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Antitumor effects of metformin are a result of inhibiting nuclear factor kappa B nuclear translocation in esophageal squamous cell carcinoma

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Esophageal squamous cell carcinoma (ESCC) is an intractable digestive organ cancer that has proven difficult to treat despite multidisciplinary therapy, and a new treatment strategy is demanded. Metformin is used for type 2 diabetes mellitus and its antitumor effects have been reported recently. Metformin exerts antitumor effects in various respects, such as inhibiting inflammation, tumor growth and epithelial-mesenchymal transition (EMT). However, few reports have described the efficacy of metformin on ESCC, and their findings have been controversial. We analyzed the antitumor effects of metformin and clarified its effects on anti-inflammation, growth suppression and EMT inhibition. Activation of nuclear factor kappa B (NF-κB), the major transcription factor induced by inflammation, was investigated by immunostaining. We found that localization of NF-κB in the nucleus was reduced after metformin treatment. This suggests that metformin inhibited the activation of NF-κB. Metformin inhibited tumor growth and induced apoptosis in ESCC cell lines. Associated with EMT, we examined cell motility by a wound healing assay and the epithelial marker E-cadherin expression of various ESCC cell lines by western blotting. Metformin inhibited cell motility and induced E-cadherin expression. In conclusion, metformin showed multiple antitumor effects such as growth suppression, invasion inhibition, and control of EMT by inhibiting NF-κB localization on ESCC. Further exploration of the marker of treatment efficacy and combination therapy could result in the possibility for novel treatment to use metformin on ESCC.

KEYWORDS
epithelial-mesenchymal transition, esophageal squamous cell carcinoma, inflammation, metformin, treatment outcome

Abbreviations: AMPK, AMP-activated kinase; DM, diabetes mellitus; EMT, epithelial mesenchymal transition; ESCC, esophageal squamous cell carcinoma; LKB1, liver kinase B1; NF-κB, nuclear factor-kappa B; TGF-β, transforming growth factor-beta.

1 | INTRODUCTION

Esophageal squamous cell carcinoma is an intractable digestive organ cancer that shows a high incidence in East Asia. It remains difficult to treat despite multidisciplinary therapy, including surgery, chemotherapy and radiotherapy. In particular, tolerance to chemotherapy and a tendency toward metastasis even when tumors are superficial are major factors making treatment difficult.[1] We have recently been conducting research focused on epigenetics and reported that microRNA-375 induced by histone acetylation had ESCC restraint activity.[2] We searched drugs which induce similar gene expression style by connectivity map and found metformin. It restrained the increase of ESCC cell lines, TE2 and T.Tn.[3]

Metformin is an oral biguanide that has been used to treat type 2 DM worldwide for a long time, and the antitumor effects of metformin have been reported recently.[4] The main target of metformin is regarded as AMPK, which has various functions. Metformin induces the activation of LKB1, which phosphorylates and activates AMPK.[5] However, details on the drug's activities remain unclear.

DM increases the risk of developing cancer, and possible biological links between DM and cancer comprise hyperinsulinemia, hyperglycemia and fat-induced chronic inflammation.[6] Patients treated with metformin have shown a lower incidence of cancer than the other treatment groups.[7] In addition, metformin reportedly affects various cancers, such as gastric cancer,[8] non-small cell lung cancer,[9] and colorectal cancer.[10] Metformin exerts its antitumor effects in various ways, including inducing cell cycle arrest,[11] enhancing the immune system,[12] inhibiting EMT,[13] inducing hypersusceptibility for chemotherapy,[14] and inhibiting inflammatory signals through NF-κB/TGF-β.[15]

Epithelial-mesenchymal transition (EMT) plays a key role in inducing stationary tumor cells to migrate and invade and metastasize to other organs.[16] E-cadherin is the most important marker of epithelial cells and is used widely to determine EMT inhibition.[17] It has recently been shown that metformin inhibits migration or invasion by affecting EMT in various tumors, such as cholangiocarcinoma,[18] cervical carcinoma,[19] thyroid cancer[20] and melanoma.[21]

Regarding the clinical antitumor effects of metformin on ESCC, Van De Voorde reported that the use of metformin was associated with an improved overall and distant metastasis-free survival rate in patients with esophageal cancer.[22] In contrast, Spierings reported that metformin use in patients receiving neoadjuvant chemo(radio) therapy for potentially curable esophageal cancer did not show better outcomes of pathological response rates or an improved overall or disease-free survival rate.[23] The antitumor effect of metformin on ESCC remains controversial. Few reports have examined the mechanism underlying the antitumor effect of metformin on ESCC.

It has already been reported that metformin affects tumor growth in ESCC through cell cycle arrest.[24,25] Furthermore, one report described the effect of EMT inhibition in ESCC, outlining the inhibitory effects of radiation exposure for EMT through TGF-β signaling.[26] These reports suggest that metformin has effects on growth inhibition as well as EMT inhibition, which is associated with invasion and metastasis, and enhancement of chemotherapy sensitivity. However, few reports have found that metformin affects EMT in ESCC, and the results of such studies are eagerly awaited.

