The inhibitory effect of anthocyanins on Akt on invasion and epithelial-mesenchymal transition is not associated with the anti-EGFR effect of the anthocyanins

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Abstract. Evidence suggests that anthocyanins inhibit EGFR and Akt activity. However, it is still unknown whether the inhibitory effect of anthocyanins on Akt is associated with the anti-EGFR effect. The effect of anthocyanins on epithelial-mesenchymal transition (EMT) has not been extensively studied. Therefore, we investigated the effects of anthocyanins from fruits of *Vitis coignetiae* Pulliat (AIMs) on EGF-induced EMT and the underlying molecular mechanisms. AIMs suppressed the invasion of A549 cells in a dose-dependent manner. AIMs inhibited the phosphorylation of Akt and EGFR, but the inhibitory effect on Akt was not derived from EGFR. EGF re-induced Akt phosphorylation at Thr308 in the AIM-treated cells, but not Akt phosphorylation at Ser473. AIMs also inhibited EMT of cancer cells. AIMs inhibited glycogen synthase kinase-3β phosphorylation and β-catenin expression that are invovled in EMT. We confirmed these findings with transforming growth factor (TGF)-β. In conclusion, these data suggest that the inhibitory effect of AIMs on Akt activity is independent of EGFR, and that AIMs suppressed invasion and migration at least in part by suppressing EMT by inhibiting Akt activity as well as EGFR. This study provides evidence that AIMs may have anticancer effects on human cancer cells.

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Introduction

Cancer still ranks first as the leading cause of death. After development of cancer, the cancer cells start to invade the vascular and lymphatic channels, and then spread to farther regional nodes and distant organs. Therefore, much attention has been paid to the control of cancer invasion and metastasis. In the progression of cancer invasion and metastasis, growth factors and cytokines play an important role. Especially, epidermal growth factor (EGF) is reported to promote tumor cell motility and invasion and to be implicated in tumor progression (1-3), and the overexpression of EGF receptor (EGFR) has shown the poor clinical outcome (4, 5). In the process of invasion, matrix matalloproteinases (MMPs) play a critical role in degradation of extracellular matrix (ECM). In addition to this degradation, epithelial cancer cells need to change to metastasize to other organs. Epithelial-mesenchymal transition (EMT) is one of the hypothetical processes for epithelial cancer cells to metastasize; it causes cancer cells to lose epithelial characteristics and acquire invasive properties and stem cell-like features (6).

Vitis coignetiae Pulliat (Meoru in Korea) has been used as a Korean folk medicine for the treatment of inflammatory disorders and cancer. Its fruit has an intense dark red hue, reflecting an abundance of anthocyanins. The anthocyanins reportedly have inhibitory effect on tyrosine kinase of EGFR (7). We reported that anthocyanins isolated from Meoru (AIMs) have anticancer property by inhibiting Akt activity (8). However, it is still unknown whether the inhibitory effects of AIMs on Akt was derived from the anti-EGFR effects of

AIMs. Moreover, few studies have been conducted regarding the effects of anthocyanins on EMT. Therefore, we investigated the effects of AIMs on cellular responses and molecular changes in EGF-treated human lung cancer cells in terms of cancer invasion and EMT.

Materials and methods

Cell culture and chemicals. A549 human lung cancer cells from the American Type Culture collection (Rockville, MD, USA) were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY, USA), 1 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 incubator. Molecular mass markers for proteins were obtained from Pharmacia Biotech (Saclay, France). Antibodies against phospho-Akt serine (Ser)473, Akt 1/2/3 (H-136), COX-2 (29), cyclin D1 (M-20), c-Myc, extracellular-regulated kinase (ERK), phospho-ERK (E-4), E-cadherin (6F9), MMP-2, MMP-9, phospho-p70S6 kinase α (Thr389), β-catenin (H-102), XIAP, Bcl-2, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho -IKB-α (Ser32/36), vimentin (D21H3), Snail (C15D3), N-cadherin, phosphorylated (p)-GSK-3β (Ser9), phospho-Akt (Thr308) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against EGFR, and phospho-EGFR were purchased from Upstate Biotech (Waltham, MA, USA). Antibody against β-actin was from Sigma (Beverly, MA, USA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin, and an enhanced chemiluminescence (ECL) kit were purchased from Amersham (Arlington Heights, IL, USA). All other chemicals not specifically cited here were purchased from Sigma Chemical (St. Louis, MO, USA).

Preparation of anthocyanins. Fruits of Meoru were collected in the middle of September 2008 at Jiri Mountain, Korea and freeze-dried and stored in dark glass containers at -20˚C until required for analysis. Anthocyanin pigments were isolated as previously described (9). The composition of anthocyanins isolated from Meoru (AIMs) was as follows: delphinidin-3,5-diglucoside: cyanidin-3,5-diglucoside:petunidin-3,5-diglucoside:delphinidin-3-glucoside:malvdin-3,5-diglucoside:peonidin-3,5-diglucoside: cyanidin-3-glucoside:petunidin-3-glucoside:peonidin-3-glucoside: malvidin-3-glucoside = 3.5:3.4:7.1:23.9:8.0:9.6:9.1:16.1:5.7:13.4.

Cell proliferation assays. For the cell viability assay, A549 cells were seeded onto 24-well plates at a concentration of $5x10^4$ cells/ml and treated with AIMs for 24 h and the number of surviving cells was counted using trypan blue exclusion methods.

Cell invasion assay. For the cell invasion assays, A549 cells were cultured in serum-free media overnight. Cells ($5x10^4$) were loaded onto pre-coated Matrigel 24-well invasion chambers (BD Biosciences, San Jose, CA, USA) with and without AIMs. Then 0.5 ml of medium containing 20% FBS was added to the wells of the plate to serve as a chemo-attractant and incubated for 24 h at 37˚C in 5% CO_2. After removing non-migrated or non-invaded cells, cells on the bottom filter surface were fixed with 10% formalin, stained with DAPI and counted.

Gelatin zymography. The gelatinolytic activities for MMP-2 and MMP-9 in the culture medium were assayed by electrophoresis on 10% polyacrylamide gels containing 1 mg/ml gelatin at 4˚C. Polyacrylamide gels were run at 120 V, washed in 2.5% Triton X-100 for 1 h, and then incubated for 16 h at 37˚C in activation buffer (50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$). After staining with Coomassie Blue (10% glacial acetic acid, 30% methanol and 1.5% Coomassie brilliant Blue) for 2-3 h, the gel was washed with a solution of 10% glacial acetic acid and 30% methanol without Coomassie Blue for 1 h. White lysis zones indicating gelatin degradation were revealed by staining with Coomassie brilliant Blue.

Wound healing assay. A549 cells were grown on 30 mm dish plate to 100% confluent monolayer and then scratched to form a 100-μm 'wound' using sterile pipette tips. The cells were then cultured in the presence or absence of AIMs (400 μg/ml) in serum-free media for 24 h. The images were recorded at 12 and 24 h after scratch using an Olympus photomicroscope.

