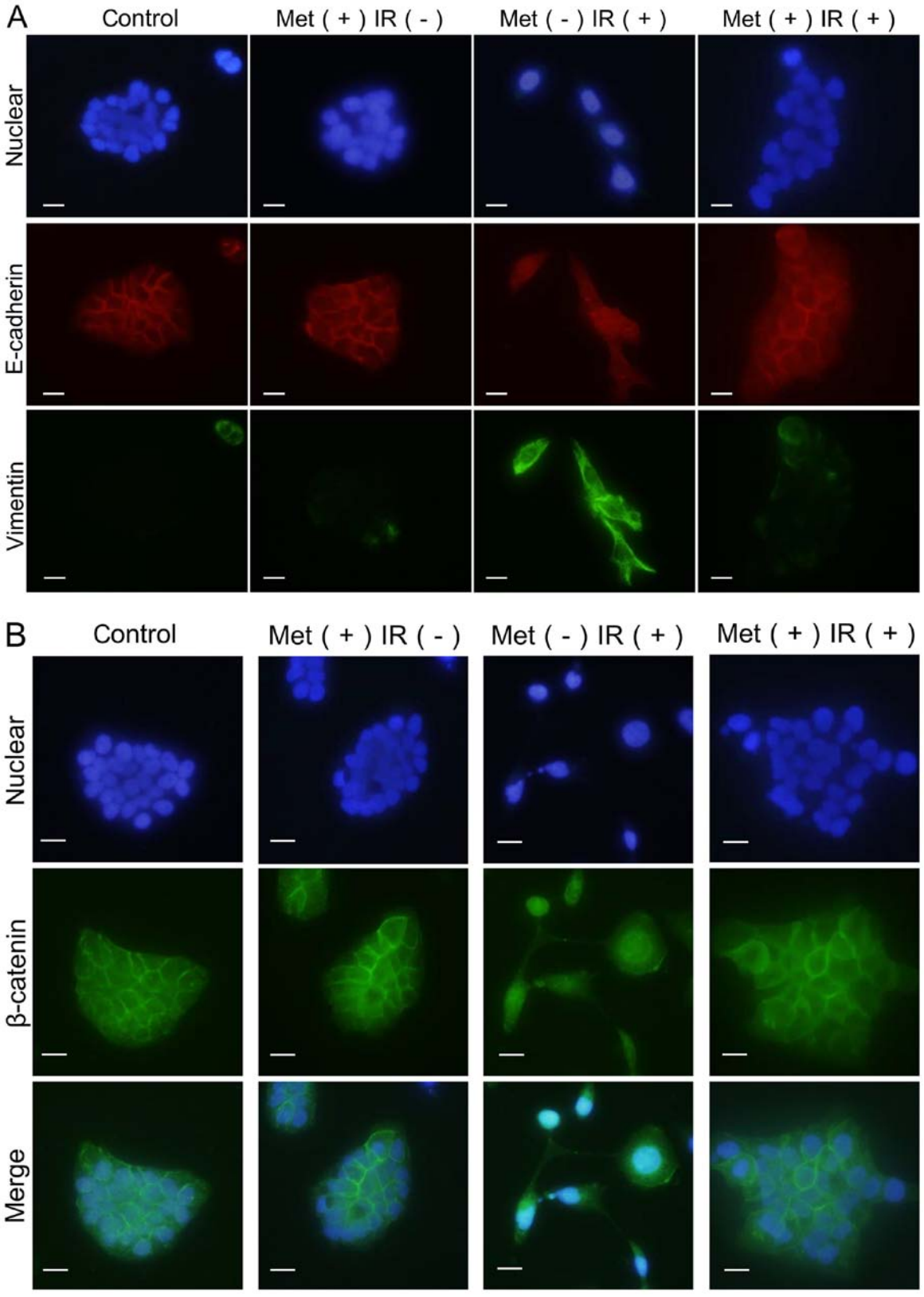

Figure 3. Immunofluorescence analysis after ionizing radiation (IR) and metformin (Met) treatments. TE-9 cells were treated with 0.5 mM metformin for 48 h and/or 2 Gy IR. Immunofluorescence analysis of E-cadherin, vimentin (A), and β-catenin (B) expression was performed. TE-9 cells were fixed and stained with bisbenzimide (blue). Expression of vimentin (green), E-cadherin (red), and β-catenin (green) was examined by immunofluorescence analysis. Representative images of multiple fields are shown for each treatment group. Scale bar, 10 μm.

Phosphorylation of mTOR was enhanced in the IR group, but it was significantly reduced in the metformin-treated group. Expression of a component of mTORc1, Raptor, was elevated in the IR group (Fig. 6C).