Given the above, we analyzed the antitumor effect of metformin, including anti-inflammation, growth suppression and EMT inhibition, and explored the mechanisms by which such effects are achieved.

2 | MATERIALS AND METHODS

2.1 | ESCC cell culture and reagents

Human ESCC cell lines were cultured in DMEM/Nutrient Mixture F-12 Ham (Sigma-Aldrich, St Louis, MO, USA) with 10% FBS in a humidified incubator at 37°C in a 5% CO_2. We used the human ESCC cell lines TE1, TE2, TE4, TE5, TE6, TE8, TE10, TE11, TE14, TE15 and T.Tn. We also used TE2-fluorescent ubiquitination-based cell cycle indicator (FUCCI) cells. T.Tn cells were provided by the Japanese Cancer Research Resources Bank. TE1, TE2, TE4, TE5, TE6, TE8, TE10, TE11, TE14 and TE15 cells were kindly provided by Dr T. Nishihira (Tohoku University, Sendai, Japan).

Metformin was obtained from Sigma Chemical Co. (St Louis, MO, USA). Penicillin and streptomycin were obtained from North China Pharmaceutical Group Corp (Shijiazhuang, China). We obtained 0.05% trypsin-EDTA and FBS from Invitrogen Life Technologies (Carlsbad, CA, USA).

Antibodies against AMPK and LKB1 were purchased from Cell Signaling Technology (Danvers, MA, USA), that against E-cadherin was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), that against NF-κB p65 and β-actin were purchased from Abcam (Cambridge, UK), and a rabbit-secondary antibody was purchased from Invitrogen.

2.2 | Cell viability assay

The inhibitory influence of cell proliferation by giving metformin was evaluated by cell viability using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cells were seeded in 96-well plates at 5×10^3 cells per well and cultured for 24 hours. Then the cells were exposed to various concentrations of metformin for 72 hours, and the antiproliferative effects were determined using a CCK-8. Absorbance was determined at 450 nm using a microplate reader.

2.3 | Tumor xenograft model

Six-week-old Balb/c nude mice were used to establish a TE2-FUCCI and TE14 tumor model. Nude mice were housed in a specific-pathogen-free facility. TE2-FUCCI and TE14 cells were harvested and injected s.c. into the dorsal area at 5×10^6 cells. The mice were randomized into a control group and a treatment group (TE2-FUCCI: 7 mice per group, and TE14: 6 mice per group). After 14 days, tumors became palpable, and mice in the treatment group were given metformin (5 mg/mice) by ip, injection, whereas those in the control group were given PBS (100 μL) 5 times a week. Treatments

were carried out for 28 days, and tumor volumes (volume = 0.5 × width^2 × length) were determined regularly.

2.4 | Apoptosis in vivo

Tumor tissues were embedded in paraffin and then sectioned at 4 μm and deparaffinized. Apoptosis was detected based on an analysis of DNA fragmentation using a TUNEL with an In situ Apoptosis Detection kit (Takara, Tokyo, Japan). The apoptotic index was defined as the number of positive cells among TE2-FUCCI tumor xenograft models in arbitrary microscopic fields (6 mice per group).

2.5 | Western blotting

Antihuman LKB1 mouse monoclonal IgG and antihuman AMPK mouse monoclonal IgG and antihuman E-cadherin rabbit polyclonal IgG were used as primary antibodies. Cells were harvested with the indicated concentrations of metformin for 24 hours and lysed with radioimmunoprecipitation assay (RIPA) buffer. Western blot analysis was done on an XV PANTERA Gel 7.5%-15% (DRC, Tokyo, Japan). Proteins in the gels were transferred electrophoretically onto nitrocellulose membranes. The membranes were incubated with primary antibodies against NF-κB p65, LKB1, AMPK, E-cadherin and β-actin. Expression of each protein was detected using secondary antibodies conjugated to peroxidase-labeled polymers. Bands were visualized using the Amersham ECL Prime Western blotting reagent (GE Healthcare Life Sciences, Uppsala, Sweden). Densitometry was done using the ImageJ software program (NIH, Bethesda, MD, USA).

2.6 | Wound healing assay

T.Tn and TE2-FUCCI cells were scratched after metformin treatment for 24 hours, harvested with the same concentration of metformin, and photographed digitally at 0 and 22 hours from scratch. The intercellular space was measured at random, and the relative value was calculated on the basis of the gap at the time when the cells were scratched.

2.7 | Cell migration and invasion assays

TE2, TE14 and T.Tn cells were used in cell migration and invasion assays. The cell migration assay was carried out using a micropore membrane filter with 8-μm pores as an insert (BD Biosciences, Franklin Lakes, NJ, USA). A total of 5 × 10^4 cells with DMEM without FBS were seeded into the 24-well insert, and DMEM containing 10% FBS was added to the lower chamber as the attractant. After 24 hours, the cells on the upper side of the membrane were removed with cotton swabs, and the membranes were fixed and stained using the Diff-Quik reagent (International Reagents, Kobe, Japan).