Western blot analysis. The concentrations of cell lysate proteins were determined by means of the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA, USA) with bovine serum albumin as a standard. For the western blot analysis, 30 μg of proteins were resolved by electrophoresis, eletrotransferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), and then incubated with primary antibodies followed by secondary antibody conjugated to peroxidase. Blots were developed with an ECL detection system.

Statistical analysis. Each experiment was performed in triplicate. The results were expressed as means ± SD. Significant differences were determined using the one-way analysis of variance (ANOVA) with Neuman-Keuls post hoc test in the cases at least three treatment groups and Student's t-test for two group comparison. Statistical significance was defined as $P<0.05$.

Results

AIMs suppressed the proliferation and invasion of A549 cells in a dose-dependent manner. At first, the growth of A549 cancer cells was assessed by trypan blue exclusion method. It revealed that the growth of A549 cells start to decline at the concentration of 200 μg/ml, and the inhibitory effects finally reached a statistical significant level at the concentration of 400 μg/ml (Fig. 1A). Next, we tested the effects of AIMs on cell invasion, because cancer cell invasion is the first step in cancer metastasis. AIMs significantly inhibited A549 cell invasion in a dose-dependent manner as measured by Matrigel invasion assays, compared to the effects of AIMs on cell growth (Fig. 1B). To verify the molecular mechanisms, we measured the secreted MMP-2 and MMP-9 by gelatin zymographic analyses using culture media because secreted MMPs are key molecules in degradation of the extracellular matrix (ECM) (10,11). As indicated in Fig. 1C, AIMs have markedly

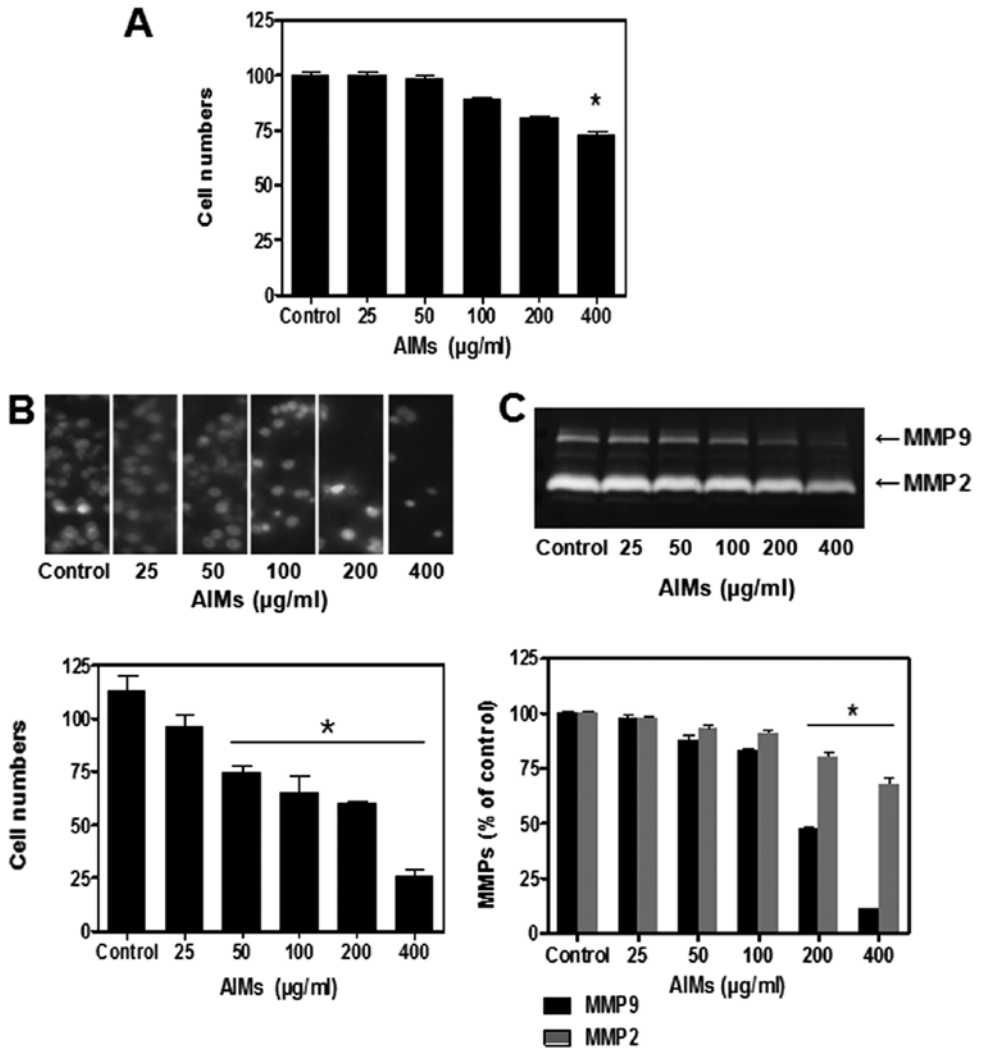

Figure 1. A dose-dependent inhibitory effect of AIMs on invasion of A549 human lung cancer cells. (A) Growth viability assay for AIMs in A549 cells. Cells were seeded at $5x10^4$ cells/ml and treated with AIMs for 24 h with the indicated concentrations (0-400 μg/ml). (B) Effects on invasion of A549 cells. A549 cells were treated with AIMs (0-400 μg/ml) for 18 h in a Matrigel-coated Transwell. (C) Secreted MMP-2 and MMP-9 proteins measured by gelatin zymography. Cells were incubated for 24 h with AIMs (0-400 μg/ml). Values represent means ± SD from four independent experiments. *P<0.05 vs. control.

suppressed the gelatinolytic activities of secreted MMP-9 in a dose-dependent manner, compared to the effects on MMP-2. These finding suggested that AIMs inhibit invasion predominantly by suppressing MMP-9 secretion.

EGF reduced the inhibitory effects of AIMs on cancer invasion other than an increase in MMP-2 and MMP-9 secretion. The human lung adenocarcinoma cell line A549 was frequently used for EGF effects on cancer cells (12). Since EGF-induced signaling is involved in cancer cell invasion (13,14), we also performed Matrigel invasion test to confirm whether EGF-promoted cell invasion. Matrigel invasion test revealed that EGF promoted cell invasion, and that AIMs inhibited the EGF-induced cell invasion (Fig. 2A). Interestingly, the degree of the anti-invasive effects of AIMs on EGF treated cells was not as strong as those on control cells. To investigate the molecular mechanisms of the augmented effects of EGF on cancer invasion as well as the anti-invasive effects of AIMs, we measured the secreted MMP-2 and MMP-9 by the gelatin zymography. Inconsistent with the results of invasion test of AIMs, the effects of AIMs on MMP-2 and MMP-9 activities was of no difference between control and EGF treated group (Fig. 2C). This result suggested that EGF-augmented invasion is not caused by the increased secretion of MMP-2 or MMP-9, but by another mechanism.

