Master's Thesis

2 - Methoxy - 4-vinylphenol induced attenuation of cell migration in pancreatic cancer cells.

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2 - Methoxy - 4-vinylphenol 은 췌장암 세포에서의 전이를 억제한다.

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CONTENTS

LIST OF FIGURES

ABSTRACT

Pancreatic cancer is the most dangerous cancer, because it is difficult to diagnose and moves easily to other organs. Also, no relevant therapeutics have been developed; so there is a need for research on the treatment agents of pancreatic cancer. 2-Methoxy-4-vinyl phenol (2M4VP) is a flavoring compound with properties such as cycle arrest and anti-inflammation. In this study, we confirmed 2M4VP anticancer effects on pancreatic cancer-cell lines, including Panc-1 and SNU-213cells. 2M4VP reduced the viability of Panc-1 cells by inhibiting the proliferation of cell nuclear antigen (PCNA) protein. 2M4VP also suppressed the migratory activity of Panc-1 and SNU-213 cells. In addition, we demonstrated that the treatment with 2M4VP effectively decreased the phosphorylation of Focal Adhesion Kinase (FAK) and AKT pathways. These results suggest that 2M4VP might be used as a pancreatic cancer treatment supplement.

1. INTRODUCTION

Pancreatic cancer has a poor prognosis and five-year survival rate of less than 5% 3). Pancreatic cancer is one of the top 5 causes of death from cancer in the western world. The main characteristics of pancreatic cancer are an early systemic metastasis and local tumor progression, and occur with 1to 2 years after surgery (2, 4–6). The unique migration activity of pancreatic cancer makes an early diagnosis and medication difficult, and increases the mortality rate of pancreatic cancer patients (7, 8). Resistance to currently available anticancer drugs also makes it more difficult to treat pancreatic cancer (9, 10). Therefore, new agents inhibiting migration activity of pancreatic cancer are required (7). In addition, a hepatocyte growth factor (HGF) that is a 90-kDa glycoprotein, secreted by mesenchymal cells has recently been noted for its role in pancreatic cancer and is known to be involved in poor prognosis. HGF was originally identified as a liver mitogen and fibroblast-derived epitheial motility factor, and is the only physiological ligand for the MET receprot tyrosine kinase. Phosphorylation of AKT was induced by HGF, and the phosphorylated AKT persisted even when E-cadherin was inhibited in pancreatic cancer cells (11–14). Therefore, it is necessary to study the substances that regulate the HGF pathway and metastasis in pancreatic cancer cells.

Buckwheat is a dicotyledonous plant common in East Asian countries (Figure 1). Buckwheat is high in protein, contains a number of amino acids. Buckwheat contains various functional substances such as rutin, isovitexin, quercetin, which have been reported to be effective in antioxidant, anti-inflammatory, and anti-cancer (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-vinylphenol (2M4VP) (16). Especially, 2M4VP is used as a fragrance and is found also in apples and peanuts (Figure 2). 2M4VP is known to stop the cell cycle induced by benzopyrene, a carcinogen, in NIH3T3 cells and to have anti-inflammatory effect by inhibiting MAPK activation induced by LPS (20, 21). In this study, we investigated the anticancer effects of 2M4VP in pancreatic cancer cell lines and found that it inhibits cell migration in pancreatic cancer cells.

Figure 1. The picture of buckwheat.

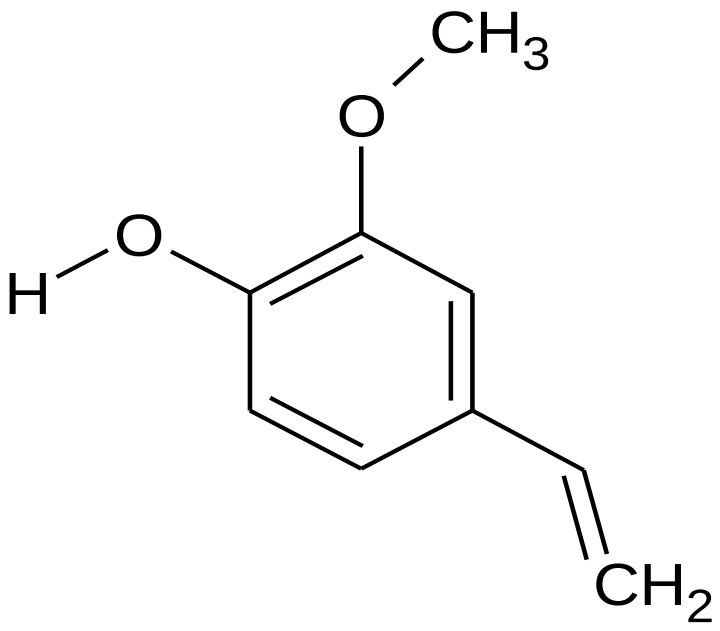

Figure 2. Structure of 2-Methoxy-4-vinylphenol.

