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Original Article

Phenethyl isothiocyanate inhibits the migration and invasion of colon cancer SW480 cells via the inhibition of matrix metalloproteinase-9

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Abstract: This study is to investigate the effect of phenethyl isothiocyanate (PEITC) on the invasion and metastasis of colon cancer. SW480 cells were cultured with PEITC for 24 h. MTT assay was used to detect cell proliferation. Wound healing assay and transwell invasion assay were used to determine cell invasion and migration, respectively. Matrix metalloproteinase (MMP)-9 activity was determined using fluorescence resonance energy transfer. The qRT-PCR was performed to measure MMP-9 mRNA expression. Western blotting was used to determine the expression of PI3K and PTEN, the phosphorylation of Akt and mTOR, and the nuclear translocation of nuclear factor-kappa B (NF-κB). NF-κB activity was tested using dual-luciferase reporter assay. Mouse model was constructed to investigate the effect of PEITC on xenograft tumor growth. SW480 cell invasion and migration were inhibited by PEITC within toxicity-free dose ranges, as demonstrated by scratch healing assay and transwell membrane assay. Molecular data showed that the effect of PEITC also inhibited the enzymatic activity and mRNA expression of MMP-9. In addition, PEITC inhibited the expression of PI3K, as well as the phosphorylation of Akt and mTOR, and up-regulated the expression of PTEN. PEITC-inhibited MMP-9 expression or activity appeared to occur via NF-κB as long as its nuclear translocation and transcriptional activity was suppressed by PEITC. Furthermore, PEITC inhibited xenograft tumor growth in mice. PEITC is a potential drug for the treatment of colon cancer metastasis. It exerts its effect by regulating PI3K/Akt and nuclear factor-kappa B pathways.

Keywords: Phenethyl isothiocyanate, SW480 cells, matrix metalloproteinase-9, PI3K/Akt, nuclear factor-kappa B

Introduction

Colon cancer is the commonest malignant tumor in gastroenterological surgery, with an incidence of over 1.2 million every year in the world [1, 2]. Similar to patients with other malignant tumors, more than 90% patients with colon cancer die of invasion and metastasis of cancer cells [3-5]. Like other malignant tumors, colon cancer cells also have invasion and metastasis. These processes include adherence of tumor cells with extracellular matrix (ECM), degradation of ECM by protease released by tumor cells, and migration of tumor cells under the guidance of chemokines [6]. In these processes, ECM plays an important barrier role during invasion and metastasis. Therefore, any materials that may disturb the dynamic balance of ECM may be related to tumor invasion and metastasis. Matrix metalloproteinases (MMPs) are a kind of enzyme that degrades all components of ECM [7]. Because MMPs play critical roles in the degradation of ECM, the proteolytic activities of MMPs are involved in the metastasis process, including cell adhesion, migration, and invasion [8]. Among all MMPs, MMP-9 (also named gelatinase B) is of the most importance [8-10]. Therefore, targeting of MMP-9 has important clinical significance in the treatment of colon cancer.

Currently, the treatment of colon cancer is still via radical surgery. However, patients who are admitted at hospital usually have middle or late

stage of colon cancer. In order to alleviate the prognosis of patients with gastric cancer and to reduce the recurrence after surgery, chemotherapy becomes very important. Chemotherapy has certain effect on late colon cancer, but is still disappointing in 5-year survival rate of cancer patients [1, 2, 6, 9, 10]. Therefore, searching for auxiliary treatment drugs with high efficiency and low toxicity becomes a research hotspot. Phenethyl isothiocyanate (PEITC), a type of isothiocyanates, widely exists in crucifers such as olives and cauliflowers. A study shows that PEITC arrests cells in G_2/M phase by binding and stabilizing β-tubulin [11]. In addition, PEITC inhibits the activity of deacetylase [12]. In vivo and in vitro toxicity tests show that PEITC has no obvious adverse reactions on normal tissues and cells [13]. However, it is still not clear whether PEITC affects the metastasis and invasion of colon cancer cells. The present study investigates the effect of PEITC on the metastasis and invasion of colon cancer SW-480 cells, as well as the underlying regulatory mechanism.