The cell invasion assay was done using a collagen type I (Corning, Corning, NY, USA)-coated micropore membrane filter with 8-μm pores (BD Biosciences). The collagen I-coated chambers were prepared as described previously.[27] Procedures of cell seeding and attractant addition were the same as in the cell migration assay, and the membrane was fixed and trained after 48 hours.

Cell migration/invasion was quantified by counting the average number of migrated/invaded cells in random high-powered fields per filter.

2.8 | Fluorescence microscopy

TE2 cells cultured on coverslips were fixed in 4% paraformaldehyde for 10 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes. After blocking with 3% FBS/PBS, cells were incubated with antihuman NF-κB p65 rabbit antibody at 37°C for 60 minutes. The cells were then incubated with Alexa Fluor 488 gout antirabbit IgG (Invitrogen) as the secondary antibody. ProLong Gold antifade reagent with DAPI (Invitrogen) was used for counterstaining of DNA.

2.9 | Statistical analyses

Data were analyzed by *t* tests conducted using the Excel software program (Microsoft, Redmond, WA, USA). $P < .05$ was considered to indicate significance.

3 | RESULTS

3.1 | NF-κB activity

Effect of metformin on the expression and intracellular localization of NF-κB on TE2 cells was examined. Total protein expression of NF-κB was decreased by giving metformin at ≥0.3 mmol/L (Figure 1A). Intracellular localization of NF-κB was evaluated by immunostaining (Figure 1B). NF-κB existed in both the cytoplasm and the nucleus in the control group, but NF-κB expression in the nucleus was lower in the group treated with 0.3 mmol/L metformin for 24 hours (Figure 1C). These results suggest that metformin affects the nuclear import of NF-κB, and inhibits activating NF-κB signaling.

3.2 | Growth inhibition

3.2.1 | Proliferation assay and AMPK and LKB1 protein expression in ESCC cell lines

Metformin inhibited cell proliferation in all ESCC cell lines (TE1, TE2, TE4, TE5, TE6, TE8, TE10, TE11, TE14, TE15 and T.Tn) in a dose-dependent way. However, there was a marked difference in the sensitivity among these cell lines (Figure 2A). AMP-activated kinase (AMPK) and LKB1 protein expression as evaluated by western blotting in the steady state varied among cell lines. However, there was no significant correlation between their expression and metformin sensitivity (Figure 2B).