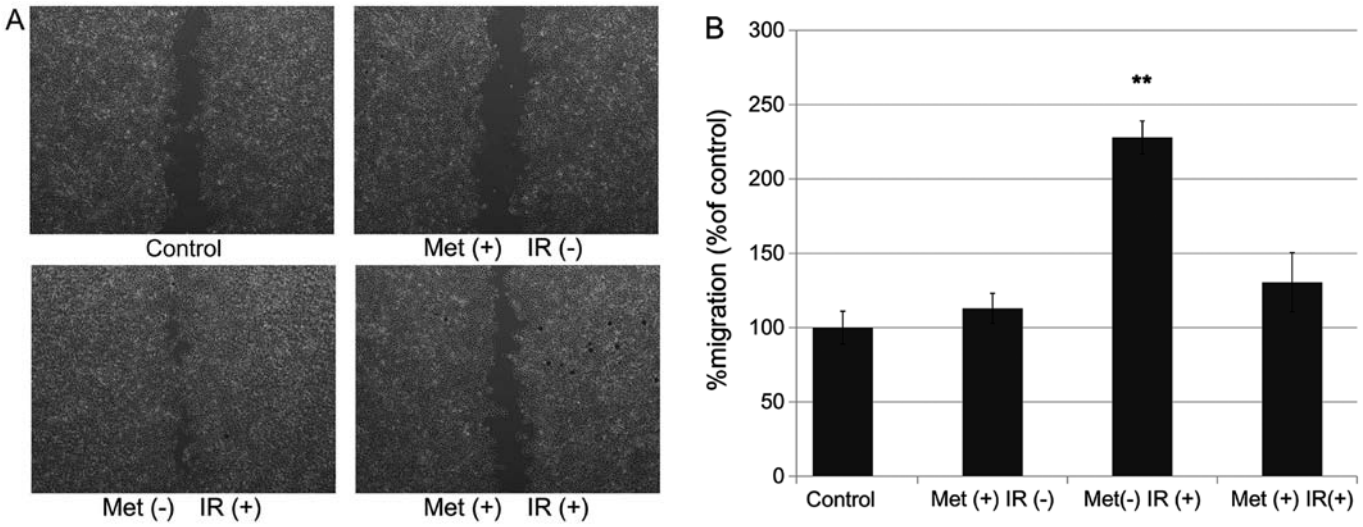

Figure 4. Effect of ionizing radiation (IR) and metformin (Met) on the migration of TE-9 cells. (A) Representative images of the migration assay (scratch wound assay). Cell monolayers were scratched with a pipette tip and then treated with 0.25 mM metformin for 48 h and then 2 Gy irradiation. Migrating cells were photographed under a phase contrast microscope. (B) Migration (% migration) was calculated by the following formula: (pretreatment wound width - width after 48 h) / pretreatment width x 100. Data are expressed as the means ± SD of triplicate experiments. **Significantly different from the control (P<0.001).