AIMs inhibited the phosphorylation of Akt and EGFR, and the inhibitory effect of AIMs on Akt was derived from the anti-EGFR activity of AIMs. It has been suggested that anthocyanins inhibit tyrosine kinase activity of EGFR (7), and that AIMs have anti-cancer effects by inhibiting phosphatidylinositol (PI)-3 kinase (PI3K)/Akt pathway (8). To determine whether the inhibitory effects of AIMs on Akt was derived from the anti-EGFR effects of AIMs as well as to investigate the molecular mechanisms of the anti-invasive effects of AIMs, we assessed the effects of AIMs on phosphorylation of Akt, p70S6K (Thr389), and ERK as well as EGFR in both EGF-treated and EGF-untreated cells. Western blot analysis revealed that AIMs suppressed EGFR phosphorylation and the downstream molecules [Akt, p70S6K (Thr389) and ERK] time-dependently (Fig. 3A). Next,

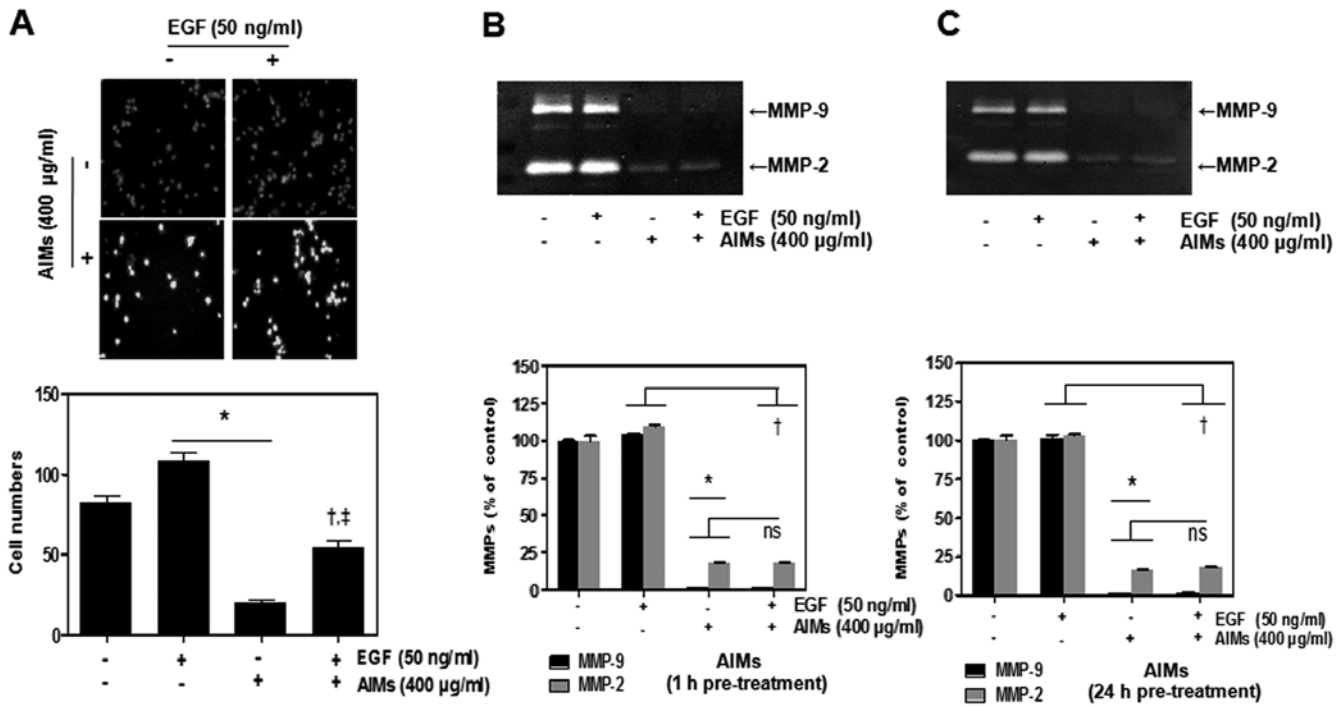

Figure 2. EGF-induced effects on cancer invasion and MMP-2 and MMP-9 secretion in A549 cells treated with or without AIMs. (A) Cells were serum-starved for 24 h with or without AIMs (400 μg/ml). Cells ($5x10^4$ cells) were loaded onto pre-coated Matrigel 24-well invasion chambers in the presence or absence of EGF (50 ng/ml). The Matrigel invasion chambers were incubated for 24 h. *P<0.05 vs. control, †P<0.05 vs. AIMs. (B and C) MMP-2 and MMP-9 protein levels were measured by gelatin zymography. Cells were incubated for 24 h without or with AIMs (1- and 24-h pre-treatment). The gelatinolytic activity was measured by densitometry and represent means ± SD from three independent experiments. *P<0.05 vs. control.

