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2-Methoxy-4-vinylphenol Attenuates Migration of Human Pancreatic Cancer Cells *via* Blockade of FAK and AKT Signaling

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Abstract. *Background/Aim: No effective therapeutics have yet been developed for pancreatic cancer. 2-Methoxy-4-vinyl phenol (2M4VP), a member of the class of phenols, has been demonstrated to have anti-inflammatory properties and cause cell cycle arrest making it an attractive candidate drug for the treatment of pancreatic cancer. Materials and Methods: The effects of 2M4VP were examined in Panc-1 and SNU-213 human pancreatic cancer cells. Results: 2M4VP had anticancer effects on pancreatic cancer cell lines, Panc-1 and SNU-213. 2M4VP reduced the viability of Panc-1 cells by inhibiting the expression of the cell nuclear antigen (PCNA) protein. 2M4VP also suppressed the migratory activity of both cell lines. In addition, treatment with 2M4VP effectively decreased the phosphorylation of Focal Adhesion Kinase (FAK) and AKT. Conclusion: 2M4VP might be used as a pancreatic cancer treatment supplement.*

Pancreatic cancer has a five-year survival rate of less than 5% (1-3). The main characteristics of pancreatic cancer are the early systemic metastasis and local tumor progression (4-6). The unique migratory activity of pancreatic cancer cells makes early diagnosis and treatment very difficult, and increases the mortality rate of pancreatic cancer patients (7, 8). Recently, it has been reported that gemcitabine, a drug used in current clinical trials, may cause metastasis at low dose in pancreatic cancers (9). Resistance to currently available anticancer drugs also makes this disease more difficult to treat (10, 11). In addition, a hepatocyte growth factor (HGF) has been noted for its role in pancreatic cancer and has been related with poor prognosis (12-14). Therefore, it is necessary to study the molecules that regulate the HGF pathway in pancreatic cancer cells.

Buckwheat is a dicotyledonous plant common in East Asian countries and contains various functional substances such as rutin, isovitexin, quercetin, which have been reported to have antioxidant, anti-inflammatory, and anti-cancer properties (15-19). In particular, buckwheat contains buckwheat flavor compounds, such as 2,5-dimethyl-4-hydroxy-3(2H)-furanone, (E-)-2,4-decadienal, and 2-Methoxy-4- vinylpenol (2M4VP). Especially, 2M4VP is used as a fragrance and is also found in apples and peanuts. 2M4VP is also known to induce cell cycle arrest by blocking the hyper-phosphorylation of retinoblastoma protein in benzopyrene-treated NIH3T3 cells and to have an anti-inflammatory effect by inhibiting mitogen-activated protein kinase (MAPK) activation (20, 21). In this study, we investigated the anticancer effects of 2M4VP in pancreatic cancer cell lines.

Materials and Methods

Cell culture and reagents. We obtained 293T, Panc-1, and SNU-213 cells from the Korean Cell Line Bank (Seoul, Repubic of Korea). The 293T and Panc-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37˚C and 5% CO_2. SNU-213 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco-BRL), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37˚C and 5% CO_2. The 2M4VP and HGF were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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Key Words: 2-methoxy-4-vinylphenol, cell proliferation, cell migration, hepatocyte growth factor.

Cell viability assay. Cell viability was measured using the WST-1(2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4- disulfophenyl)-2H-tetrazolium) assay (Boechringer Mannheim, Mannheim, Germany). Cells were seeded at a density of 2.5×104 per well in 24-well plates. After incubation for 24 h, they were treated with 2M4VP for 72 h at 37˚C. Then, WST-1 solution was added to the cells and incubated for 15 min at room temperature. The absorbance was measured at 450 nm using a microplate reader.

Flow cytometry analysis. For apoptosis analysis, Panc-1 and 293T cells were seeded in 6-well plates. After 24 h, they were treated with 2M4VP for 72 h. Cells were then collected and incubated with Annexin V-FITC and PI (FITC Annexin V apoptosis detection kit, BD Pharmigen, San Diego, CA, USA). The apoptotic cells were detected by flow cytometry (LSRFortessa, BD Pharmigen).