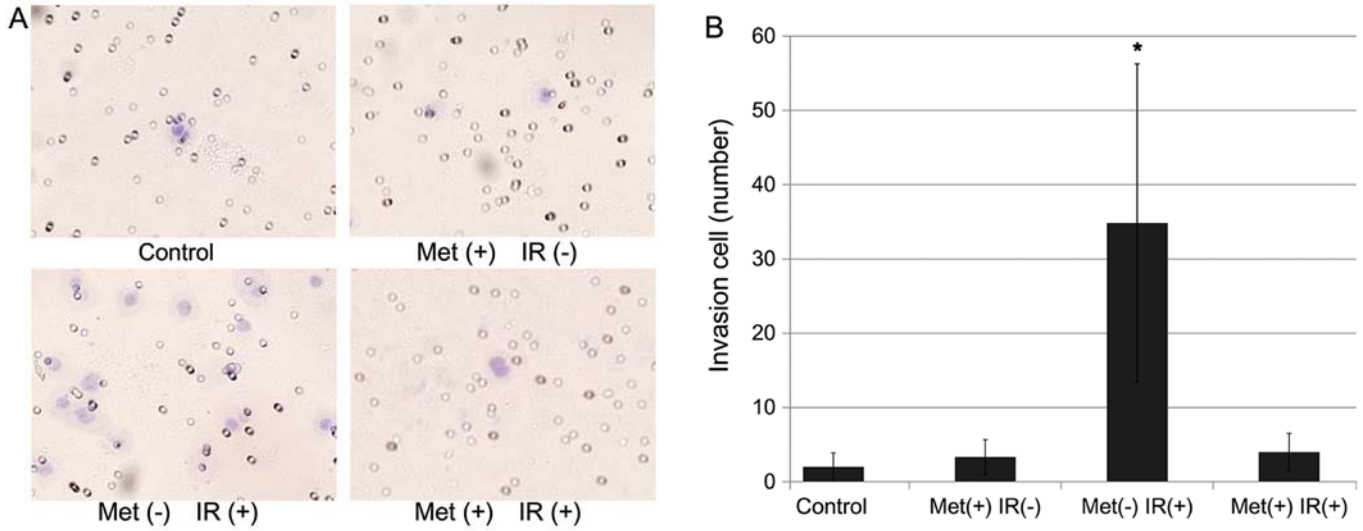

Figure 5. Effect of ionizing radiation (IR) and metformin (Met) on the invasion of TE-9 cells. (A) Representative images of the invasion assay. Cells were treated with 0.25 mM metformin or the vehicle for 48 h. At 48 h after irradiation (2 Gy), the invasive ability of the cells was examined by a Matrigel invasion assay. (B) Invaded cells on the lower surface of the filter were counted. Data are expressed as the means ± SD of triplicate experiments. *Significantly different from the control (P=0.003).

Discussion

This study showed that IR induces EMT and promotes migration and invasion potential in TE-9 ESCC cells and metformin inhibits IR-induced EMT.

In several studies, IR has induced EMT that occurs at the time of initiation of metastasis, leading to cancer progression, and is associated with the development of resistance to radiotherapy (10,11). In this study, we found that TE-9 cells changed their morphology to a spindle shape and lost cell-cell adhesion after IR exposure. Immunofluorescence and immunoblot analyses showed that IR induced expression of mesenchymal markers vimentin and N-cadherin as well as nuclear localization of β-catenin in TE-9 cells. These results indicate that a sub-lethal dose of IR can induce EMT in ESCC. We have already confirmed these phenomena using several other ESCC cell lines (data not shown). Although IR did not reduce the total amount of epithelial marker E-cadherin, the distribution of E-cadherin was changed from the cell membrane to the cytoplasm. During the EMT process, expression of E-cadherin is repressed through transcriptional reduction. E-cadherin translocates from the cell surface by endocytosis and is degraded by a proteasome (26). Cytoplasmic localization of E-cadherin without a decrease in the total protein amount shown in this study might suggest the degradation process of E-cadherin during EMT. Furthermore, we found upregulation of transcription factors, including Slug, Snail, and Twist, which regulate EMT (12). The effect of TGF-β on EMT has been