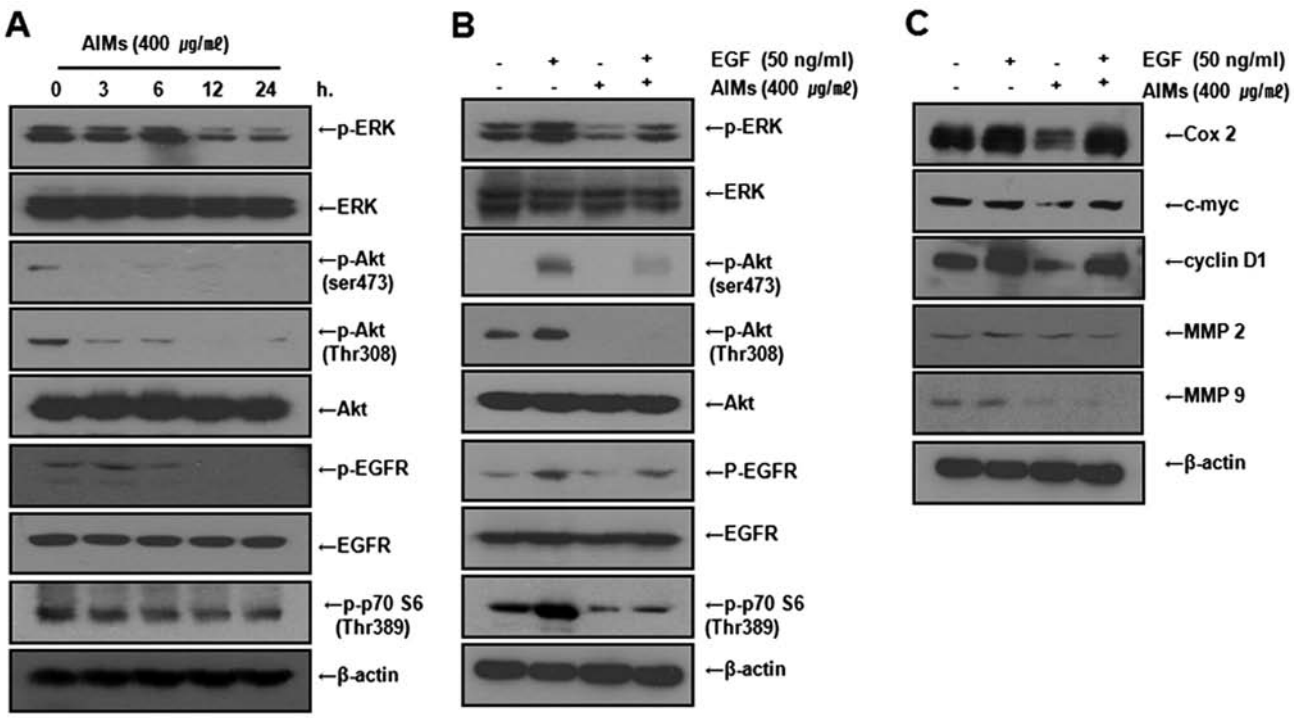

Figure 3. The inhibitory effects of AIMs on the phosphorylation of Akt and ERK as well as EGFR. Western blot analysis for phospho-Akt (Thr308, and Ser473), Akt, phospho-ERK, ERK, phospho-EGFR, and EGFR in A549 cells. (A) Cells ($5x10^4$ cells) were pretreated with AIMs (400 μg/ml) for the indicated times and lysed. Equal amounts of the cell lysate were separated by SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies and detected by an ECL detection system. (B) Cells ($5x10^4$ cells) were pretreated with AIMs (400 μg/ml) for 1 h and then treated with EGF (50 ng/ml) for 24 h. (C) Western blot analysis for COX-2, c-Myc, cyclin D1, MMP-2 and MMP-9 in A549 cells. Cells ($5x10^4$ cells), either left untreated or pretreated with AIMs (400 μg/ml) for 1 h and then were exposed to EGF (50 ng/ml) for 24 h. Whole-cell extracts were prepared, and 30 μg of the whole-cell lysate was analyzed by western blot analysis. The results are representative of two independent experiments.

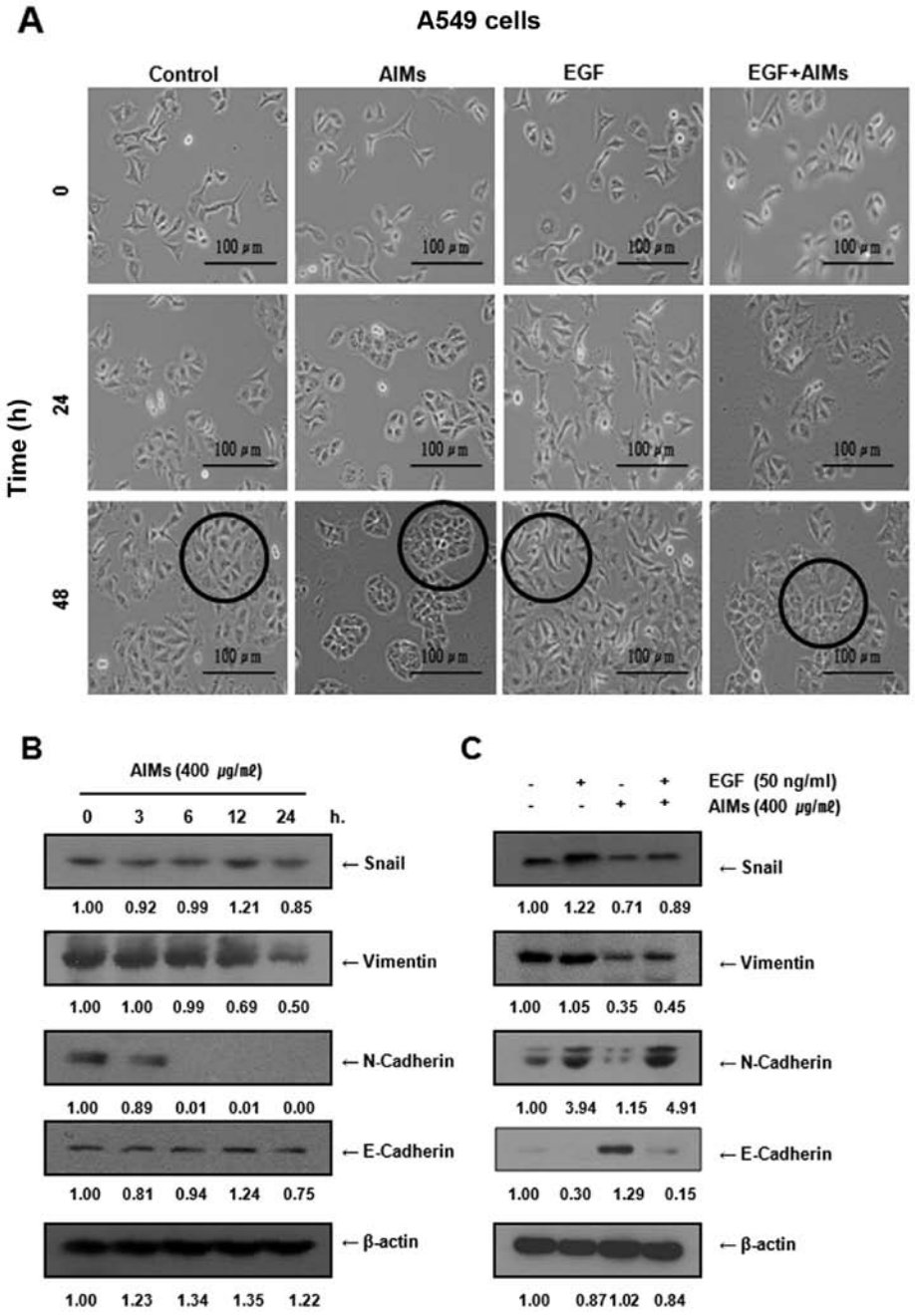

Figure 4. Effects of AIMs on EMT in either EGF-treated or EGF-untreated cells. (A) Cells were pretreated with AIMs (400 μg/ml) for 1 h and then treated with EGF (50 ng/ml) for 24 h. Data are representative of three independent experiments. (B and C) Western blot analysis for Snail, vimentin, N-cadherin and E-cadherin. Cells ($5x10^4$ cells) were pretreated with AIMs (400 μg/ml) for the indicated times and lysed. Equal amounts of the cell lysate were separated by SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies and detected by an ECL detection system. The results are representative of two independent experiments. The expression of the indicated proteins were measured by densitometry and expressed as relative ratio.

we assessed the effects of EGF on these molecules. Western blot analysis revealed that EGF induced phosphorylation of EGFR and the downstream molecules in control cells, and also augmented EGFR and ERK phosphorylation in AIM-treated cells (Fig. 3B). However, EGF did not augment Akt phosphorylation (Thr308) in AIM-treated cells even though it slightly reduced the inhibitory effects of AIMs on Akt (Ser473) and p70S6K which is linked to mTORC1 activation in AIM-treated cells. These findings raised the possibility that the inhibitory effect of AIMs on Akt phosphorylation on Thr308 is not derived from anti-EGFR effect of AIMs. Next, we tested the effects on downstream effector molecules of EGFR involved in cell proliferation and invasion. EGF moderately increased the gene expressions involved in cell proliferation (COX-2, c-Myc and cyclin D1) with an increase in EGFR activity (phosphorylation of EGFR) in either AIM-treated cells or AIM-untreated cells (Fig. 3B and C), but it did not significantly increase the expression of MMP-2 and MMP-9 in AIM-treated cells (Fig. 3C). These finding suggested that AIMs might suppress MMP-2 and MMP-9 expression by inhibiting Akt rather than by inhibiting