Cell migration assay. The filter was pre-coated with 1 μg/μl fibronectin (Sigma-Aldrich, St. Louis, MO, USA), and then 500 μl RPMI were added to the lower chamber. The suspended cells in the upper chambers were treated with HGF for 30 min and then with 2M4VP for 6 h at 37˚C in serum-free medium. Then, cells were fixed using 4% paraformaldehyde (Biosesang, Seongnam, Republic of Korea) and stained using 0.1% crystal violet solution. Finally, the solution was eluted with 10% acetic acid and absorbance was measured at 560 nm using a microplate reader.

Western blot assay. Cells were lysed in M-PER lysis buffer (Thermo science, Bonn, Germany) containing a protease inhibitor cocktail (Roche), 2 mM sodium vanadate, 30 mM sodium pyrophosphate, and 100 mM sodium fluoride. After total protein quantification, proteins were separated by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK). Membranes were blocked with 5% skim milk in TBST followed by incubation overnight at 4˚C with primary antibodies, such as phospho-FAK (Tyr397), phospho AKT (Ser473), proliferating cell nuclear antigen (PCNA), and GAPDH (Cell signaling technology, Beverly, MA, USA), that were diluted 1:1000 in TBST. Membranes were then washed four times with TBST and incubated for 1 h with secondary antibodies (Merck Millipore, Germany) that were diluted 1:4000 in TBST. Membranes were then washed with TBST and the protein bands were detected by the ECL kit (Biosesang).

Statistical analysis. Error bars represent±SEM. Statistical analysis was performed using one-way ANOVA, two-way ANOVA, and student's *t*-test (SPSS, Chicago, IL, USA). $p<0.05$ was considered to indicate significant differences.

Results

2-Methoxy-4-vinyl phenol inhibited the proliferation of Panc-1 cells. WST-1 assay was used to analyze the effect of different concentrations of 2M4VP on the proliferation of Panc-1 and SNU-213 cells. Panc-1 cells showed a significant ($p<0.001$) decrease in viability after treatment with 2M4VP. In contrast, SNU-213 cell proliferation was not statistically significantly affected by 2M4VP. Moreover, treatment with 100 μM 2M4VP did not affect the viability of control 293T cells, indicating that 2M4VP at a 100 μM concentration was not cytotoxic (Figure 1A). Cells were treated with 2M4VP and analyzed by flow cytometry to determine whether the reduction in viability of Panc-1 cells was due to apoptosis or to the inhibition of proliferation. Figure 1B shows that apoptosis did not occur in 2M4VP-treated Panc-1 cells and control 293T cells. Next, to investigate the effect of 2M4VP on cell proliferation, the expression of PCNA was examined. PCNA was downregulated in Panc-1 cells after treatment with 2M4VP, but no significant changes were observed in SNU-213 and control cells (Figure 1C). These results showed that 2M4VP inhibited proliferation of Panc-1 pancreatic cancer cells.

2M4VP inhibited metastasis of Panc-1 and SNU-213 cells by regulating p-FAK and p-AKT. Panc-1 and SNU-213 cells were treated with 0, 10, or 100 μM of 2M4VP for 6 h and analyzed using a transwell assay. Panc-1 cell migration was reduced by about 15% after treatment for 6 h with 10 μM 2M4VP (Figure 2A). In SNU-213 cells, 2M4VP inhibited migration by about 9% at a concentration of 10 μM and about 17% at a concentration of 100 μM (Figure 2B). These results suggest that 2M4VP inhibits metastasis of pancreatic cancer cells and is more effective in Panc-1 than in SNU-213 cells. To identify the mechanism of this inhibition, cells were treated with 2M4VP (0, 10, 100 μM) for 24 h and analyzed for the phosphorylation levels of FAK (Tyr 397) and AKT (ser473) (Figure 3). p-FAK and p-AKT levels were reduced following treatment of Panc-1 cells with 2M4VP. However, p-FAK and p-AKT levels did not change in 2M4VP-treated control cells.

