Phenethyl Isothiocyanate Inhibits Migration and Invasion of Human Gastric Cancer AGS Cells through Suppressing MAPK and NF-κB Signal Pathways

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Abstract. Cell motility involves metastasis suppressors and other regulators that play an important role in tumor invasion and metastasis. Phenethyl isothiocyanate (PEITC), found in dietary cruciferous vegetables, has been found to exhibit antitumor properties and therefore is of special interest for the development of chemopreventive and chemotherapeutic agents for human cancers. Here, we report that in addition to its function as an anticancer agent, and PEITC can inhibit migration and invasion through the extracellular signal-regulated kinases 1/2 (ERK1/2), protein kinase C (PKC) and nuclear factor-kappaB (NF-κB) signaling pathways in human gastric cells. The results from wound healing and Boyden chamber assays (migration and invasion) indicated that PEITC exhibited an inhibitory effect on the migration and invasion of AGS cells. Results from Western blotting examination demonstrated that PEITC exerted an inhibitory effect on the ERK1/2, mitogen-activated protein kinase kinase 7 (MKK7), MAP kinase kinase kinase 3 (MEKK3), son of sevenless 1 (SOS1), PKC, Ras homolog gene family, member A (Rho A) and urokinase-type plasminogen activator (uPA), causing the inhibition of matrix metallopeptidase-2 (MMP-2) and -9 then followed by the inhibition of invasion and migration of GAS cells in vitro. PEITC also inhibited Ras, growth factor receptor-bound protein 2 (GRB2), vascular endothelial growth factor (VEGF), focal adhesion kinase (FAK), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), causing inhibition of cell proliferation of AGS cells. Results from real-time PCR showed that PEITC inhibited the gene expressions of MMP-2, -7 and -9, FAK and RhoA after PEITC treatment for 24 and 48 h of AGS cells. Taken together, these findings may provide insight into new mechanisms and functions of PEITC in migration and invasion of human gastric cancer AGS cells. Our data imply that molecular targeting of PKC leading to the inhibition of MMP-2 and -9 might be a useful strategy for the inhibition of migration and invasion of human gastric cancer.

Stomach cancer is the fourth most common cancer and the second most common cause of cancer death in the world (1). In Taiwan, about 10 people per 100 thousand die annually from stomach cancer, based on reports from Department of Health, R.O.C. (Taiwan) in year 2008. Although many treatments including surgery, radiation, chemotherapy, or combination of radiotherapy with chemotherapy have been used for patients, the mortality of stomach cancer remains high. It was reported that dietary intake of cruciferous vegetables may reduce the risk of various types of malignancies and organic isothiocyanates are important components of such vegetables (2, 3). Phenethyl isothiocyanate (PEITC) has been shown to possess chemopreventive activity (4). PEITC inhibits cytochrome P450...
(CYP) enzymes and induces phase II detoxification enzymes and apoptosis in HT-29 cells (5). In an animal model, PEITC inhibited 4-(methylmercaptosulfonyl)-1-(3-pyridyl)-1-butene-induced pulmonary neoplasia (6, 7) and also inhibited azoxymethane-induced colonic aberrant crypt foci formation (8).

Metastasis of cancer cells is associated with various steps and cytophysiological changes such as changed adhesion between cells and the extracellular matrix (ECM) and this disrupted intercellular interaction and the ECM degradation are associated with tumor invasion and migration. Overexpression of matrix metalloproteases (MMPs) and urokinase-type plasminogen activator (uPA) are associated with the metastasis of cancer cells. Much evidence has shown that MMPs are involved in the invasion and metastasis of various tumor cells (9-11). It was reported that MMP-2 and MMP-9 are capable of degrading most ECM components that form the basal membrane (12-14). Several studies demonstrated that inhibition of MMP expression or enzyme activity can be used as targets for preventing cancer metastasis (15-16).