Materials and methods

Cells

SW480 cell line was obtained from Cancer Research Institute, Central South University and cultured using RPMI-1640 medium supplemented with 10% fetal bovine serum under 37°C and 5% CO_2. The cells were cultured in the presence of dimethyl sulfoxide (control group) or 10, 30 and 50 μmol/L PEITC (purity ≥ 95%, LKT Laboratories Inc., St. Paul, MN, USA; experimental groups) for 24 h.

Animals

Specific pathogen-free class BALB/c (nu/nu) male nude mice (6-8 weeks old; body weights 16-18 g) were obtained from the Department of Animals, Nanhua University (Lot No. 201203-02). Log-phase SW480 cells (5×10^6) were injected subcutaneously into right axilla of each mouse, which was then fed in specific pathogen-free environment. Tumor diameter was measured every 2 or 3 days. When the diameter of tumors reached 0.5-0.6 cm, PEITC (0.5, 1 and 5 μmol/g body weight) was administered by gastric lavage every 2 days for a total of 7 times. The mice had free access to food and water. The body weight and tumor weight were measured 20 days after the last gastric lavage. All animal experiments were conducted according to the ethical guidelines of Southern Medical University.

Methylthiazolyldiphenyl-tetrazolium (MTT) assay

After incubation with DMSO or PEITC, 30 μL methylthiazolyldiphenyl-tetrazolium bromide (1 g/L) was added onto SW480 cells for another incubation for 2 h. The supernatants were discarded before addition of 200 μL DMSO with thorough mixing. The absorbance (A570) was measured using a microplate reader (Synergy HT, Biotek, Winooski, VT, USA) for the calculation of proliferation inhibition rate.

Fluorescence resonance energy transfer

After treatments, the supernatants were subject to determination of MMP-9 activity using SensoLyte® 490 MMP-9 Assay Kit (Ana Spec, Fremont, CA, USA). Fluorescence intensity was measured using a fluorescence reader (Synergy HT, Biotek, Winooski, VT, USA) with excitation and emission wavelengths being 340 nm and 490 nm, respectively. The relative MMP-9 activity of experimental groups is calculated by comparing with control group.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). QRT-PCR was performed using One Step PrimeScript RT-PCR kit (Takara, Dalian, China). PCR amplification conditions were as follows: initial denaturation at 94°C for 30 s, annealing at 65°C (MMP-9) or 59°C (GAPDH) for 30 s, elongation at 72°C for 30 s, repeated for 40 (MMP-9) or 35 (GAPDH) cycles. The primers for MMP-9 were 5'-CGCTACCACCTCGAACTTTG-3' and 5'-GCCATTCACGTCGTCCTTAT-3' (product: 196 bp). The primers for GAPDH were 5'-TCACCATCTTCCAGGAGCGA-3' and 5'-CACAATGCCGAAGTGGTCGT-3' (product: 700 bp). The products were then subject to 2% agarose gel electrophoresis before analysis using Image J software (http://rsb.info.nih.gov/ij/).

Wound healing assay

The cells (5×10^6) were seeded onto 6-well plates and cultured under 37°C for 6 h. When reaching confluence, the cell sheets were scratched using a 200 μL pipette tip. Debris was

removed by extensive washing with phosphate-buffered saline and the cells were further incubated with medium containing PEITC for another 24 h. Then, the images of the cells were taken under a microscope for the calculation of migration rate. Migration rate was calculated by the function: migration rate = (original scratch distance-present scratch distance)/original scratch distance × 100%. The measurements of scratch distance were performed in triplicate.