2M4VP inhibited the hepatocyte growth factor-induced metastasis of Panc-1 cells. Hepatocyte growth factor (HGF) is known to induce metastasis in various cancers, such as colorectal, colon, prostate, and pancreatic cancer. To find out whether 2-methoxy-4-vinyl phenol (2M4VP) inhibits HGF-induced metastasis, pancreatic cancer cells were first treated with 2M4VP for 30 min and then treated with HGF (10 ng/ml) for 30 min. As expected, treatment of Panc-1 cells with HGF (10 ng/ml) resulted in about 40% increase in metastasis, but 2M4VP treatment inhibited the migration induced by HGF to levels of untreated cells (Figure 4A). In addition, we found that the levels of p-AKT increased with HGF treatment and decreased to untreated levels by pretreatment with 2M4VP (Figure 4B). These results suggest that 2M4VP reduces the phosphorylation levels of HGF-induced AKT and inhibits metastasis of Panc-1 cells.

Discussion

2M4VP is an aromatic compound that has anti-inflammatory effects by inhibiting NO production and also inhibits cell-cycle activation induced by the carcinogen benzopyrene (20, 21). However, there are no studies on the specific effect of

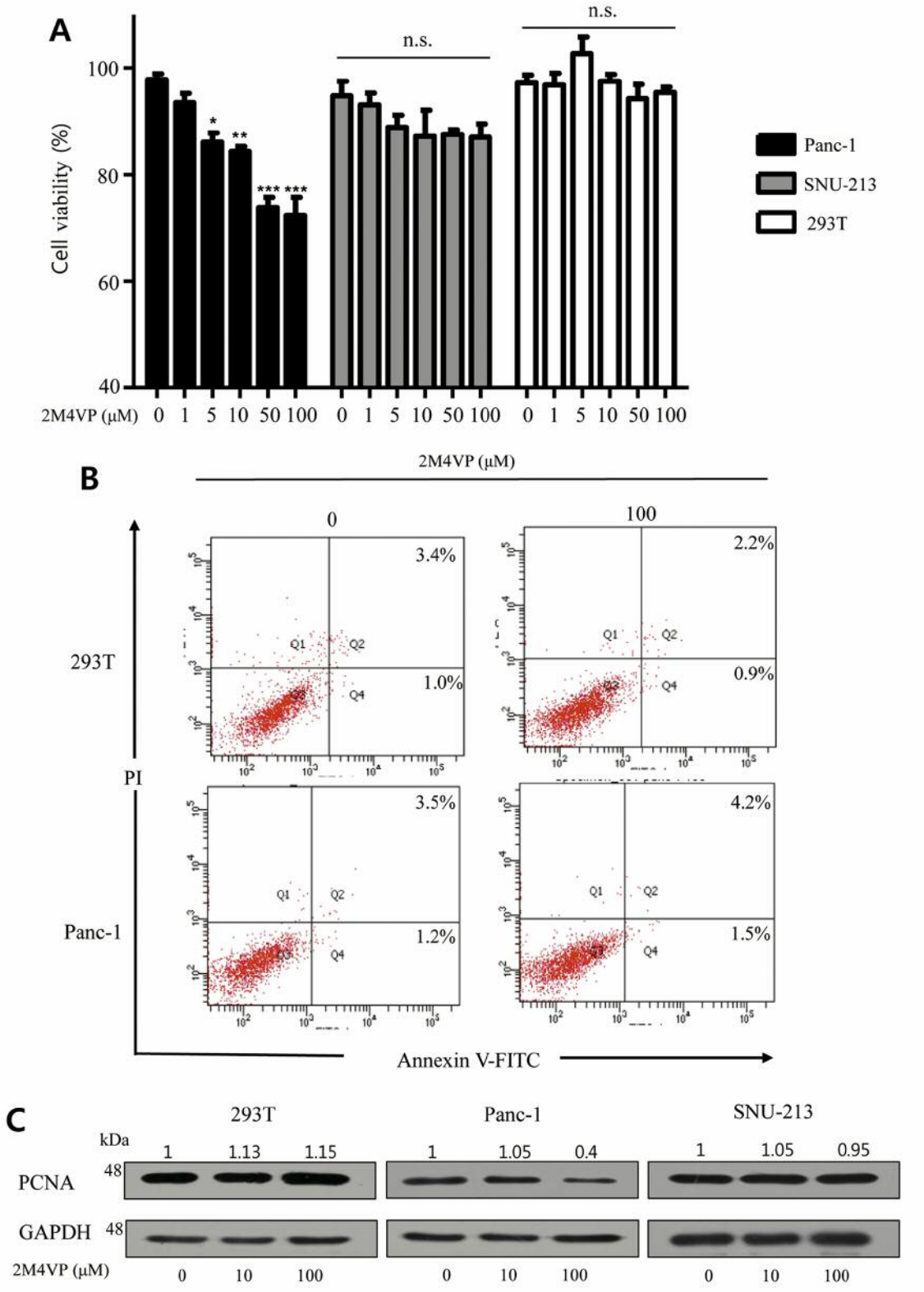

Figure 1. *Effect of 2M4VP treatment on pancreatic cancer cell viability and expression of PCNA. (A) WST-1 assay performed after treatment of Panc-1, SNU-213, and 293T cells with 2M4VP (0, 5, 10, 50, 100 μM). (B) Flow cytometry analysis of Panc-1 and 293T cells after treatment with or without 2M4VP. (C) The expression of PCNA through western blot after treatment with 2M4VP for 50 h in Panc-1, SNU-213, and 293T cells. The relative band intensities of PCNA/GAPDH were measured using ImageJ software. (p<0.05; stars indicate a significant difference vs. 0, *p<0.05, **p<0.01, ***p<0.001).*