Many experiments have demonstrated that PEITC can induce apoptosis in many types of human cancer cells (17-21), but there is no information to show whether PEITC inhibits the migration and invasion of stomach cancer cells. Therefore, in the present study, we investigated the effect of PEITC on the migration and invasion of human stomach cancer AGS cells in vitro.

**Materials and Methods**

**Chemicals and reagents.** PEITC, dimethyl sulfoxide (DMSO), potassium phosphates, propidium iodide and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco (Invitrogen, Carlsbad, CA, USA). Primary antibodies used for Western blotting analysis were obtained as follows: antibodies for MMP-2, MMP-9, inducible nitric oxide synthase (iNOS), nuclear factor-kappaB (NF-κB) p65, cyclooxygenase-2 (COX-2), uPA, mitogen-activated protein kinase kinase 7 (MEKK7), MAP kinase kinase 3 (MEKK3), focal adhesion kinase (FAK), extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun NH2-terminal kinases 1/2 (JNK1/2), p-p38, Ras, growth factor receptor-bound protein 2 (GRB2), vascular endothelial growth factor (VEGF), Ras homolog gene family, member A (Rho A), Rho-associated coiled-coil-containing protein kinases 1 (ROCK1), son of sevenless 1 (SOS1), phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC) and β-Actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and diluted before use.

**AGS cell line.** Human gastric cancer AGS cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, R.O.C.). Cells were cultured with 88% RPMI-1640 medium with 2 mM L-glutamine and supplemented with 10% FBS, and 1% penicillin-streptomycin (100 Units/ml penicillin and 100 μg/ml streptomycin) and were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

**Wound-healing assay.** AGS cell migration was examined by using a wound-healing assay. In brief, AGS cells (1×10⁵ cells/well) were placed for 24 h in six-well plates and at confluence a wound was made with a pipette tip followed by washing with serum-free medium to remove cell debris. They were then photographed under phase-contrast microscopy (time=0) and then incubated in media with or without PEITC (0.01 and 0.25 μM) at 37°C and 5% CO₂ and allowed to migrate into the wound area for up to 24 h at 37°C. Then cells were gently washed with phosphate buffered saline (PBS) and the wound area photographed under phase-contrast microscope (22). Experiments were performed in triplicate.

**In vitro migration and invasion assays.** The migration of AGS cells was also measured by chemotactic directional migration by using a 24-well Transwell insert. The 8 μm pore filters (Millipore, MA, USA) were coated with 30 μg type I collagen (Millipore) for 1 h and AGS cells (10⁴ cells/0.4 ml RPMI-1640 medium) were placed in the upper chamber with or without PEITC (0.25 or 0.50 μM) and allowed to undergo migration for 24 and 48 h. The non-migrated cells in the upper chamber were removed with a cotton swab. The filters were stained with 2% crystal violet. Migrated cells adherent to the underside of the filter were counted and photographed under a light microscope at ×200 (23-24).

The invasion of AGS cells was measured using Matrigel-coated transwell cell culture chambers (8 μm pore size) as previously described (23, 25). After cells were cultured for 24 h in serum-free RPMI-1640 medium, they were collected and resuspended in serum-free medium, and then were placed in the upper chamber of the transwell insert (5×10⁴ cells/well) and treated with 0.5% DMSO (as a control) or PEITC (0.25 or 0.50 μM). RPMI-1640 medium containing 10% FBS was placed in the lower chamber. All cells in each treatment were incubated for 24 or 48 h at 37°C in a humidified atmosphere with 95% air and 5% CO₂. The non-invasive cells maintained in the upper chamber were removed by wiping with a cotton swab and the invasive cells were fixed with 4% formaldehyde in PBS and stained with 2% crystal violet in 2% ethanol. The cells (invasive cells) in the lower surface of the filter which penetrated through the Matrigel were counted and photographed under a light microscope at ×200 (23). Each treatment, including control, was assayed twice and three independent experiments were performed.