2. MATERIALS AND METHODS

2.1 Cell culture and reagents

We obtained a human embryonic kidney cells 293T, Panc-1, and SNU-213 human pancreatic cells from Korean Cell Line Bank (Seoul, 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, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), and 5% at 37℃. SNU-213 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco-BRL), 100 U/mL penicillin, 100 μg/ml streptomycin (Invitrogen), and 5% at 37℃. The 2M4VP and HGF were purchased Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

2.2 Cell viability assay

We measured cell viability using the WST-1(2-(4-iodophenyl)-3-(4-nitro phenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) solution (Boechringer Mann heim, Mannheim , Germany). Cells were seeded 2.5×10^{4} in 24-well plates. After cells were incubated for 24 h, we were treated with 2M4VP for 72 h at 37℃. Each well was added to a final concentration of 10% WST-1 solution after 15 min incubation at room temperature. The absorbance was measured at 450 nm using the microplate reader.

2.3 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. After collecting the cells, we incubated the cells with Annexin V-FITC and PI (FITC Annexin V apoptosis detection kit, BD pharmigen). The apoptotic cells were detected by flow cytometry (LSRFortessa, BD).

2.4 Cell migration assay

The filter was pre-coated 1 μg/μl fibronectin (Sigma-Aldrich, St. Louis, MO, USA), and then 500 μl RPMI was added to the lower chamber. The suspended cells were treated with 2M4VP in serum-free medium in the upper chambers. Panc-1 cells were incubated for 6 h at 37℃, and SNU-213 cells were incubated for 6 h at 37℃. Then these cells were fixed using 4% paraformaldehyde (Biosesang, Seongnam, Korea) and then stained using 0.1% crystal violet solution. Finally, we measured the absorbance of the solution eluted with 10% acetic acid at 560 nm using a microplate reader. HGF treatment was done for 30 min before the 2M4VP incubation for 6 h.

2.5 Western blot assay

Cells were lysed in M-PER lysis buffer (Thermo science, Bonn, Germany) containing protease inhibitor cocktail (Roche), 2mM sodium vanadate, 30mM sodium pyrophosphate, and 100mM 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. Primary antibodies, such as phospho-FAK (Tyr397), phospho AKT (Ser473), PCNA, and GAPDH (Cell signaling technology, USA), were diluted 1:1000 in TBST and incubated overnight at 4℃. Secondary antibodies (Merck Millipore, Germany) were diluted in TBST 1:4000 and incubated for 1h. The protein bands were detected by the ECL kit (Biosesang).

2.6. Statistical analysis

Error bars represent ±SEM. We did statistical analysis by one-way ANOVA, two-way ANOVA, and student's t-test. $p < 0.05$ was considered to indicate significant differences.

3. RESULTS

3.1 Proliferation inhibition of 2-Methoxy-4-vinyl phenol in Panc-1 cells

We evaluated 2-Methoxy-4-vinyl phenol (2M4VP) anticancer effects in pancreatic cancer by means of a WST-1 assay in 2M4VP-treated Panc-1 and SNU-213 cells with various concentrations. Panc-1 cells showed a significant decrease in viability when treated with 2M4VP. On the other hand, SNU-213 cells and Capan-1 cells appeared no statistically significant difference. In control 293T cells, 100 μM 2M4VP treatment did not change viability, indicating that 2M4VP at 100 μM concentration was not cytotoxic (Figure 3). Cells were treated with 2M4VP and analyzed by flow cytometry to find out whether the reduction in viability of Panc-1 cells was due to apoptosis or to inhibition of proliferation. Figure 4A shows that apoptosis did not occur in 2M4VP-treated Panc-1 cells, SNU-213 cells and 293T cells. Next, to investigate cell proliferation, the expression of PCNA was examined. PCNA was downregulated in Panc-1 cells when treated with 2M4VP, but no significant changes were seen in SNU-213 and control cells (Figure 4B). These results show that 2M4VP inhibits proliferation in Panc-1 pancreatic cancer cells.