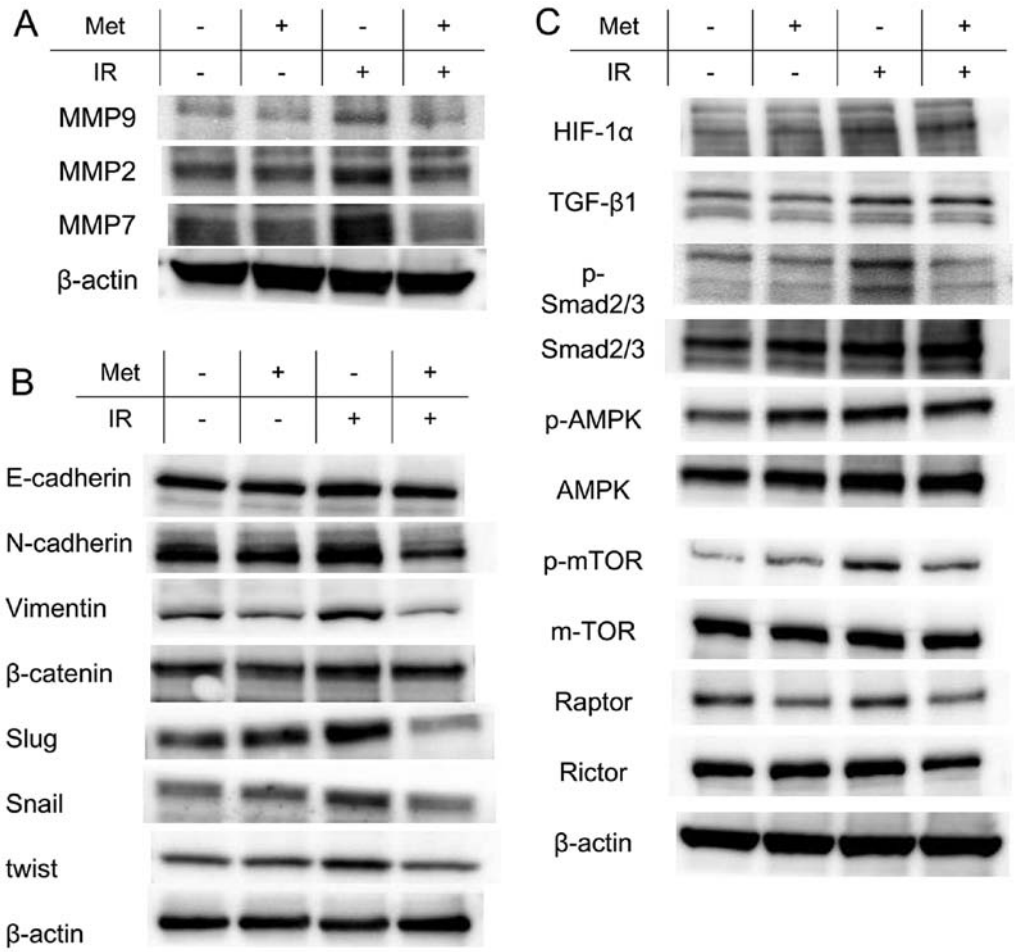

Figure 6. Effects of ionizing radiation (IR) and metformin (Met) on the expression of MMPs and EMT-related factors. Cells were pretreated with 0.25 mM metformin or the vehicle for 48 h. Soluble lysates from untreated or irradiated cells were prepared at 48 h after irradiation (2 Gy) and analyzed for expression of MMPs (A), EMT markers and EMT-associated transcription factors (B), and EMT-associated signaling factors (C) by immunoblot analysis.

identified in a variety of cancer types (27,28). We confirmed that TGF-β stimulation also induced EMT by morphological changes, enhancement of mesenchymal markers, and β-catenin translocation to the nucleus in TE-9 cells (data not shown).