**Western blotting analysis.** AGS cells (1×10⁶ cells/well) were cultured in 6-well plates and grown for 24 h. PEITC was added to cells in each well at a final concentration of 2.5 μM, while DMSO (solvent) alone was added to control cells. All cells in the wells were incubated at 37°C for 0, 6, 12, 24 and 48 h. The cells were then collected and resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 0.1% Triton X-100 for sonication and centrifugation at 13,000 g for 10 min at 4°C to remove cell debris. The supernatant was collected and total protein concentration of each sample was determined using a Bio-Rad protein assay kit (Hercules, CA, USA) with bovine serum albumin (BSA) as the standard. SDS gel electrophoresis and Western blotting were conducted to determine the effects of PEITC on protein levels of MMP-2, MMP-9, iNOS, NF-κB p65, COX-2, uPA, MKK7, MEKK3, FAK, ERK1/2, JNK1/2 and p-p38, Ras, GRB2, VEGF, RhoA, ROCK1, SOS1, PI3K, PKC and β-actin as described previously (26-27).
Student’s changes were derived using the comparative CT method. PCR system was used for each assay in triplicate and expression fold-
primers as shown in Table Ι. Applied Biosystems 7300 Real-Time
Master Mix (Applied Biosystems) and 200 nM of forward and reverse
cDNA reverse-transcribed as described above, 2X SYBR Green PCR
95˚C, and 40 cycles of 15 s at 95˚C; 1 min at 60˚C using 1 μl of the
RNA samples were reverse-transcribed at 42˚C with High
Capacity cDNA Reverse Transcription Kit for 30 min according to
2.5 μM for 24 and 48 h. Cells were collected and total RNA was
incubated with PEITC for 24 h, respectively. AGS cells were also tented for their ability to invade through
a filter coated with Matrigel on treatment with PEITC for 24
and invasion in AGS cells. The breakdown of biological barriers such as the
basement membrane which requires activation of proteolytic
digestion of ECM, proteolytic cleavage or destruction of the ECM, and cell
treatment, the inhibition ranged from 37-54% (Figure 2D).
Significant inhibition of cell invasion occurred in a
dose-dependent manner, as shown in Figure 2C and D. The
percentage inhibition at 0.25 μM PEITC was 53-55% for 24
and 48 h treatments and at 0.50 μM PEITC for 24 and 48 h
treatment, the inhibition ranged from 37-54% (Figure 2D).

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<tr>
<td>homo MMP-2  F</td>
<td>CCCAGACAGGTGATCTTGAC</td>
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<tr>
<td>R</td>
<td>GCTTGGAGGGAAGAAGTTG</td>
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<td>homo MMP-7  F</td>
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<td>R</td>
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<td>homo FAK    F</td>
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<td>ACACCCACTCCTCCACCTT</td>
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<td>R</td>
<td>TAGCCAAATCCGTGTCATACC</td>
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**Real-time PCR of MMP-2, -7, and -9, FAK and Rho A.** AGS cells
(1×10⁶ cells/well) were cultured in 6-well plates and grown for 24 h.
PEITC was added to cells in each well for a final concentration of 2.5 μM for 24 and 48 h. Cells were collected and total RNA was extracted using Qiagen RNeasy Mini Kit as described previously (25, 28). RNA samples were reverse-transcribed at 42˚C with High Capacity cDNA Reverse Transcription Kit for 30 min according to
the protocol of the supplier (Applied Biosystems, Foster City, CA, USA). Quantitative PCR conditions were: 2 min at 50˚C, 10 min at 95˚C, and 40 cycles of 15 s at 95˚C; 1 min at 60˚C using 1 μl of the
cDNA reverse-transcribed as described above, 2X SYBR Green PCR
Master Mix (Applied Biosystems) and 200 nM of forward and reverse
primers as shown in Table I. Applied Biosystems 7300 Real-Time
PCR system was used for each assay in triplicate and expression fold-
changes were derived using the comparative CT method.