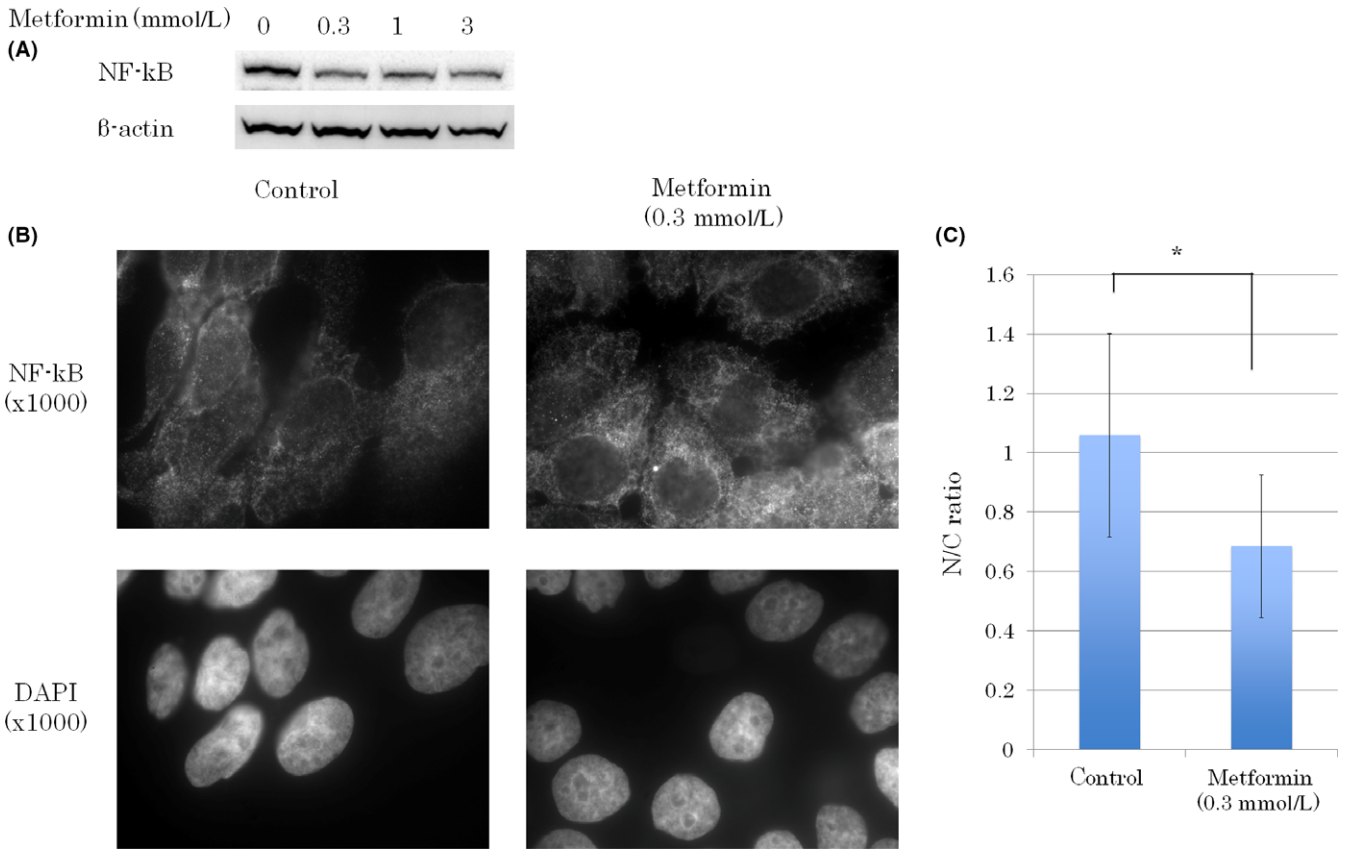


FIGURE 1 Metformin affects the intracellular localization of nuclear factor kappa B (NF-κB). A, Western blotting analysis of the NF-κB protein expression on TE2 cells treated with various concentrations of metformin (0, 0.3, 1, 3 mmol/L) for 24 h. Expression level of β-actin was used as an endogenous control. B, Local existence of NF-κB in cells was evaluated by immunostaining. In the metformin group, 0.3 mmol/L metformin was given for 24 h. Images of immunohistochemistry staining with NF-κB (upper) and the nucleus stained by DAPI (lower) are shown. (upper left, lower left) Control cells. (upper right, lower right) Metformin-treated cells. Magnification, ×1000. C, Brightness in the cytoplasm and nucleus of each cell was quantified using the Image J program, and the nuclear/cytoplasmic (N/C) ratio was calculated (n = 10). NF-κB expression in the nucleus was significantly lower in the metformin-treated group than in the control group (*P = .012)

3.2.2 | Tumor growth and apoptosis in vivo

Relative tumor volume was significantly smaller in the metformin-treated group than in the control group in both TE2-FUCCI (*P = .033) (Figure 3A) and TE14 cell lines (**P = .031) (Figure 3B). There was no marked difference in bodyweight change of the mice in the control group and in the metformin-treated group for both TE2-FUCCI (Figure 3C) and TE14 (Figure 3D). In the TE2-FUCCI xenograft model, apoptotic cells were detected by TUNEL stain (brown nuclear cells: positive cells; green nuclear cells: negative), and there were significantly more apoptotic cells in the tumors in the metformin-treated group than in the control group (*P = .0257) (Figure 3E,F).

3.3 | EMT inhibition

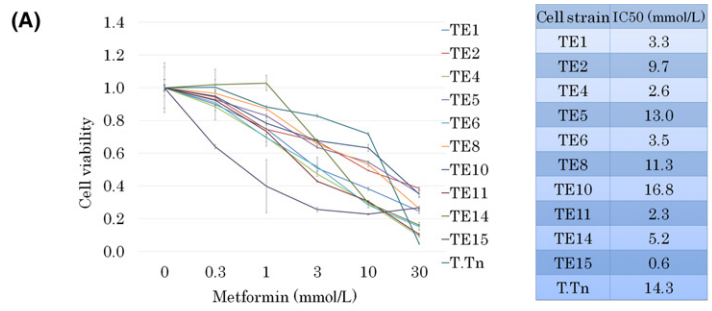
Epithelial-mesenchymal transition (EMT) is associated with malignancy and increased cell motility, based on migration and invasion ability. The wound healing assay and cell migration/invasion assay were useful for evaluating cell motility.

3.3.1 | Wound healing assay

Cell migration in the high-concentration metformin-treated group (5 or 10 mmol/L) was significantly lower than in the control group, but the migration in the low-concentration metformin-treated group (1 or 3 mmol/L) did not show a significant difference from the control group (Figure 4A,B). These results suggest that metformin inhibited TE2-FUCCI and T.Tn cell motility in a dose-dependent way. Furthermore, a larger proportion of cells were in G0/G1 in the high-concentration metformin-treated group than in the control group.

3.3.2 | Cell migration/invasion assay

In the migration assay, the number of cells penetrating the membrane of TE2 cells was significantly and dose-dependently decreased by metformin (Figure 5A; *P = .01 for 0 vs 3 mmol/L and **P = .01 for 0 vs 5 mmol/L). In contrast, the number of cells penetrating the membrane was slightly less but not significantly so for T.Tn cells (Figure 5B; *P = .88 for 0 vs 3 mmol/L and **P = .16 for 0 vs 5 mmol/L).