Transwell invasion assay

Matrigel was thawed at 4°C overnight and diluted with serum-free medium (dilution 1:1). The mixture (50 μL) was evenly smeared into the upper Transwell chamber (Corning, New York, NY, USA) and incubated at 37°C for 60 min. After solidification, 100 μL cell suspension (10^6 cells/ml) from each group were seeded into the upper chamber containing 200 μL serum-free medium. In addition, 500 μL complete medium was added into the lower chamber. After 24 h, the chamber was removed. After being fixed with paraformaldehyde for 10 min, the membrane was stained using 0.1% crystal violet for 20 min. After the cells in the upper chamber were wiped off, the chamber was visualized under a microscope for imaging of several random fields (200×). The number of transwell cells was calculated for the evaluation of cell invasion ability. Invasion inhibitory rate was calculated using the function: invasion inhibitory rate = (the number of cells crossing the membrane in control group-the number of cells crossing the membrane in treatment group)/the number of cells crossing the membrane in control group × 100%. The number of cells crossing the membrane was counted in three individual wells.

Western blotting

The expression of phosphoinisitide-3-kinase (PI3K) and phosphatase and tensin homologue (PTEN), the phosphorylation of Akt and mammalian target of rapamycin (mTOR), and the nuclear translocation of nuclear factor-kappa B (NF-κB) were determined using Western blotting assay. Cell total protein and nucleoprotein were extracted using NE-PER Nuclear and Cytoplasmic Extraction kits (Thermo Scientific, Waltham, MA, USA). A total of 100 μg protein was separated using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (Millipore, Billerica, MA, USA) before blocking with Tris-buffered saline and Tween 20 containing 5% skimmed milk for 1 h. Then, the membrane was incubated with primary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight. After extensive washing, the membrane was incubated with secondary antibodies labeled with horseradish peroxidase (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h. The immunoreactive bands were visualized by enhanced chemiluminescence (Amersham plc, Amersham, UK).

Dual-luciferase reporter assay

NF-κB luciferase reporter gene (SABioscicence, Qiagen, Venlo, the Netherlands) was transfected into SW480 cells using x-tremeGENE HP (Roche, Basel, Switzerland). After 18 h, cells were incubated with different concentrations of PEITC for another 24 h. Using Renilla fluorescence as internal reference, dual-luciferase reporter assay was performed by Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's manual.

Statistical analysis

The results were analyzed using SPSS 17.0 software (IBM, Armonk, NY, USA). The data were given in means ± standard deviation. Statistical analysis was carried out using unpaired Student's t-test. *P* value less than 0.05 was considered statistically significant.

Results

PEITC inhibits the migration and invasion of SW480 cells in a dose-dependent manner

To test SW480 cell migration and invasion, wound healing assay and transwell invasion assay were performed. The data showed that the migration rate of SW480 cells was significantly reduced by PEITC, with higher concentrations of PEITC resulting in lower migration rate ($P < 0.05$) (**Table 1**). In addition, the invasion inhibitory rate of SW480 cells was significantly enhanced by PEITC, with higher concentrations of PEITC leading to higher invasion inhibitory rate ($P < 0.05$) (**Table 1**). These results suggest that PEITC inhibits the migration and invasion of SW480 cells in a dose-dependent manner.

Table 1. Effect of PEITC on SW480 cell migration and invasion

Groups	Migration rate (%)	Invasion inhibitory rate (%)
Control	78.08 ± 3.14	8.13 ± 0.48
PEITC (10 μmol/L)	64.27 ± 5.14*	27.61 ± 1.01*
PEITC (30 μmol/L)	51.57 ± 4.39*	38.28 ± 0.69*
PEITC (50 μmol/L)	43.81 ± 3.61*	51.47 ± 1.37*

Note: Data were expressed as means ± standard deviation (n = 3). *P < 0.05 compared with control.

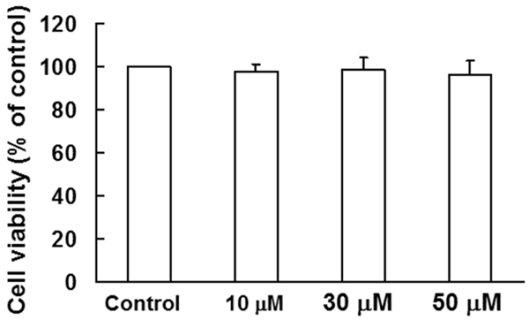

Figure 1. Effects of PEITC on SW480 cell viability and growth. SW480 cells were treated with various concentrations (10-50 μM) of PEITC for 24 h. Cell viability was analyzed by MTT assay. The results represented the average of three independent experiments ± S.D.