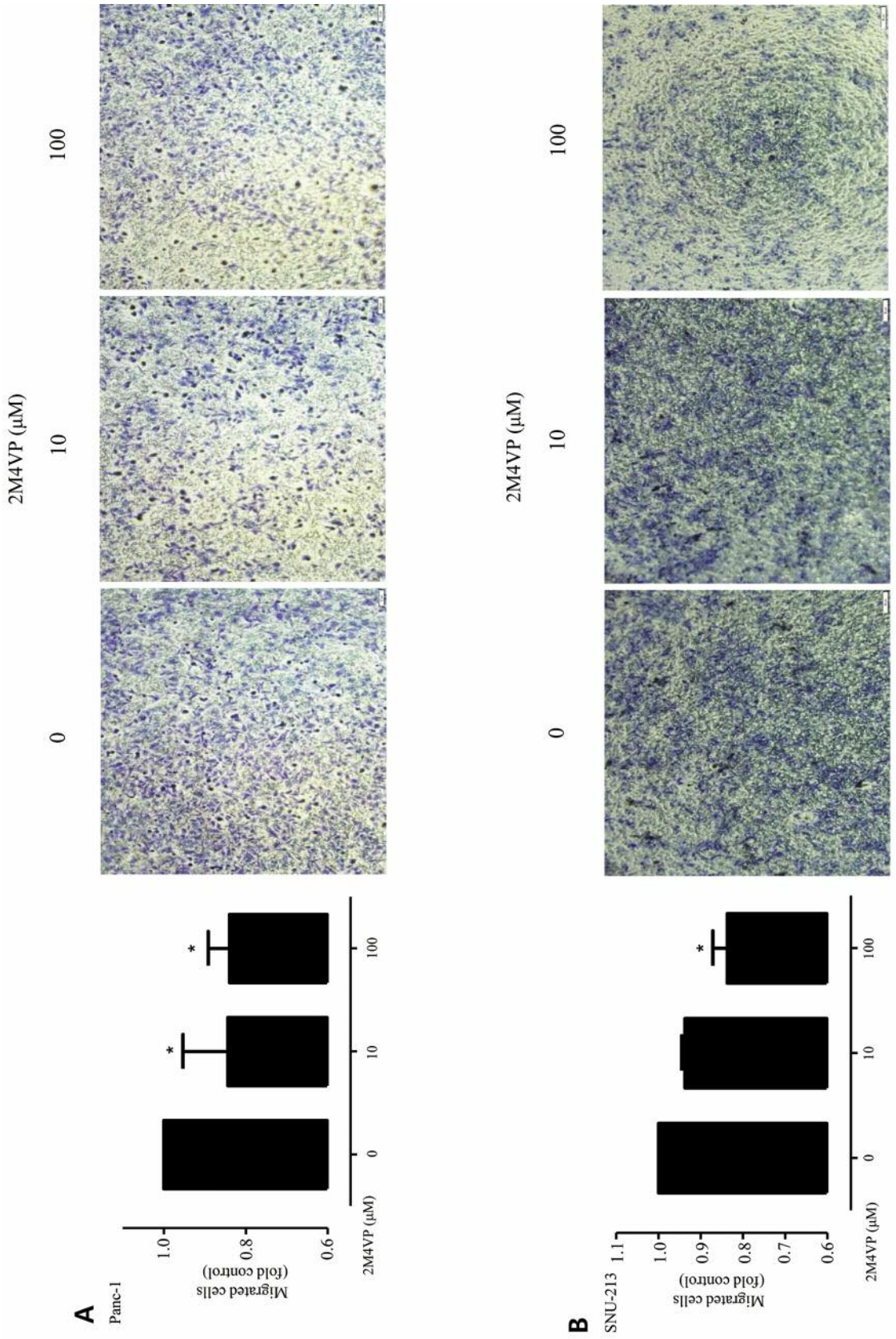

Figure 2. *The effect of 2M4VP on the migration of Panc-1 and SNU-123 cells. (A) Left, migration activities of Panc-1 cells after treatment with 2M4VP for 6 h. Right, a representative image of Transwell migration assay in Panc-1. (B) Left, migration activities of SNU-213 cells after treatment with 2M4VP for 6 h. A representative image of Transwell migration assay in SNU-213 cells ($p<0.05$; stars indicate a significant difference vs. 0, *$p<0.05$).*