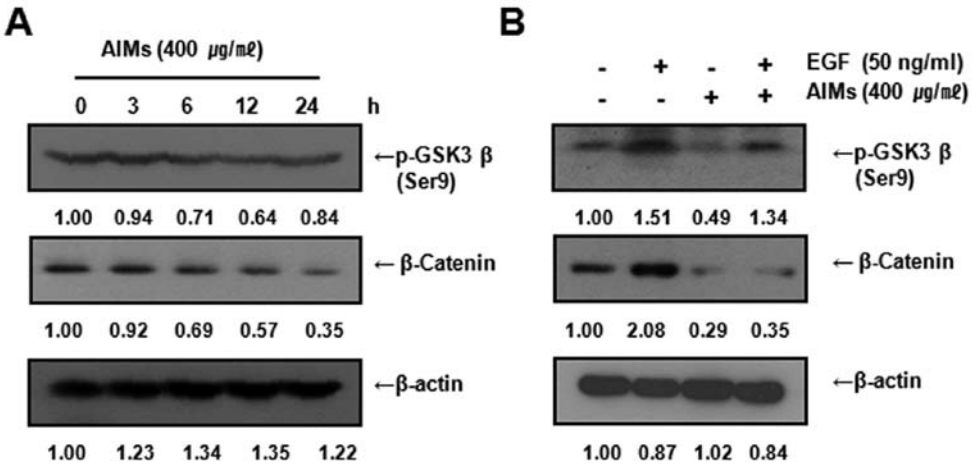

Figure 5. Effects of AIMs on β-catenin and phosphorylation of Ser9 in glycogen synthase kinase-3β (GSK-3β). Western blot analysis for β-catenin and phospho-GSK-3β (Ser9). (A) Cells (5x10^4 cells) were pretreated with AIMs (400 μg/ml) for the indicated times and lysed. Equal amounts of the cell lysate were separated by SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies and detected by an ECL detection system. (B) Cells (5x10^4 cells) were pretreated with AIMs (400 μg/ml) for 1 h and then treated with EGF (5 ng/ml) for 24 h. The results are representative of two independent experiments. The expression of the indicated proteins were measured by densitometry and expressed as relative ratio.

EGFR even though EGF-augmented invasion may abide by EGFR activity through activation of Akt signaling as well as ERK signaling.

AIMs inhibited epithelial-mesenchymal transition (EMT) of A549 cells, but not completely in EGF-treated cells. To determine whether EGF induced EMT of A549 cells, and whether AIMs inhibit EGF-induced EMT, we observed the cell morphology of A549 cells for 24 and 48 h after the treatment of EGF alone or in combination with AIMs. The cell morphology revealed that EGF induced morphological changes of elongation of A549 cells, and that AIMs could not completely prevent the morphological changes (Fig. 4A). To confirm this finding in the molecular level, we next assessed changes in EMT biomarkers after the treatment. Western blot analysis revealed that AIMs suppressed mesenchymal markers such as Snail, vimentin, N-cadherin and induced E-cadherin, an epithelial marker in either EGF-treated or untreated cells (Fig. 4B and C). Consistent with the morphology results, AIMs could not completely suppress the EGF effects on the expression of EMT markers (Fig. 4C) while EGF increased EGFR activity in A549 cells (Fig. 3B). These results indicated that EGF reduced the inhibitory effect of AIMs on EMT with an increase in EGFR activity although AIMs inhibited EMT in either EGF-treated or untreated cells. These findings suggest that EGF-induced EMT may also abide by EGFR activity.

AIMs inhibited EMT at least in part by inhibiting the expression of GSK-3β phosphorylation (Ser9) and β-catenin. β-catenin also plays an important role in EMT in cancer (15,16). In the degradation of β-catenin, when Wnt signaling is not activated, it is degraded by GSK-3-induced phosphorylation, and the enzymatic activity of GSK-3 is regulated by phosphorylation of certain GSK-3 residues. Phosphorylation of GSK-3β on Ser9 is induced by Akt activation, and results in inhibition of GSK-3 (17). Therefore, we assessed the changes in the expression of β-catenin, and p-GSK-3β (Ser9) after AIM treatment. Western blot analysis revealed that AIMs suppressed β-catenin and Ser9 time-dependently (Fig. 5A). Next to determine whether the inhibitory effect of AIMs on β-catenin and GSK-3β are influenced by EGF, we assessed the expression of β-catenin and Ser9 after EGF treatment in either AIM-treated or untreated cells. Western blot analysis revealed that the inhibitory effects of AIMs on Ser9 and β-catenin attenuated by EGF while AIMs clearly inhibited the expression of β-catenin and Ser9 (Fig. 5B). These results suggest that AIMs inhibited EMT, at least in part, by inhibiting the expression of GSK-3β phosphorylation (Ser9) and β-catenin.

AIMs suppress EMT and invasion by suppressing Akt activity as well as EGFR activity, but the inhibitory effects of AIMs on Akt are not derived from EGFR. To give more convincing evidence that AIMs inhibit EMT by inhibiting Akt activity as well as EGFR activity, we assessed the effects of inhibition of EGFR downstream pathways (ERK pathway and PI3K/Akt pathway) on cell migration with the EGF-stimulated cells. Wound healing tests reveled that AIMs partially inhibited the EGF-augmented cell migration just as AIMs inhibited EGF-augmented EMT. The PI3K inhibitor Ly 294002 and the ERK inhibitor PD 98059 both augmented the inhibitory effects of AIMs on migration of A549 cells treated with EGF (Fig. 6A and B), suggesting each of the two downstream pathways of EGFR (ERK pathway and PI3K/Akt pathway) are contributing to EGF-augmented cancer cell migration. Next, using the PI3K inhibitor and the ERK inhibitor, we also assessed the effects of AIMs and/or EGFR on EMT by western blot analysis. It revealed that EGF activated the two downstream pathways in both AIM-treated and AIM-untreated cells, and that the ERK inhibitor PD 98059 or the PI3K/Akt inhibitor LY 294002 inhibited EMT in EGF-treated cells in both AIM-treated and AIM-untreated cells (Fig. 6C). These finding indicated that EGF cannot induce the Akt phosphorylation (Ser473), even though EGF treatment can partially reverse the inhibitory effect of AIMs on EGFR and re-induce Akt phosphorylation