**Results**

**Effects of PEITC on migration of AGS cells.** To determine the effect of PEITC on cell migration, AGS cells were incubated with different concentrations of PEITC for 24 h, then wound healing assay was carried out; the results are shown in Figure 1. An apparent and gradual increase of cells in the denuded zone was observed by light microscopy. The quantitative data in Figure 1B indicate a significant inhibition of cell migration and this was also time-dependent.

**Effect of PEITC on migration and invasion of AGS cells.** To further examine the effect of PEITC on cell migration, AGS cells were incubated with different concentrations of PEITC for 24 and 48 h in a migration assay. Results from the migration assay are shown in Figure 2A and B, indicating that PEITC had a significant inhibitory effect on cell migration at concentrations between 0.25-0.50 μM and the percentage inhibition was at 44-45% and 38-47% when cells were incubated with PEITC for 24 and 48 h, respectively. AGS cells were also tented for their ability to invade through a filter coated with Matrigel on treatment with PEITC for 24 and 48 h. Significant inhibition of cell invasion occurred in a dose-dependent manner, as shown in Figure 2C and D. The percentage inhibition at 0.25 μM PEITC was 53-55% for 24 and 48 h treatments and at 0.50 μM PEITC for 24 and 48 h treatment, the inhibition ranged from 37-54% (Figure 2D).

**Effect of PEITC on levels of proteins associated with migration and invasion in AGS cells.** In order to determine the effects of PEITC on the protein levels associated with migration and invasion in AGS cells, Western blotting was applied after the cells were exposed to PEITC. The results are shown in Figure 3 and indicate that PEITC reduced levels of MMP-2 and MMP-9 (Figure 3A) iNOS, COX-2 and uPA (Figure 3B),

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<td>homo GAPDH  F</td>
<td>ACACCCACTCCTCCACCTT</td>
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MMP, Matrix metalloproteinase; FAK, focal adhesion kinase; RhoA, RAS homologue gene family member A; ROCK1, Rho-associated coiled coil-containing kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward primers; R, reverse primers.

**Discussion**

Cancer cell metastasis involves tumor cell adhesion to the ECM, proteolytic cleavage or destruction of the ECM, and cell migration through the resultant defect. In the present study, we demonstrate that PEITC reduced invasion and migration of AGS cells. The breakdown of biological barriers such as the basement membrane which requires activation of proteolytic enzymes is a critical step for invasion and metastasis (29, 30). Of these basement membrane-degrading enzymes, the MMPs play an important role in tumor angiogenesis, metastasis and growth factor release from the ECM (26). MMP-2 (72-kDa gelatinase A) and MMP-9 (92-kDa gelatinase B) are associated with the invasive metastatic potential of tumor cells.
Figure 1. Effects of PEITC on the migration of AGS cells: Wound-healing migration assay. AGS cells were placed on the plate and a wound line was made with a tip. The cells were incubated with or without 0.25 and 0.50 μM of PEITC for 0, 24 and 48 h. A: Cell migration was assessed by microscopy at the indicated time points (×100). B: The mean number cells in the denuded zone were shown when compared with control group at 0 h. Each bar represents the mean±S.D. (n=3). *Significantly different from the control (0 μM) at p<0.05.
Figure 2. Effect of PEITC on migration and invasion of AGS cells. AGS cells were treated with PEITC for 24 and 48 h. Cell migration was measured in a Boyden chamber assay with A: polycarbonate filters (pore size, 8 μm); and C: polycarbonate filters were precoated with Matrigel. Migration (B) and invasion (D) ability of AGS cells were quantified by counting the number of cells that invaded the underside of the porous polycarbonate membrane under microscopy and represent the average of three experiments. *p<0.05 compared with the untreated control (dose 0 μM).
It was reported that inhibition of MMP expression or enzyme activity can be used as targets for preventing cancer metastasis (15-16). Thus, the present of cell migration provides many molecular targets for the development of therapeutic agents to inhibit cancer metastasis.