Cell strain	IC50 (mmol/L)
TE1	3.3
TE2	9.7
TE4	2.6
TE5	13.0
TE6	3.5
TE8	11.3
TE10	16.8
TE11	2.3
TE14	5.2
TE15	0.6
T.Tn	14.3

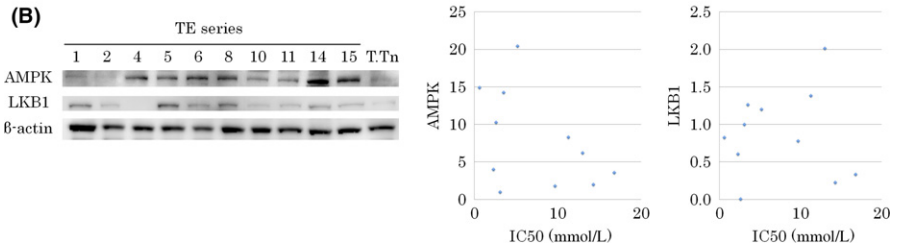


FIGURE 2 Proliferation assay and AMP-activated kinase (AMPK) and liver kinase B1 (LKB1) protein expression in esophageal squamous cell carcinoma (ESCC) cell lines. A, Proliferation assay using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Metformin was given to ESCC cells for 72 h. B, Western blotting analysis of AMPK and LKB1 protein expression at steady state in these ESCC cell lines

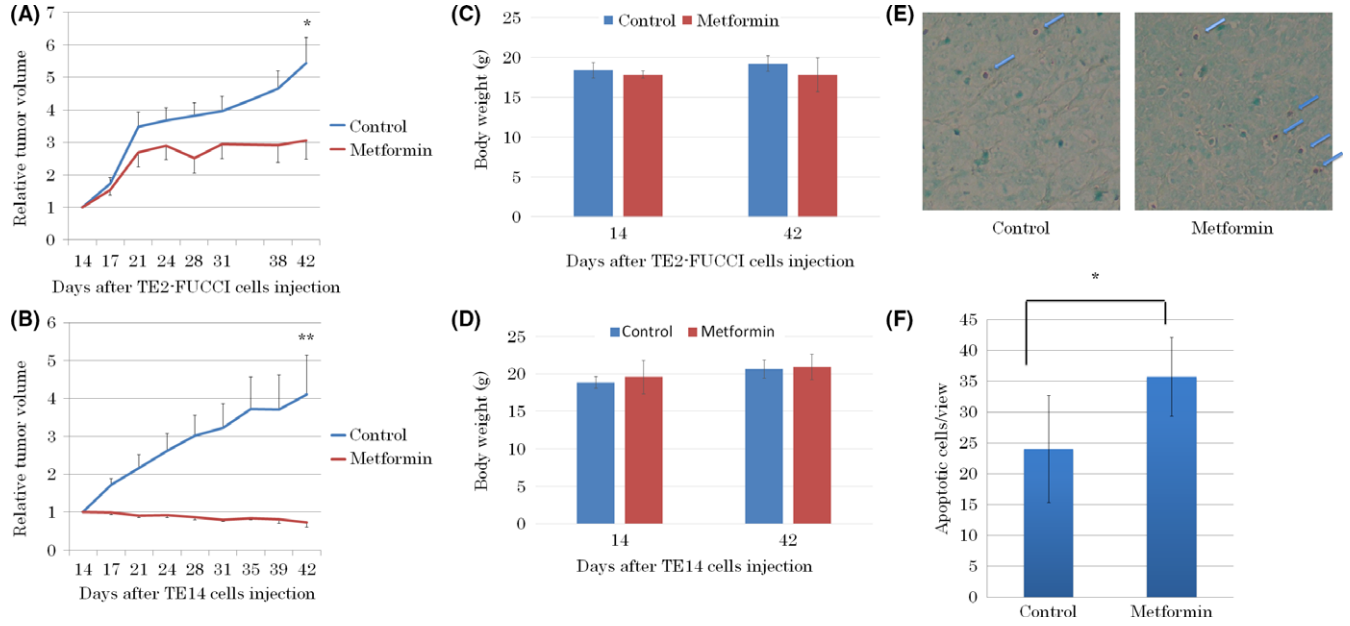


FIGURE 3 Tumor growth and apoptosis in vivo. Relative tumor volume in the xenograft model of (A) TE2-FUCCI (n = 7) and (B) TE14 (n = 6). In both the TE2-FUCCI and TE14 models, relative tumor volume was significantly smaller in the metformin-treated group than in the control group (TE2-FUCCI: *P = .033, TE14: **P = .031). Bar, SE. Bodyweight of the xenograft model at days 14 and 42 after the injection of (C) TE2-FUCCI (n = 7) and (D) TE14 (n = 6) is shown. There were no significant differences between the control group and the metformin-treated group. Bar, SD. E, In the TE2-FUCCI xenograft model, the apoptotic cells were detected by TUNEL stain (brown nuclear cell: positive; green nuclear cell: negative). F, Average number of apoptotic cells per view in the TE2-FUCCI xenograft model (n = 6). There were significantly more apoptotic cells in the tumors in the metformin-treated group than in the control group (*P = .0257). Bar, SD

In the invasion assay, the number of cells penetrating the TE2 cell membrane coated with collagen-I was also decreased by metformin in a dose-dependent way (Figure 5C; *P = .23 for 0 vs 3 mmol/L and **P = .04 for 0 vs 5 mmol/L). In contrast, the number of cells penetrating the membrane was slightly less but not significantly so for T.Tn cells (Figure 5D; *P = .18 for 0 vs 3 mmol/L and **P = .13 for 0 vs 5 mmol/L).