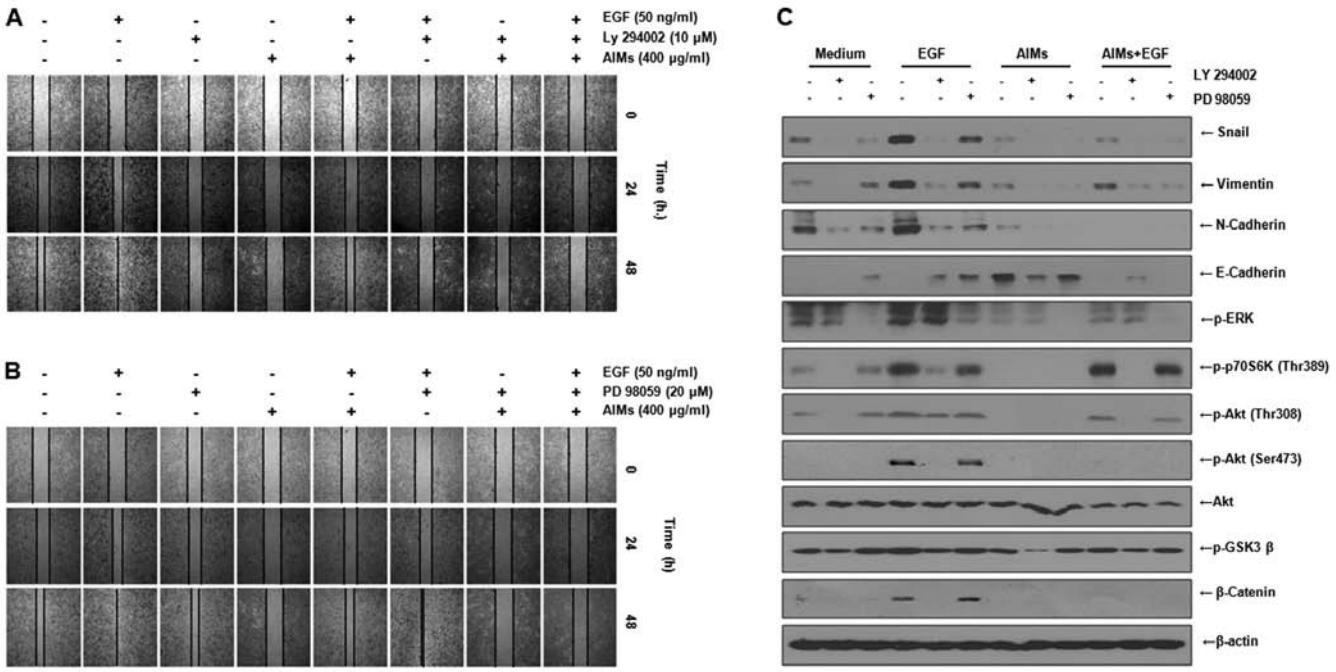

Figure 6. The effects of AIMs on EGFR downstream pathways (ERK pathway and PI3K/Akt pathway) as well as cancer migration and EMT. (A and B) Cells were grown to 100% confluent monolayer on 30-mm cell culture dishes coated with collagen and then serum-starved for 24 h with or without EGF (50 ng/ml) and/or indicated agents for 48 h. A linear scratch was made on the culture dish through the cell layer using a pipette tip. (A) Cells were pretreated with AIMs (400 μg/ml) and/or Ly 294002 (10 μM) for 1 h and then treated with EGF (50 ng/ml) for 48 h. (B) Cells were pretreated with AIMs (400 μg/ml) and/or PD 98059 (20 μM) for 1 h and then treated with EGF (50 ng/ml) for 48 h. (C) Cells were pretreated with AIMs (400 μg/ml) and/or indicated agents for 1 h and then treated with EGF (50 ng/ml) for 24 h. Whole-cell extracts were prepared and 30 μg of the whole-cell lysate was analyzed by western blot analysis with the indicated antibodies. The results are representatives from two independent experiments.

(Thr308) and p-p70S6K (Thr389) in AIM-treated cells. These findings suggested that and AIMs might suppress Akt activity by inhibiting Akt phosphorylation on Ser473 apart from inhibiting EGFR activity.

Taken together, these data also support the finding that cancer migration and EMT in EGF-treated cells abide by EGFR activity through the two EGFR downstream pathways (ERK pathway and PI3K/Akt pathway), and that the inhibitory effect of AIMs on Akt activity may be independent of anti-EGFR activity.

AIMs inhibit transforming growth factor (TGF-β)-induced EMT in A549 cells, indicating the inhibitory effect of AIMs on Akt activity is independent of EGFR activity. To confirm that the inhibitory effects of AIMs on Akt pathway is independent of EGFR, we investigated the effects of AIMs on TGF-β-induced invasion and EMT along with assessing the changes of EMT biomarkers, because TGF-β is a well known cytokine involved in invasion and EMT, as well as in augmenting Akt activity independent of EGFR activity. Firstly, to confirm whether TGF-β also induces EMT of A549 cells and augments cancer cell invasion, we observed morphological changes and assessed the invasive activity of A549 cells after TGF-β treament; TGF-β induced elongation of A549 cells (Fig. 7A) and augemented invasion of the cancer cells (Fig. 7B). AIMs inhibited the morphological changes to EMT and the invasive activity even in TGF-β-treated or TGF-β-untreated cells, but TGF-β also reduced the effects of AIMs on EMT and invasive activity similarly to EGF (Fig. 7A and B). We also measured the expression of the MMPs in AIMs-treated A549 cells by the gelatin zymography analysis. TGF-β augmented the expression of MMP-2 and MMP-9 in both AIM-treated and untreated cells, and the pattern of the inhibition of MMP-2 and MMP-9 by AIMs was similar to those shown by EGF; the inhibitory effect of AIMs on MMP-2 and MMP-9 was not influenced by TGF-β treatment (Fig. 7C). The protein expression of MMP-2 and MMP-9 revealed by western blot analysis showed similar pattern as the gelatinolytic activity (Fig. 7D). Additionally, to confirm TGF-β-driven EMT was not associated with EGFR activity, but rather with Akt activity, we assessed the effects of AIMs on phosphorylation of Akt and ERK as well as EGFR. It revealed that TGF-β induced Akt phosphorylation, but not EGFR phosphorylation, and that AIMs suppressed the Akt phosphorylation (Ser473) even in TGF-β-treated cells (Fig. 7E). TGF-β re-induced Akt phosphorylation (Thr308) and the downstream molecule p70S6K, which is linked to mTORC1 activation in AIM-treated cells. These findings indicated that AIMs have an inhibitory effect on Akt, independent of anti-EGFR effects of AIMs. We also assessed the changes in the molecules involved in EMT. Western blot analysis revealed that AIMs suppressed mesenchymal markers such as Snail, vimentin, N-cadherin and induced E-cadherin, which is an epithelial marker in control cells (Fig. 7F). However, TGF-β increased mesenchymal biomarkers as well as GSK-3β phosphorylation and β-catenin expression in both AIM-treated and AIM-untreated cells. These results confirmed that the inhibitory effect of AIMs on Akt activity is independent of EGFR activity, and AIMs still suppressed Akt phosphorylation on