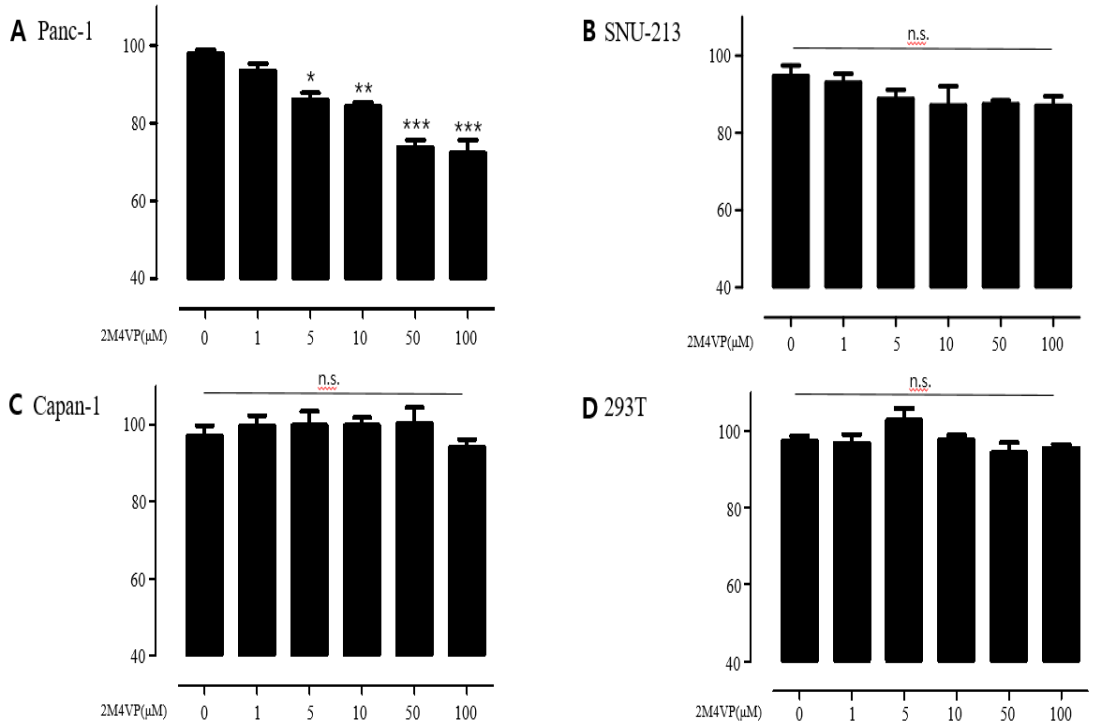

Figure 3. 2M4VP treatment on pancreatic cancer cells. WST-1 assay were performed after treatment with 2M4VP (0, 5, 10, 50, 100 μM) on Panc-1 (A), SNU-213 (B), Capan-1 (C) and 293T (D) cells. ($p < 0.05$; stars indicate a significant difference vs. 0, *$p < 0.05$, **$p < 0.01$, ***p < 0,001).