To clarify the mechanisms of EMT induced by IR, we focused on HIF-1α expression in this study. Radiation causes hypoxia in cancer cells and the surrounding microenvironment (6,7). Hypoxia induces various cytokines and growth factors including HIF-1α in cancer cells. These cytokines increase angiogenesis, self-renewal, and cancer stem cell-like characteristics (6,7,29). We found that IR induced HIF-1α and TGF-β expression. Subsequently, phosphorylation of Smad2/3 was increased. TGF-β family proteins signal through Smads that combine with DNA sequence-specific transcription factors to activate or repress transcription. The Smad pathway, which, in the case of TGF-β, is mediated by Smad2 and Smad3 in combination with Smad4, is considered to be the major TGF-β family signaling pathway and accounts for the many changes in gene expression observed in response to TGF-β family proteins (12,27,30). However, various EMT pathways have been reported to be induced by TGF-β. These are called non-Smad signaling pathways and include activation of Erk Map kinase, Rho GTPases and PI3 kinase/Akt pathways. These pathways have been linked to TGF-β-induced EMT through their regulation of distinct processes such as cytoskeleton organization, cell growth, survival, migration and invasion (12,27). Through PI3 kinase/Akt pathways, mTOR/S6 kinase 1 is activated in the mammary gland cells of mice by TGF-β-induced EMT (12,30). In this study, we found that IR induced mTOR phosphorylation, suggesting activation of non-Smad signaling pathways. These results indicate that IR induces EMT through activation of TGF-β family signaling pathways including Smad and non-Smad pathways in TE-9 cells.

As a result of mesenchymal transition, β-catenin (the lining protein of E-cadherin) changes its localization from the cell membrane to the nucleus. The transcription of cell invasion-related protein MMP-7 has been reported to be controlled by nuclear translocation of β-catenin (31). We also found enhanced expression of MMPs, including MMP7, after IR exposure. We confirmed enhancement of the invasive potential and migratory capacity of esophageal cancer cells by IR using scratch wound and Matrigel invasion assays. It has been reported that radiation-induced EMT causes radiation resistance and enhances the migration-invasive potential with increases of MMPs in several types of cancer cells (9,11,14,32).

We found that metformin inhibited IR-induced EMT in association with the invasive and migratory phenotypes

of ESCC cells. To elucidate the mechanism by which metformin inhibited EMT enhanced by IR, we investigated cell morphology and alterations of EMT-associated proteins by metformin after IR. The cells treated with metformin did not show the EMT-like morphological changes caused by IR. The enhancements in the expression of mesenchymal markers (vimentin and N-cadherin), EMT-associated transcription factors (Snail, Slug, and Twist), and MMPs after IR were suppressed by metformin. However, increased expression of HIF-1α and TGF-β by IR was not inhibited by metformin. Therefore, metformin might suppress radiation-induced EMT by inhibition of TGF-β signaling pathways. The fact that metformin suppressed radiation-induced Smad2/3 and mTOR phosphorylation support this hypothesis. Recently, several studies have indicated that metformin suppresses proliferation, migration, and EMT by inhibition of mTOR signaling (33,34). Metformin has also been reported to suppress EMT induced by TGF-β administration in breast and lung cancer cells (23,24,35).