Our results showed that PEITC inhibited the migration and invasion of AGS cells through the examinations of wound healing assay and migration and invasion assays and these effects occurred in dose- and time-dependent manners. Furthermore, we found that PEITC reduced the level of proteins associated with migration and invasion, such as MMP-2 and MMP-9, iNOS, COX-2, uPA, MKK7, MEKK3, FAK, ERK1/2, JNK, Ras, GRB2, VEGF, RhoA and ROCK1, but increased protein levels of NF-κB p65, p-p38 and PI3K.
Overexpression of MMPs such as MMP-2 (32, 33), MMP-7 (34, 35) and MMP-9 (36) has been observed in gastric cancer. But our result did not show such an effect of PEITC on MMP-7 protein level.

However, our results also showed that PEITC suppressed MMP-2, MMP-7, MMP-9, FAK and RhoA gene expressions, which may suppress the PKC/MAPK and PI3K/AKT/NF-κB cascades with consequent suppression of migration and invasion by human gastric cancer AGS cells.

Highly metastatic tumors have been shown to overexpress MMP-2, and MMP-9 can be stimulated by TNF-α (37) or growth factors such as VEGF, Epidermal growth factor (EGF) and transforming growth factor beta (TGF-β) (38-39), or Ras oncogene (5, 40) through activation of different intracellular signaling pathways. Results from the present study indicate that PEITC inhibited VEGF and Ras, which may have led to the inhibition of MMP-7 expression. Other investigators also showed that the activation of PKC led to translocation of the protein to membranes and led to control of the expression of MMP-9 through modulating the activation of transcription factors including NF-κB or SP-1 through MAPK and PI3K signaling pathways (40-42). Our results also showed that PEITC promoted the levels of NF-κB in AGS cells.

In the present study, we observed that PEITC inhibited not only phosphorylation of JNK but also VEGF, MMP-9 and uPA expression, suggesting that inhibition of JNK by PEITC is involved in the inhibition of VEGF, MMP-9 and uPA expression. These results indicate that inhibition of JNK activity contributes to the decrease in migration of PEITC-treated AGS cells. The results in the present study also showed that the regulation of NF-κB, and downstream of the PI3K/AKT and MAPK (ERK1/2, p38 and JNK) pathways might be involved in PEITC suppression of MMP-9 expression and invasion in AGS cells. The activation of NF-κB is involved in the induction of the MMP-9 gene associated with the invasion and metastasis of tumor cells (37, 43). We also found that PEITC reduced the JNK and PKC levels (Figure 4). It was reported that resveratrol suppresses MMP-9 expression in phorbol myristate acetate (PMA)-induced human Caski cells by blocking JNK and PKCδ signal transduction (44).

Our results also showed that PEITC reduced the level of uPA, which has been shown to play a major role in the decomposition of basement membranes. The activation of the uPA/uPAR/plasmin proteolytic network is involved in tumor invasion and dissemination of various malignancies (45-46). Thus, the presence of uPA in tumor tissues is a potential prognostic factor, and the levels of uPA and uPAR expression serve as prognostic markers in various malignancies. Several
reports indicated that ERK1/2, p38 and JNK play a central role in regulating the expression of MMPs and uPA (47-49). Thus, inhibition of the MAPK pathway might have the potential to prevent invasion, and metastasis for a wide range of tumor types. Other signal pathways, such as the PI3K/AKT signal transduction pathway regulating cell metastasis of prostate cancer PC-3 cells, are closely associated with the development and progress of various tumors (49). In the present study, PEITC also reduced JNK1/2. Future studies are needed to explore the relationship between the expression of MMPs and phosphorylation of JNK in gastric cancer cells treated with PEITC.

In summary, the molecular mechanism of PEITC appears to be via down-regulation of PKC and then blocking of MAPK signaling pathways through NF-κB, as well as uPA, which then led to the inhibition of MMP-2 and MMP-9 (Figure 5). The present results may be relevant to the therapeutic targeting of invasion and migration of gastric cancer cells.

Acknowledgements

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