Decreased numbers of migrating and invading cells were not a consequence of reduced proliferation

To exclude the possibility that decreased numbers of migrating and invading cells were a consequence of reduced proliferation, we performed viability assays using SW480 cells treated with the same concentration that was used in the migration and invasion assays for 24 h. The data showed no significant difference between the treated cells and control cells (**Figure 1**). The results suggest that decreased numbers of migrating and invading cells were not a consequence of reduced proliferation.

PEITC down-regulates MMP-9 in SW480 cells in a dose-dependent manner

To measure the effect of PEITC on MMP-9 in SW480 cells, qRT-PCR and MMP-9 activity test were carried out. The data showed that higher concentrations of PEITC resulted in lower levels of MMP-9 mRNA (**Figure 2A**). With the increase of PEITC concentrations, the relative enzymatic activity of MMP-9 was gradually inhibited ($P < 0.05$) (**Figure 2B**). These results indicate that

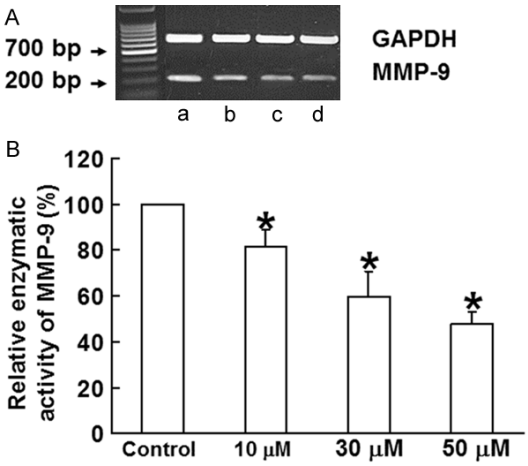

Figure 2. Effect of PEITC on mRNA expression and enzymatic activity of MMP-9. A. MMP-9 mRNA was determined by qRT-PCR; B. Relative enzymatic activity of MMP-9. a. Control; b. 10 μmol/L PEITC; c. 30 μmol/L PEITC; d. 50 μmol/L PEITC. *P < 0.05 compared with control.

PEITC down-regulates MMP-9 in SW480 cells in a dose-dependent manner.

PEITC inhibits the PI3K/AKT pathway in SW480 cells

To investigate the role of PEITC in PI3K/AKT pathways in SW480 cells, Western blotting assay was used. The data showed that the levels of PI3K (P85) were reduced after PEITC treatment (**Figure 3A**). In addition, treatment with PEITC decreased the levels of phosphorylated Akt (p-Akt) and phosphorylated mTOR (p-mTOR), but had no effect on Akt and mTOR (**Figure 3B** and **3C**). In addition, PEITC treatment up-regulated the expression of PTEN in SW480 cells (**Figure 3D**). These results suggest that PEITC inhibits the PI3K/AKT pathway in SW480 cells.

PEITC inhibits the NF-κB pathway in SW480 cells

To test the effect of PEITC on NF-κB pathway in SW480 cells, we performed Western blotting and dual-luciferase reporter assay. Western blotting data showed that translocation of P65 subunit of NF-κB was inhibited by PEITC (**Figure 4A**). In addition, PEITC (10, 30 and 50 μmol/L) inhibited the activity of NF-κB transcription, with PEITC (50 μmol/L) having the strongest inhibitory effect (42% reduction; $P < 0.05$) (**Figure 4B**). These results indicate that PEITC inhibits the NF-κB pathway in SW480 cells.