Originally, metformin was found to inhibit complex I of the mitochondria respiratory chain and increase the proportion of AMP in cells. Furthermore, it reduces blood glucose and improves glucose tolerance and insulin resistance by activation of AMPK (15). Increasing evidence suggests that the antitumor effect of metformin might be linked to its direct and indirect mechanisms. The indirect mechanisms of metformin, which are considered as insulin dependent, include enhancement of insulin sensitivity and reductions in the blood levels of glucose and insulin, resulting in the inhibition of the insulin/insulin-like growth factor-1 signaling pathway, and eventually resulting in the inhibition of tumor cell growth. Its direct mechanisms, which are considered as insulin independent, include primarily activation of AMPK, a central energy sensor, in turn leading to inhibition of the mTOR signaling pathway. Specifically, when the level of cellular ATP decreases, resulting in an increase in the ratio of AMP/ATP, AMPK is activated, contributing to inactivation of the Akt/mTOR signaling mediated through the inhibition of mTOR by activation of LKB1, a known tumor suppressor. The inhibition of mTOR activity leads to the inhibition of glucose synthesis in the liver and protein synthesis, contributing to the inhibition of cell growth/proliferation of tumor cells mediated through the reduction in the bioavailability of intracellular energy and nutrients such as glucose and amino acids. Moreover, metformin can directly inhibit tumor cell growth/proliferation via modulation of the cyclin D1-medicated cell cycle and the expression of tumor suppressor p53 in various tumor cell types including pancreatic and breast cancers (20-22). In this study, we found the 0.5 mM metformin, which do not have a direct inhibitory effect on ESCC cell growth, suppressed IR-induced EMT, leading to less cell invasion after IR.

IR activates AMPK acutely in epithelial cancer cell cultures to transduce signals through a DNA damage response-mediated ataxia teleangiectasia-mutated-AMPK-p53-p21cip1 axis, and mediate cell cycle arrest and radiosensitization (36,37). However, IR also activates molecular pathways of tumor radioresistance, such as the Akt/mTOR pathway (38). mTOR stimulates growth of cellular biomass, proliferation, and resistance to cytotoxic agents (20). As shown in this study, a sub-lethal dose of IR induced mTOR phosphorylation and EMT. Metformin activated AMPK by phosphorylation and suppressed phosphorylation of mTOR induced by IR.

Most *in vitro* studies investigating anticancer effect of metformin have used metformin at a dose between 1 and 10 mM (165-1650 mg/l), which is beyond the therapeutic plasma levels (0.465-2.5 mg/l or 2.8-15 μM) (19). We examined the effects of metformin on cell proliferation and cytotoxicity. It was found that metformin showed no significant cytostatic effect at 0.25-0.5 mM. In this study, the inhibitory effect of metformin against radiation-induced EMT was examined at a higher concentration than the clinically therapeutic plasma levels. Recently, it has been reported that low-dose metformin (2.5 μM-5 mM) inhibits proliferation and enhances radiosensitivity in lung cancer cells (37). Further study should be performed to clarify the possibility of clinical usage of metformin by examining its effect at lower doses.

Metformin has a long track record as a diabetic drug, and its efficacy and side-effects have been fully discussed previously (15). Its most common toxicity is mild-to-moderate gastrointestinal discomfort, usually self-limited and ameliorated by a graduated ramp up in dose. The most serious toxicity of metformin, lactic acidosis, is rare, occurring once per 100,000 years of use (39). Metformin alone is rarely associated with hypoglycemia. According to a recent review, the reported risks of hypoglycemia for metformin users vary between 0 and 21%. Because metformin does not directly stimulate insulin secretion, the hypoglycemic risk may be lower than for that of other oral antidiabetic drugs (39).

Prospective data regarding metformin use in non-diabetic cancer patients are beginning to emerge (40,41). Completion of these studies, as well as planned and ongoing studies on the metastatic and adjuvant setting in breast, prostate, pancreatic and endometrial cancers (clinicaltrials.gov), will provide important additional information on the anticancer effects of metformin and help elucidate the clinically important mechanisms of metformin action in diabetic and non-diabetic cancer patients.

In this study, metformin inhibited IR-induced invasion and migration of esophageal cancer cells by inhibition of EMT via suppression of TGF-β signaling pathways. Metformin might be beneficial to use during radiation therapy for esophageal cancer to prevent invasion and metastasis caused by IR-induced EMT. To confirm the clinical availability of metformin in radiotherapy, further studies are needed.

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