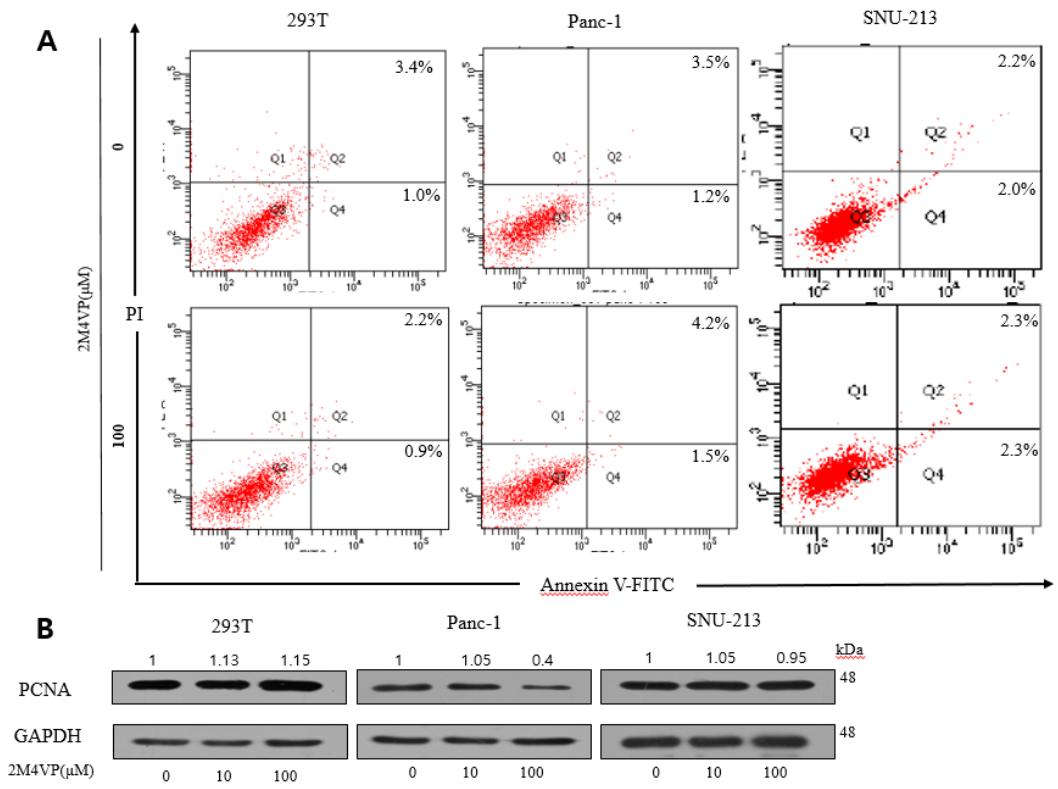

Figure 4. 2M4VP inhibited cell proliferation. (A) Flow cytometry analysis of Panc-1, SNU-213, and 293T cells with or without 2M4VP treatment. (B) The expression of PCNA through Western blot after treating 2M4VP for 50 h in Panc-1, SNU-213, and 293T cells. The relative band intensities of PCNA/GAPDH was measured using Image J software

3.2 2-Methoxy-4-vinyl phenol inhibits metastasis by regulating p-FAK and p-AKT in Panc-1 and SNU-213 cells

To evaluate the anticancer effects of 2-methoxy-4-vinyl phenol (2M4VP), 2M4VP was incubated with Panc-1 and SNU-213 cells at 0, 10, and 100 μM for 6 h using a transwell assay. Panc-1 cell migration was reduced by about 15% after treatment for 6 hours by 10 μM 2M4VP (Figure 5A). In SNU-213 cells, 2M4VP inhibited the migration by about 9% at 10 μM 2M4VP and about 17% at 100 μM 2M4VP (Figure 5B). The Capan-1 cells were reduced by about 35% treatment for 12 hours by 100 μM 2M4VP (Figure 5C). These results suggest that 2M4VP inhibits the metastasis of pancreatic cancer cells and is more effective in Panc-1 than in SNU-213 and Capan-1. To identify a mechanism for inhibiting metastasis through protein phosphorylation, Panc-1 cells were treated with 2M4VP (0, 10, 100 μM) for 24 hours. The treatment reduced the phosphorylation levels of FAK (Tyr 397) and AKT (ser473) (Figure 6). However, the phosphorylation level of FAK and AKT did not change in 2M4VP-treated control cells.