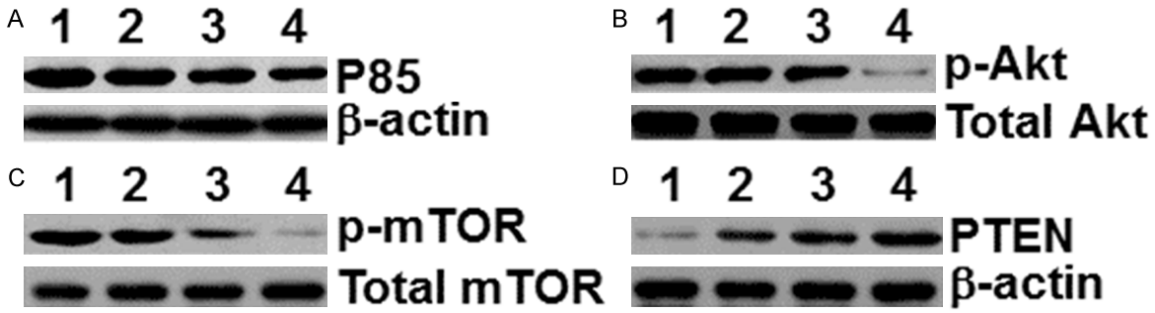

Figure 3. Effect of PEITC on PI3K/AKT pathways in SW480 cells. (A) P85, (B) Phosphorylated Akt (p-Akt) and total Akt, (C) Phosphorylated mTOR (p-mTOR) and total mTOR, and (D) PTEN expressions were measured with Western blotting. A total of 100 μg protein was separated using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (Millipore, Billerica, MA, USA) before blocking with Tris-buffered saline and Tween 20 containing 5% skimmed milk for 1 h. Then, the membrane was incubated with primary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight. After extensive washing, the membrane was incubated with secondary antibodies labeled with horseradish peroxidase (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h. The immunoreactive bands were visualized by enhanced chemiluminescence (Amersham plc, Amersham, UK).

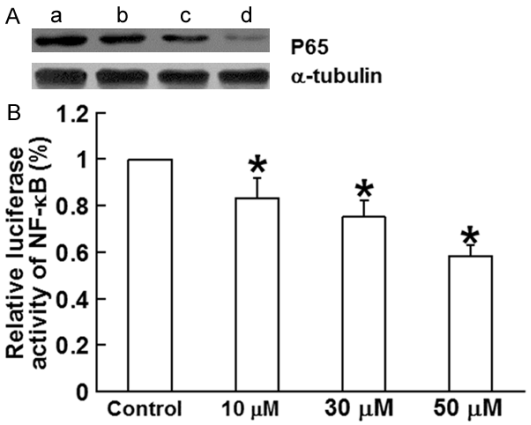

Figure 4. Effect of PEITC on NF-κB pathway in SW480 cells. A. Western blotting analysis of P65 nuclear translocation; B. Luciferase activity of NF-κB. Luciferase reporter gene (SABioscicence, Qiagen, Venlo, the Netherlands) was transfected into SW480 cells using x-treme GENE HP (Roche, Basel, Switzerland). After 18 h, cells were incubated with different concentrations of PEITC for another 24 h. Using Renilla fluorescence as internal reference, dual-luciferase reporter assay was performed by Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). NF-κB, nuclear factor-kappa B. a. Control; b. 10 μmol/L PEITC; c. 30 μmol/L PEITC; d. 50 μmol/L PEITC. *P < 0.05 compared with control.

PEITC inhibits xenograft tumor growth in vivo in a dose-dependent manner

To study the effect of PEITC on tumor growth, we constructed xenograft tumors in BALB/c nude mice. The data showed that PEITC significantly inhibited tumor weight, with higher doses of PEITC corresponding to higher inhibition rate (**Table 2**). The results suggest that PEITC inhibits xenograft tumor growth *in vivo* in a dose-dependent manner.

Discussion

During the invasion of tumor cells, degradation of ECM by proteolytic enzymes such as MMP-9 is the key to tumor cell invasion and metastasis. Studies show that PEITC inhibits the metastasis of prostate cancer, breast cancer and lung cancer [14, 15]. However, the effect of PEITC on colon cancer is not totally clear yet. The present study demonstrates that PEITC within toxicity-free concentration ranges significantly inhibits the invasion and migration of SW480 cells within, reduces cells that adhere to matrix membrane, and significantly inhibits gelatinase activity and MMP-9 expression, finally inhibiting tumor growth. These observations demonstrate that PEITC exerts its anti-invasion effect by inhibiting the expression and activity of MMP-9.