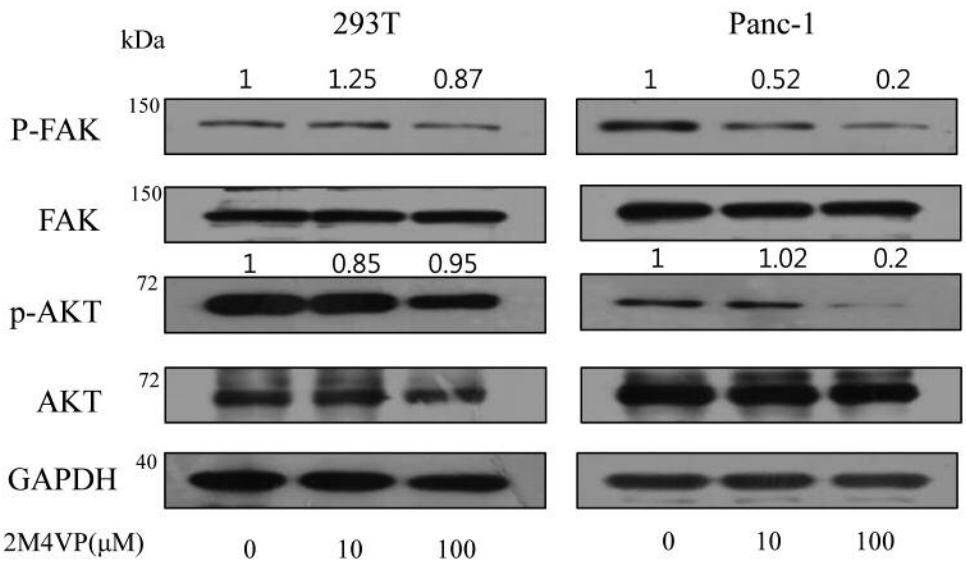

Figure 3. *The expression and phosphorylation analysis of FAK and AKT in 293T and Panc-1 cells. The relative band intensities of p-FAK/FAK and p-AKT/AKT were measured using ImageJ software.*

2M4VP on cancer cells. In this study, we found that 2M4VP inhibited cell proliferation and metastasis in pancreatic cancer cells.

PCNA is known to regulate cell cycle and proliferation by tetramerization with cyclins and p21 (22). In this study, 2M4VP treatment reduced the expression of PCNA by more than 50% in Panc-1 cells. Therefore, it can be suggested that 2M4VP inhibits Panc-1 cell proliferation by inhibiting PCNA expression. This result is similar to that of a previous report where matrine, a compound isolated from the legume, suppressed cell proliferation by inhibiting the expression of PCNA (23).

FAK is a non-receptor tyrosine kinase that activates downstream signaling pathways, such as proto oncogene tyrosine-protein kinase Src (Src) and phosphatidylinositol-3-kinase (PI3K) /AKT (24). FAK has been reported to be overexpressed in malignant tumors and is known to play an important role in cell survival, proliferation, migration, and invasion (24-27). In previous studies, we have reported that flavonoids, such as quercetin and kaempferol, are effective in inhibiting pancreatic cancer metastasis by inhibiting FAK phosphorylation (6, 10). Similarly, 2M4VP inhibited metastasis through the FAK pathway, thereby reducing the phosphorylation levels of FAK.

Hepatocyte growth factor (HGF) and its receptor c-MET are of considerable interest because they are closely related to various human cancers. Many HGF/c-MET pathway inhibitors have been developed and evaluated in the clinic (28). These agents include MET antagonists, neutralizing antibodies against HGF, and inhibitors of downstream signaling pathways. Phytochemicals including wihthaferin A and carnosol, block HGF/c-MET activation and attenuate migration in AsPC-1 pancreatic cancer cells, suggesting that phytochemicals are effective potential candidate therapeutics (29, 30). The PI3K/AKT pathway is known to be activated when HGF binds to its receptor, and to control invasion and metastasis of cancer cells (11, 12). We found that HGF-induced cell migration in Panc-1 cells was reduced dose-dependently following treatment with 2M4VP. The induced AKT phosphorylation was also decreased. Since 2M4VP inhibits the translocation of NF-κB p65 into the nucleus (21), it is expected that 2M4VP regulates the HGF/FAK/PI3K/Akt/NF-kb pathway in pancreatic cancer cells.

In conclusion, 2M4VP inhibited proliferation and metastasis in Panc-1 cells and blocked HGF-mediated metastasis. 2M4VP can be potentially used as an inhibitor of metastasis of pancreatic cancer cells.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

DH Kim performed the experiments and drafted the main manuscript. SI Han, UH Oh, and B Go assisted with the experiments. DH Kim, CS Kim, and YH Jung analyzed and interpreted data. JH Kim and J Lee supervised the project.