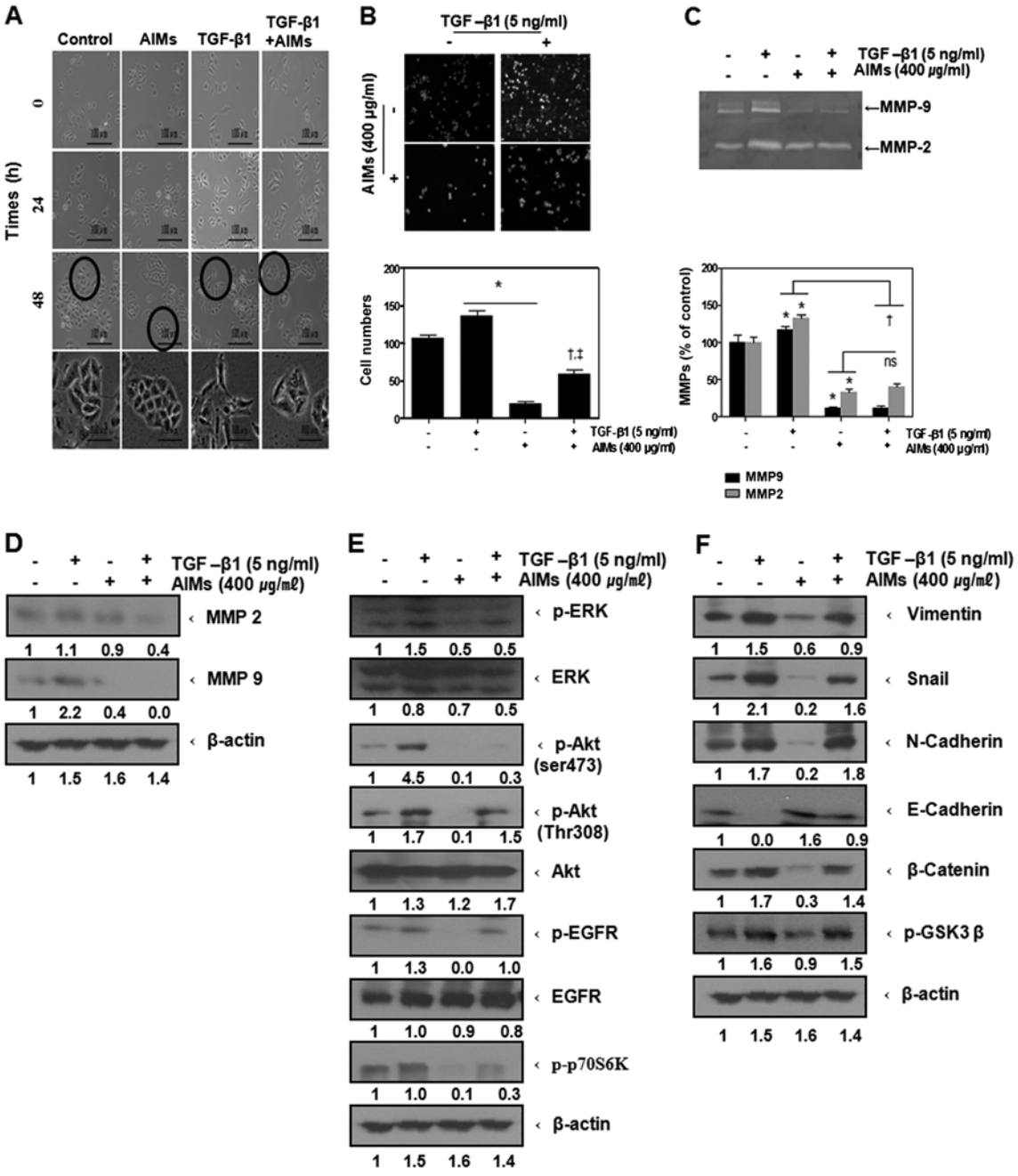

Figure 7. Inhibitory effects of AIMs on TGF-β-induced invasion and EMT in A549 cells. (A) Cells were pretreated with AIMs (400 μg/ml) for 1 h and then treated with TGF-β (5 ng/ml) for 24 h. Data are representative of three independent experiments. (B) Cells were serum-starved for 24 h with or without AIMs (400 μg/ml). Cells ($5x10^4$ cells) were loaded onto pre-coated Matrigel 24-well invasion chambers in the presence or absence of TGF-β (5 ng/ml). The Matrigel invasion chambers were incubated for 24 h. *P<0.05 vs. control, †P<0.05 vs. AIMs. (C) MMP-2 and MMP-9 protein levels were measured by gelatin zymography. Cells were incubated for 48 h without or with AIMs. Values represent means ± SD from three independent experiments. *P<0.05 vs. control. (D-F) Cells ($5x10^4$ cells), either left untreated or pretreated with AIMs (400 μg/ml) for 24 h and then were exposed to TGF-β (5 ng/ml) for indicated times. Whole-cell extracts were prepared, and 30 μg of the whole-cell lysate was analyzed by western blot analysis for (D) MMP-2 and MMP-9, (E) phospho-Akt (Thr308 and Ser473), Akt, phospho-ERK, ERK, phospho-EGFR and EGFR, and (F) Snail, vimentin, N-cadherin and E-cadherin in A549 cells. The results are representative of two independent experiments. The expression of the indicated proteins were measured by densitometry and expressed as relative ratio.

Ser473 even in TGF-β-treated cells whereas the inhibitory effects of AIMs on TGF-β-induced EMT was limited.

Discussion

This study was designed to answer the question whether the inhibitory effects of AIMs on Akt activity were associated with anti-EGFR effects on cancer invasion, and whether AIMs have anti-EMT effects. Here, we investigated the effects of AIMs on cellular responses and molecular changes involved in cancer invasion and EMT in human lung cancer cells treated with EGF or TGF-β. We demonstrated that AIMs suppressed PI3k/Akt and EGFR pathway independently in a dual suppression mode, and that AIMs suppressed invasion