Giving metformin significantly decreased the migration/invasion ability of TE2 cells, but this tendency was not clearly observed in T.Tn cells.

3.3.3 | E-cadherin expression

E-cadherin is expressed by epithelial cells and so is an important marker of EMT. We first evaluated the effect of metformin on the induction of E-cadherin expression by western blotting. In TE2 cells, metformin induced E-cadherin protein expression in a dose-dependent method (Figure 6A). T.Tn cells were similarly affected by metformin, but in TE14 cells, metformin did not increase the expression of E-cadherin (Figure 6B). Finally we examined E-cadherin expression in TE1, TE4, TE5, TE6, TE8 and TE11 cells. In TE1, TE8 and TE11

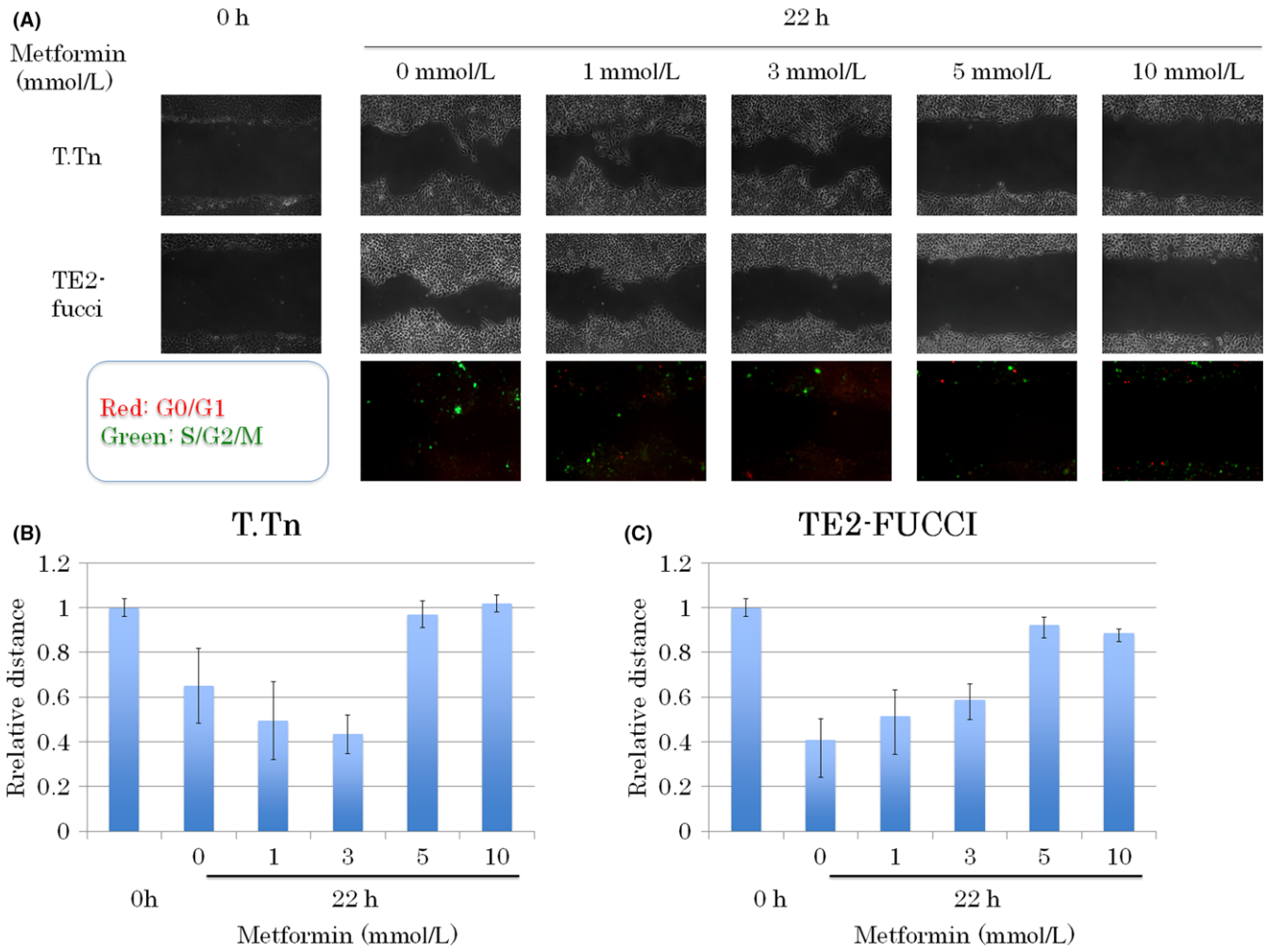


FIGURE 4 Wound healing assay. A, T.Tn and TE2-FUCCI cells were treated with metformin and captured after 22 h from scratch. Magnification, ×200. Relative distance of the intracellular space is shown (n = 7). Bar, SD. Cell strains: (B) T.Tn, (C) TE2-FUCCI