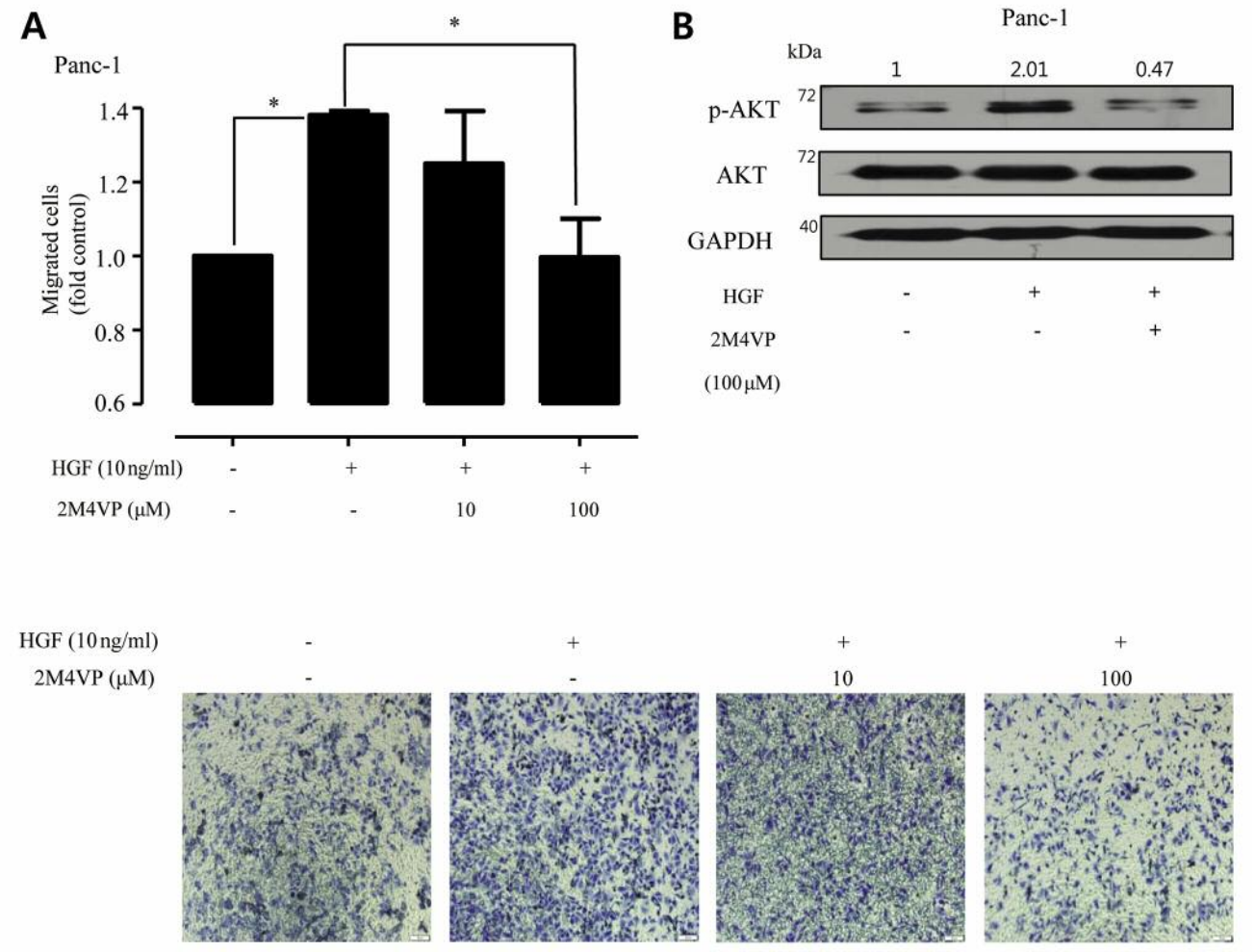

Figure 4. *Inhibition of hepatocyte growth factor (HGF)-induced metastasis by 2M4VP and its effect on the expression and phosphorylation of AKT in Panc-1 cells. (A) Quantification of the effect of 2M4VP on HGF-induced migration in Panc-1 cells. Below, representative image of the Transwell migration assay in Panc-1 cells. (B) Effect of 2M4VP on the expression and phosphorylation of AKT in HGF-treated Panc-1 cells. The relative band intensities of p-AKT/AKT were measured using ImageJ software (p<0.05; stars indicate a significant difference vs. 0, *p<0.05).*

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