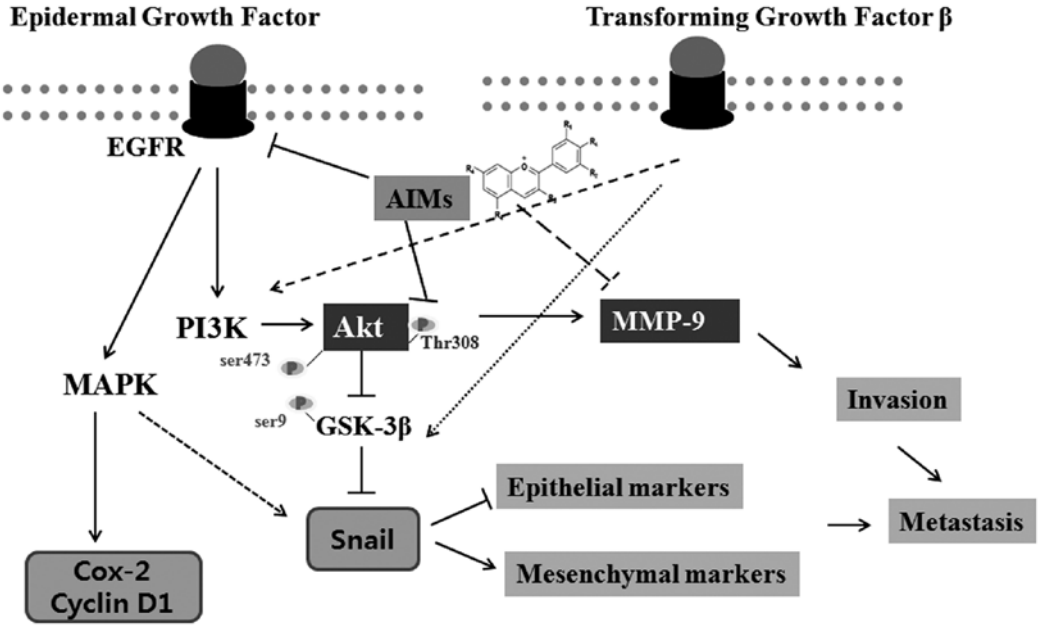

Figure 8. Schematic representation of the inhibitory effects of AIMs on invasion and EMT in A549 human lung cancer cells. EGF activates PI3K/Akt and MAPK pathways. AIMs inhibited invasion and EMT by suppressing the activation EGFR and the downstream pathways (ERK and Akt), but the inhibitory effect of AIMs on Akt was not dependent on the inhibition of EGFR, and is possibly derived from the inhibition of Akt phosphorylation (Thr308). AIMs also inhibit phosphorylation of GSK-3β (Ser9) that is regulated by Akt activity. By inhibiting EGFR and Akt activity in a dual mode, AIMs inhibit invasion and EMT either induced by EGF or TGF-β, which are well-known factors involved in invasion and EMT. AIMs also inhibited the effector downstream molecules (COX-2 and cyclin D1) that are involved in cell proliferation. This study suggests that AIMs inhibit the invasion and EMT of A549 human lung cancer cells by dual inhibition of Akt and EGFR activity.

and migration at least in part by suppressing EMT. A previous report showed that anthocyanins have anti-EGFR (7,18) and anti-Akt activities (19). Here, we firstly demonstrated that the anti-Akt activity was independent of anti-EGFR activity of anthocyanins in cancer invasion and EMT by using inhibitors for each EGFR downstream pathway (ERK pathway and PI3K/Akt pathway), EGF or TGF-β stimulation.

In this study, AIMs predominantly suppressed MMP-9 rather than MMP-2, which was consistent the previous findings which our colleagues demonstrated in HT-29 human colon cancer cells (9). EMT has attracted a great deal of attention as a potential mechanism for tumor cell metastasis (20). Snail is a well-known transcription factor that promotes EMT by repressing the expression of E-cadherin which is important in cell adhesion. With E-cadherin, cancer cells are tightly bound to each other and to stromal cells. To metastasize, cancer cells need to change in order to gain mesenchymal phenotype. Snail is degraded and exported from the nucleus by GSK-3β (21). Here, we demonstrated that EGF induced Snail expression and suppressed E-cadherin expression, and that AIMs inhibited the EGF effects on Snail and E-cadherin. These effects of AIMs on EGF- and TGF-β-induced EMT were also confirmed in HeLa human uterine cancer cells treated by TNF (data not shown).

However, there are still several findings that are not clearly demonstrated. Both EGF and TGF-β enhanced cancer invasion without any further induction of MMP-2 and MMP-9 in the AIM-treated cells (Figs. 2A and B, 7B and C). This finding indicated that there are other mechanisms for EGF- and TGF-β-augmented cancer cell invasion rather than the secretion of MMPs. Here, we demonstrated that one of the mechanisms of EGF- and TGF-β-augmented cancer cell invasion was EMT with assessing the changes in cell morphology and EMT biomarkers. We suggested that AIMs might suppress the secretion of MMP-2 and MMP-9 by inhibition of Akt signaling which is well known to regulate MMP-9 expression (22) because of the reports demonstrating that anthocyanins inhibit MMP-2 and MMP-9 by suppression of PI3K/Akt (19). However, this study also suggests that there is a possibility that AIMs inhibit MMP-9 by another mechanism that is not influenced by either EGFR or Akt activity because EGF and TGF-β activated Akt activity and EGFR activity in AIM-treated cells.

The effect of AIMs on EMT were different between EGF-treated cells and TGF-β-treated cells; AIMs partially inhibited EGF-induced EMT, but not TGF-β-induced EMT although AIMs inhibited Akt phosphorylation on Ser473 in both EGF- and TGF-β-treated cells. The difference can be explained by the way TGF-β induces EMT because TGF-β also induces EMT by SMAD-dependent signaling which does not involve Akt activation (23-25).

EGF stimulated the MMPs only slightly compared to those derived from TGF-β. There are some reports that EGF can induce MMP-9 (26), but in others show that the extent of EGF-induced MMP-9 secretion is far less than that induced by TGF-β or tumor necrosis factor (TNF), and that EGF can even inhibit MMP-9 expression in some cancer cells (27,28). The latter finding is consistent with our findings. Another merit of the finding that EGF did not increase MMP-2 and MMP-9 expression give us more valid evidence that EGF did augmented invasion by induction of EMT but not by induction of MMPs.

We determined whether the inhibitory effect of AIMs on GSK-3β was derived from that on Akt. Previous studies demonstrated that Akt and GSK-3β signaling pathway are involved in EMT (29), and that GSK-3β signaling pathway is dependent on Akt pathway and other signaling such as WNT signaling (29,30). In addition, EGFR signaling pathway is also involved in GSK-3 pathway (31). GSK-3 is encoded by two known genes, GSK-3α and GSK-3β. The site of serine or threonine phosphorylation determines the activity of GSK-3; phosphorylation of Ser9 in GSK-3β significantly decreases the activity of GSK-3. The phosphorylation of Ser9 in GSK-3β is regulated by Akt (17). Therefore, we assessed phosphorylation of Ser9 in GSK-3β to look at GSK-3 activity regulated by Akt. GSK-3 activty is closely linked to β-catenin activation which is important in EMT. In this study, we found that AIMs suppressed p-GSK-3β (Ser9) and β-catenin, and that EGF and TGF-β enhanced GSK-3β activity by suppressing phosphorylation of Ser9 in GSK-3β. These results support that the inhibitory effects of AIMs on GSK-3β activity is derived from the anti-Akt effects. Up to now, few studies has been performed regarding the effects of anthocyanins on GSK-3. There is a report that cyanidin-3-glucoside upregulated p-GSK-3β (Ser9) in a neuroblastoma cell line (32). The result was opposite to ours. However, there is another study supporting our results, which showed that the extract of skin of Muscadine grape containing abundant anthocyanins suppressed p-GSK-3β (Ser9) (33). In that our anthocyanins were isolated from fruit, it is likely that the latter study would be more similar to ours.

Although we demonstrated that EGF and TGF-β re-induced into AIM-treated cells the p70S6K linked to mTORC-1 signaling activation with Akt phosphorylation on Ser473, but not on Thr308, this finding is different from the rescent finding that EGFR-mediated activation of mTORC-1 signaling is by Akt phosphorylation on Ser473, not Akt phosphorylation on Thr308. However, this study demonstrated that the inhibition of both mTORC1 and mTORC2 activity lead to activation of EGFR followed by mTORC-1 signaling activation through Akt phosphorylation on Thr308. However, it does not indicate that EGF induced Akt activation should follow the same pathway. There is one report showing exactly the same pattern of Akt activation by EGF stimulation with or without sirolimus (rapamycin) treatment (34).

In conclusion, this study suggests that the inhibitory effect of AIMs on Akt activity is independent of that on EGFR, and that AIMs suppressed invasion and migration, at least in part, by suppressing EMT by inhibiting Akt activity as well as EGFR (Fig. 8). This study provides evidence that AIMs have anti-metastatic effects on human lung cancer by inhibition of both PI3k/Akt and EGFR pathways involved in invasion and EMT.

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