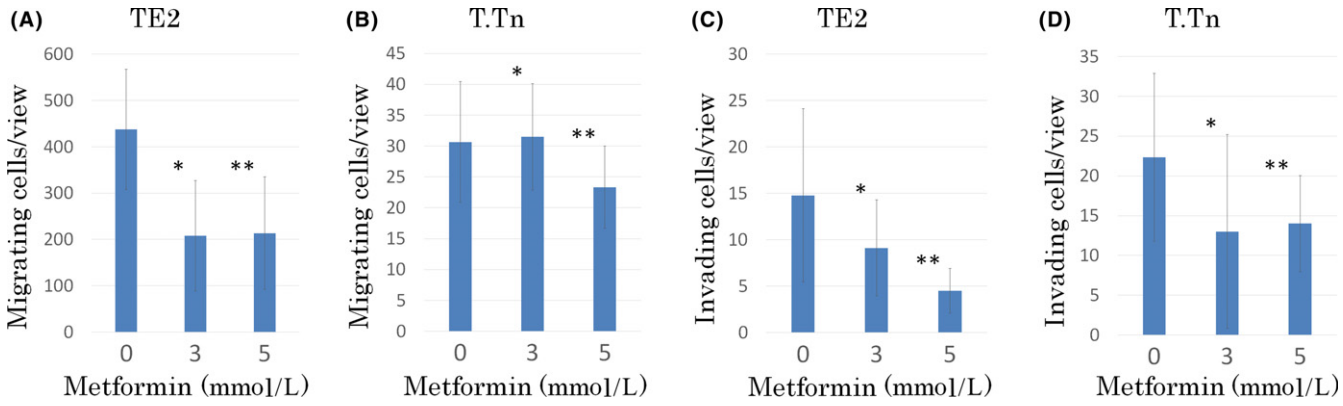


FIGURE 5 Cell migration/invasion assay. A cell migration assay was carried out. The migrating cells were fixed and stained at 24 h after seeding. A, TE2 cells, **P* = .01 (0 vs 3 mmol/L), and ***P* = .01 (0 vs 5 mmol/L). B, T.Tn cells, **P* = .88 (0 vs 3 mmol/L), and ***P* = 0.16 (0 vs 5 mmol/L). A cell invasion assay was carried out. The invading cells were fixed and stained at 48 h after seeding. C, TE2 cells, **P* = .23 (0 vs 3 mmol/L), and ***P* = .04 (0 vs 5 mmol/L). D, T.Tn cells, **P* = .18 (0 vs 3 mmol/L), and ***P* = .13 (0 vs 5 mmol/L)

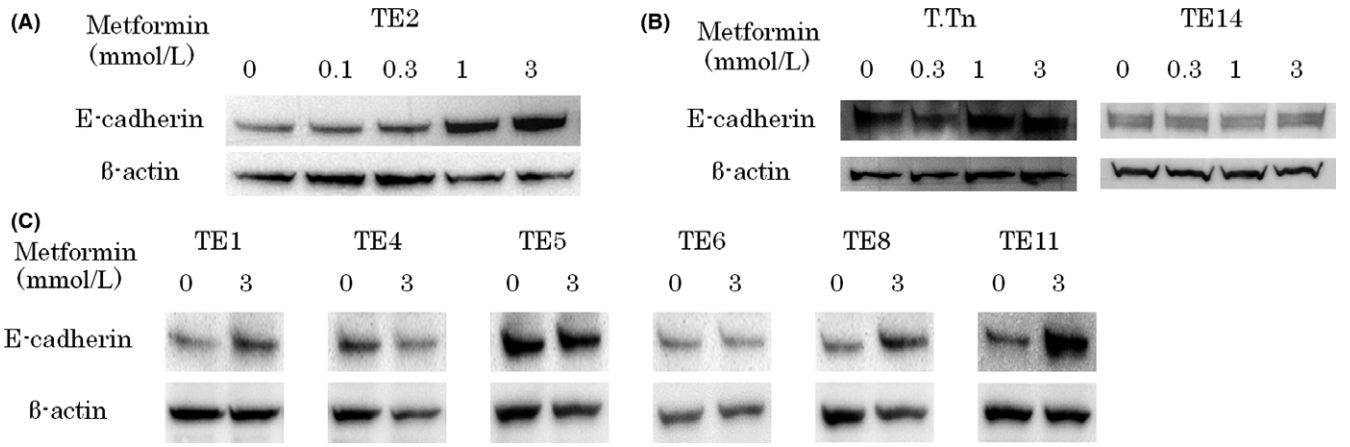


FIGURE 6 Western blotting analysis of E-cadherin protein expression in esophageal squamous cell carcinoma (ESCC) cell lines. A, In TE2 cells, expression of E-cadherin at each metformin dosage (0, 0.1, 0.3, 1, 3 mmol/L) for 24 h was examined, and the expression level of β-actin was used as an endogenous control. B, In T.Tn and TE14 cells, expression of E-cadherin at each metformin dosage (0, 0.3, 1, 3 mmol/L) was evaluated. C, In another several ESCC cell strains, E-cadherin expression at the control and metformin dosage (3 mmol/L) is shown

cells, E-cadherin expression was increased, but the other ESCC cell lines did not show a similar tendency (Figure 6C).