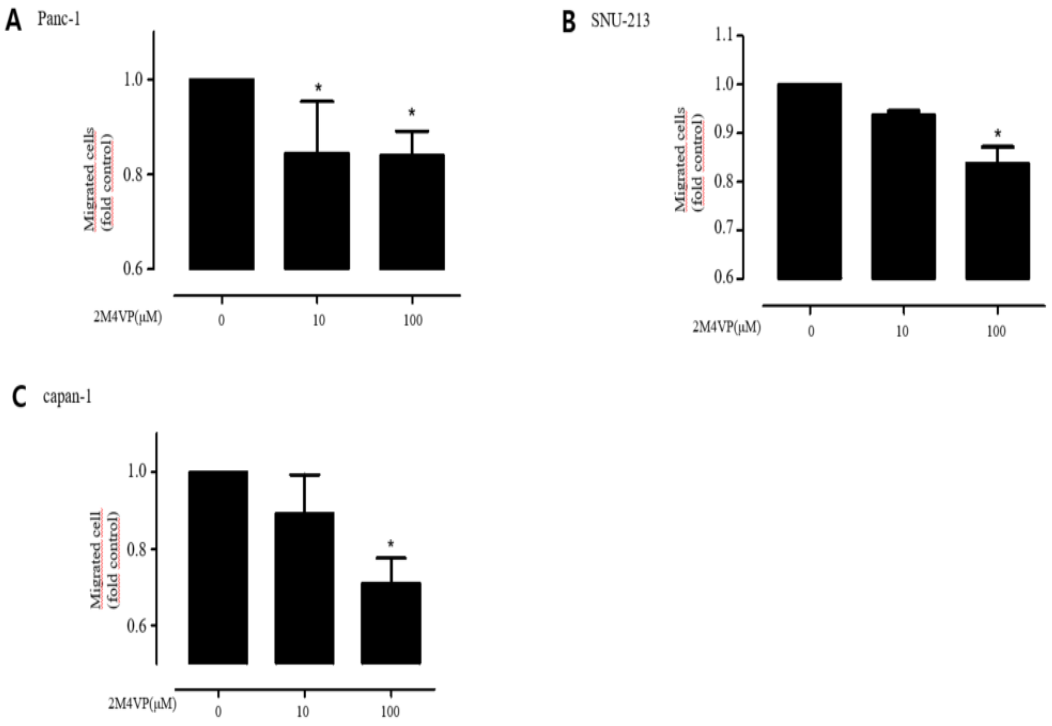

Figure 5. 2M4VP migration inhibition effect. (A) Migration activities on Panc-1 cells after treatment of 2M4VP for 6 h. (B) Migration activities on SNU-213 cells after treatment of 2M4VP for 6 h. (C) Migration activities on Capan-1 cells after treatment of 2M4VP for 12 h.

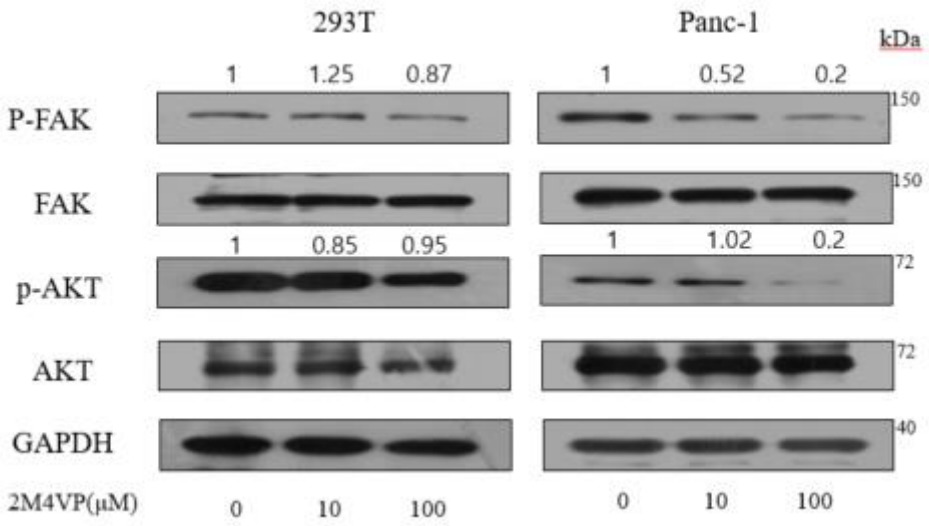

Figure 6. The expression and phosphorylation analysis of FAK and AKT in 293T and Pacn-1 cells. The relative band intensities of p-FAK/FAK and p-AKT/AKT were measured using Image J software.

3.3 Panc-1 inhibits hepatocyte growth factor-induced metastasis by 2M4VP.

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 the HGF-induced metastasis, pancreatic cancer cells were first treated with 2M4VP for 30min 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 a 40% increase in metastasis, but 2M4VP treatment inhibited the migration induced by HGF, maintaining the same level of metastasis as in untreated cells (Figure 7A). In addition, we found that the phosphorylation level of AKT increased with HGF treatment and decreased to untreated levels by pretreatment with 2M4VP (Figure 7B). These results suggest that 2M4VP reduces the phosphorylation level of HGF-induced AKT and inhibits metastasis in Panc-1 cells.