PI3K is a kind of lipid kinase that regulates multiple physiological processes by activating Akt (protein kinase B). It locates in cytoplasm by inducing $P21^{WAF1}$, and exerts its anti-apoptotic effect by deactivating proapoptotic factors BAD and caspase-9. In addition, activated Akt promotes cancer cell invasion and migration by inducing the expression of MMPs [16]. mTOR is one of the downstream target molecules that are regulated by Akt. It leads to ECM reconstruction by inducing the secretion of MMP-2 and MMP-9, resulting in tumor cell metastasis

Table 2. Effect of PEITC on the growth of xenograft tumors

Groups	Body weight (g)	Tumor weight (g)	Inhibition rate (%)
Control	19.45 ± 2.31	1.63 ± 0.41	N/A
PEITC (0.5 μmol/g weight)	20.02 ± 1.73	1.31 ± 0.29*	19.63
PEITC (1 μmol/g weight)	18.45 ± 2.04	0.82 ± 0.19*	49.69
PEITC (5 μmol/g weight)	17.38 ± 2.26	0.62 ± 0.13*	61.96

Note: Data were expressed as means ± standard deviation (n = 6). *P < 0.05 compared with control.

[17]. PTEN is a lipid phosphatase that has dual specificity. It inhibits Akt activity by negatively regulating PI3K/AKT pathway. A study shows that MMP-9 mRNA levels is positively correlated to PI3K/AKT/mTOR pathway, but is negatively correlated with PTEN [18]. The present study also demonstrated that PEITC increased the expression of PTEN and inhibited PI3K (P85) pathway and the phosphorylation of Akt and mTOR. Because of its anti-function on PI3K/Akt/AP-1, PEITC might, in turn, have a significant impact on the mechanism to inhibit MMP-mediated cellular events in SW480 cells.

NF-κB is an important target molecule downstream of Akt that participates in the regulation of MMP-9 expression. It is closely related to inflammation, tumor cell proliferation, invasion and migration [19]. In addition, NF-κB is constantly active multiple types of tumor cells, and induces the expression of several anti-apoptotic proteins that leads to chemoradiotherapy tolerance [20]. Therefore, inhibition of NF-κB activity can promote therapeutic effects of anticancer drugs. The present study showed that PEITC effectively inhibited the transcriptional activity of NF-κB and its nuclear translocation. Since a binding site for NF-κB exists in the promoter region of MMP-9 [21], the inhibitory effect of PEITC on NF-κB activity might be the reason for the down-regulation of MMP-9 expression. However, further investigation is required to confirm the effects of PEITC on other factors that are important for the transcriptional activation of NF-κB, including IκBα ubiquitination and p65 serine 536 residue phosphorylation.

In conclusion, the present study demonstrates that PEITC inhibits SW480 cell invasion and tumor growth, possibly through the inhibition of the expression and activity of MMP-9, which are achieved by up-regulating PTEN expression and inhibiting PI3K/Akt pathway and NF-κB activity. Therefore, PEITC might become a potential auxiliary drug for the treatment of colon cancer. The anti-invasive effects of PEITC on SW480 cells might occur by inhibiting the degradation of IκBα protein expression to reduce NF-κB translocation and NF-κB DNA-binding activities, leading to down-regulation of MMP-9 expression. PEITC might also inhibit the slug-E-cadherin pathway to suppress cancer cell invasion. Previous research indicates that slug also mediates MMP-9 expression in oral cancer [22]. However, the interaction between slug and MMP-9 in PEITC-mediated inhibition of the invasive ability of SW480 cells should be further investigated. With clarification of signal transduction mediators and transcriptional factors involved in the anti-invasive process of PEITC on human colon cancer cell lines, it might be possible to develop specific mediators to inhibit undesired cell invasion.

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Disclosure of conflict of interest

None.

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