4 | DISCUSSION

In the present study, we found that metformin affected tumor malignancy through action on tumor growth and EMT, and these effects may have been induced by inhibiting NF-κB activity. Cell cycle arrest and TGF-β signaling have already been reported as antitumor mechanisms of metformin, but NF-κB also participates in the antitumor effects of metformin.

It has been reported that metformin inhibits cell proliferation on ESCC cell lines, but our evaluation of the effects on proliferation of various ESCC cell lines showed that metformin sensitivity varied among cell lines. Surprisingly, there was no significant correlation between metformin sensitivity and expression of AMPK or LKB1. Other determinatives and various molecular functions may influence metformin sensitivity of cancer cells. Although the main target of metformin is thought to be AMPK, it reportedly also acts on other pathways, such as the MAPK pathway and NF-κB.[28] These pathways may also be activated by metformin apart from AMPK activation. Whether or not the antitumor effect of metformin differs depending on the cancer type remains unclear, but the answer may involve the pathway that is more likely to be activated.

It has been reported that tumor growth is inhibited and apoptosis is induced by metformin in vivo and, in our study, a similar tendency was shown. Furthermore, TE14 cells were more sensitive to metformin than TE2 cells in vitro, and the tumor size of TE14 cells was smaller than that of TE2-FUCCI cells. Metformin sensitivity may therefore be correlated in vitro and in vivo.

Based on these in vitro and in vivo results, metformin appears to affect tumor size by inhibiting cell proliferation and inducing apoptosis.

Expression of E-cadherin, the most important marker of epithelial cells, was induced by metformin in various ESCC lines, but sensitivity to metformin differed among those cell lines. Expression of LKB1 or AMPK did not show clear correlations with metformin sensitivity. As mentioned earlier, this result also suggests that other factors may exist that influence the sensitivity to metformin.

Various reports have examined the mechanisms by which metformin affects EMT, such as through TGF-β or mammalian target of rapamycin (mTOR).[20,26] It has also been reported that NF-κB induced EMT and reduced E-cadherin expression.[29] NF-κB is an important nuclear factor that is associated with various physiological phenomena, including acute or chronic inflammation,[30] cell proliferation, suppressing apoptosis,[31] and the immune response.[32] NF-κB is known to be constantly active in many malignant tumors and is a central signaling hub in inflammation-mediated tumor promotion and metastasis.[33] In ESCC, clinical correlation of NF-κB expression and prognosis has been examined, and ESCC patients with NF-κB-positive tumors have shown poor survival.[34]

Because metformin exerts multiple effects, including inhibiting tumor growth and EMT and inducing apoptosis, correlation of metformin and NF-κB, which also has various physiological functions, has received attention. There are some reports that metformin inhibits NF-κB in several cancers, such as pancreatic tumor,[15] bladder cancer,[35] and cholangiocarcinoma.[36] This is the first report to find that metformin inhibits NF-κB in ESCC. Carcinogenesis occurs in multiple phases as tumor initiation, promotion and progression. NF-κB induces inflammation, which is a very important aspect of tumor promotion. Therefore, metformin may inhibit tumor promotion by inactivating NF-κB.

There are many signals that are associated with EMT.[37] Transforming growth factor-beta (TGF-β), which strongly promotes EMT, was reported to be inhibited by metformin in ESCC cell lines. However, in our study, mRNA expression of TGF-β showed no significant

change on treatment with metformin (data not shown), suggesting that some other EMT-inducing signal is likely inhibited by metformin. Change in intracellular localization of NF-κB by metformin may show that metformin exerts its antitumor effect by inhibiting EMT through NF-κB.

As is often pointed out, the concentration at which metformin exerts its effects differs between in vivo and in vitro. We reported that 5 mg/mouse (250 mg/kg) of metformin was given to mice, giving a blood concentration of metformin of 5 μmol/L.[38] The blood concentration of metformin in patients taking metformin is reported to be 10 μmol/L.[39] The metformin concentration that showed an in vitro effect was much higher than that showing an effect in vivo. Therefore, we must confirm whether or not the results obtained in vitro reflect the effects in vivo. Differences in the metformin concentration can result in a high glucose concentration in the medium. It has been reported that the concentration of glucose in solid tumor is ≤0.5 mmol/L,[40] and cells cultured at low glucose concentrations have greater metformin sensitivity for cell viability than those cultured at high glucose concentrations.[41] As a result, metformin may act specifically on solid tumor cells instead of on normal cells, and a high glucose concentration may mask the antitumor effects of metformin to some extent in vitro.

Although the concentration of metformin given was higher than that delivered in vivo, the antitumor effects that metformin showed in vitro in this report seem sufficient to result in the same outcome in vivo, as these effects were recognized even under the influence of high glucose concentrations. In this report, medium with a normal glucose concentration was used, but medium with a low glucose concentration may be more suitable for examining the antitumor effect in vivo.

In our study, metformin showed antitumor effects by inhibiting cell proliferation, tumor growth and EMT and inducing apoptosis in ESCC cell lines and xenograft models. These effects may have been induced by inhibiting NF-κB activation on ESCC. Therefore, metformin may be useful for treating ESCC, and further studies are expected.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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