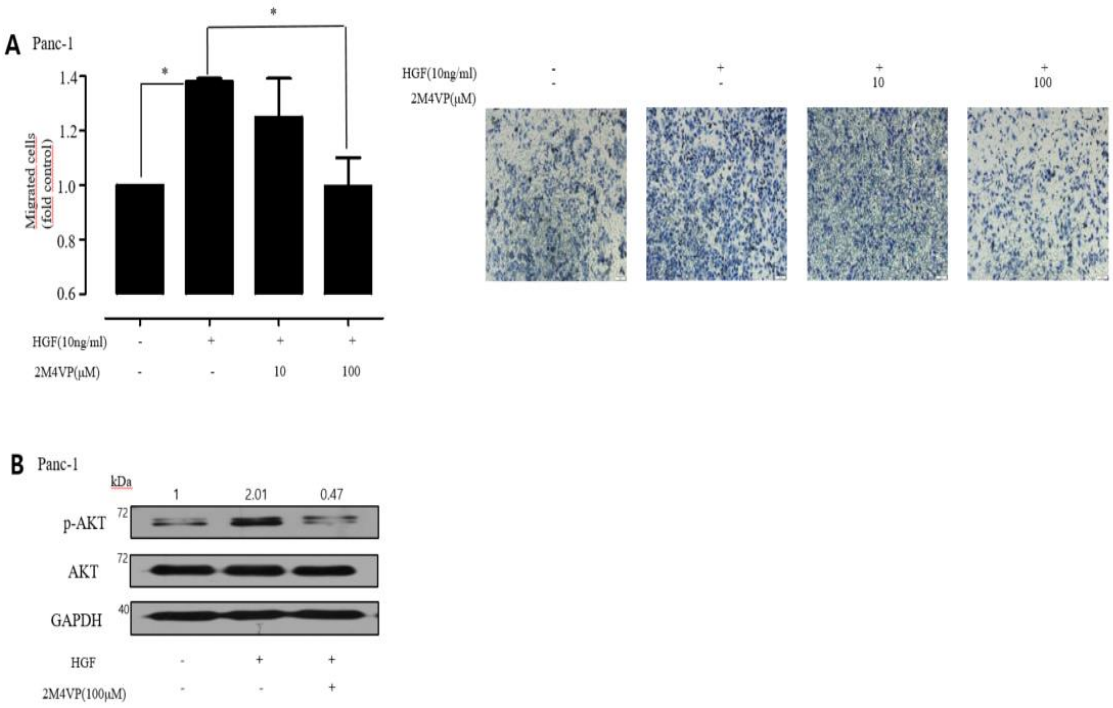

Figure 7. Effect of 2M4VP on HGF-induced migration in Panc-1 cells (A).
(B) Effect of 2M4VP on the expression and phosphorylation of AKT in HGF-treated Panc-1 cells. The relative band intensities of p-AKT/AKT was measured using Image J software. ($p < 0.05$; stars indicate a significant difference vs. 0, $*p < 0.05$).

4. DISCUSSION

2-methoxy-4-vinylpenol (2M4VP) is an aromatic compound that has an anti-inflammatory effect by inhibiting NO production and that inhibits cell-cycle activation induced by a carcinogen, benzopyrene (20, 21). However, there have been no studies on the specific relationship between 2M4VP and cancer cells. In this study, we found that 2-methoxy-4-vinyl phenol (2M4VP) has anticancer activity in inhibiting cell proliferation and metastasis in pancreatic cancer cells. Proliferating cell nuclear antigen (PCNA) is known to regulate the cell cycle and cell proliferation by tetramerization with cyclin and p21 (22). In our experiment, 2M4VP treatment reduced the expression of PCNA by above 50% in Panc-1 cells. It can be expected that 2M4VP inhibits Panc-1 cell proliferation by inhibiting PCNA expression. This result is similar to that of a previous report that matrine, a compound isolated from the legume, suppresses cell proliferation by inhibiting the expression of PCNA and its related genes(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 level of FAK. The PI3K/AKT pathway is known to be activated when hepatocyte growth factor (HGF) binds to the receptor, which control the

invasion and metastasis of cancer cells (11, 12, 28). HGF-induced cell migration in Panc-1 cells was reduced dose-dependently following treatment with 2M4VP. The induced AKT phosphorylation was also decreased. In conclusion, 2M4VP inhibited proliferation and metastasis in Panc-1 and blocked HGF-mediated metastasis. This study predicts that 2M4VP can be used as a reagent to inhibit metastasis of pancreatic cancer.

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그 동안 저에게 도움을 주신 모든 분들께 다시 한 번 감사의 인사를 드립니다.