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CXCR4 and PTEN are involved in the anti-metastatic regulation of anethole in DU145 prostate cancer cells

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ABSTRACT

Anethole has been known to have chemopreventive activities as a suppressor of the incidence and multiplicity of both invasive and noninvasive carcinomas. The goal of this study was to understand the anti-metastatic effect of anethole through C-X-C chemokine receptor type 4 (CXCR4)/tumor suppressor phosphatase and tensin homologue (PTEN) axis in DU145 prostate cancer cells. Anethole reduced both of the RNA level and the protein level of CXCR4 in a dose-dependent manner without cytotoxicity. Anethole also reduced the expression of CXCR4 and prolonged the expression of PTEN in DU145 prostate cancers. The phosphorylation of AKT and phosphatidylinositol-3kinase (PI3K) were decreased with anethole. The inhibition metastatic effect of anethole was arisen from down-regulating CXCR4 and up-regulating PTEN. Morphologically, anethole significantly inhibited the invasion of DU145 cell and down-regulated the activities of matrix-metalloproteinase (MMPs) in a dose-dependent manner. However, anethole didnot decrease the phosphorylation of PI3K and AKT while PTEN was silenced. Furthermore, the CXCR4 inhibition of anethole was not caused to proteasomal or lysosomal of CXCR4.

Taken together, anethole demonstrated to act as the CXCR4 antagonist and as the PTEN activator which resulted to PI3K/AKT-mediated inhibition of the metastatic prostate cancer progressions.

1. Introduction

Chemokines are a family of pro-inflammatory cytokines and known to involve the metastasis causing migration and chemoattraction. In prostate cancer, chemokine CXC has been reported to be expressed strongly which concerned to tumor proliferation and local invasion [1,8]. Most of all, CXCR4/CXCL12 had been well established as a key role in the metastasis of many cancer types including prostate cancer. Furthermore, CXCR4/CXCL12 axis is correlated with other malignant cellular processes, such as tumor progression, angiogenesis by stimulation of PI3K/AKT, JAK/STAT, and other signaling pathways [3].

Anethole [1-methoxy-4-(1-propenyl) benzene] is an aromatic compound that occurs widely in nature, and revealed various biological activities as anti-oxidant [7], lipid-peroxidation inhibitor [15] and hydroxyl radical scavenger [17]. Additionally, anethole and its analogues exhibited the chemopreventive activities by suppressing the incidence and multiplicity of both invasive and non-invasive adenocarcinomas [11]. We also reported anti-metastatic activity of anethole in highly-metastatic HT-1080 human fibrosarcoma tumor cells [5]. In the present study, the anti-metastatic potent of anethole on prostate cancer was investigated which focused on PTEN and CXCR4 signaling. All study was designed to investigate the effect of anethole on the constitutive expression CXCR4 in DU145 prostate cells since the chemokine receptor that plays a critical role in tumor cell invasion and metastasis. The expression of CXCR4 in prostate cancer was analyzed by Western blot and reverse transcriptional PCR. The phosphorylation of PI3K and AKT and expression of PTEN were analyzed by Western blot. We explored the inhibitory effect of anethole on cell invasion and the activities of matrix-metalloproteinase (MMPs) by zymography. The PTEN loss and restoration of PI3K/AKT were analyzed by transfection of siRNA PTEN.

2. Materials and methods

2.1. Chemicals and antibodies

RPMI 1640 medium, fetal bovine serum and penicillin–streptomycin were purchased from WelGENE (Daegu, Korea). 3-(4,5-

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dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Triton X-100, SDS, aprotinin, leupeptin, pepstatin, sodium azide, sodium fluoride, sodium orthovanadate, iodoacetamide, phenylmethylsulfonyl fluoride, sodium chloride, Tris–HCl, Tween 20, gelatin, and DMSO were from Sigma chemical Co. (St. Louis, MO). Bio-Rad DC Protein assay kit II was obtained from Bio-Rad Laboratories (Hercules, CA). Matrigel was from BD Biosciences (Bedford, MA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Life Science (Amersham, UK). PTEN was purchased from SantaCruz (SantaCruz, CA). p-AKT, p-PI3K, AKT, and PI3K were purchased from Cell Signaling Technology (Beverly, MA). CXCR4 was purchased from ABCam (Cambridge, MA). ß-actin was purchased from Sigma chemical Co. Goat anti-mouse IgG HRP and goat anti-rabbit IgG HRP conjugated secondary antibody from purchased from vector (San Francisco, CA). X-ray films were from Agfa-Gevaert (Belgium). 8-μm pore polycarbonate membranes were purchased from neuroprobe (Gaithersberg, MD). Lactacystin and chloroquine were obtained from Millipore (Billerica, MA).

2.2. Cell culture

DU145, PC-3, and LNCaP, the human prostate cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 g/L sodium bicarbonate, 1% penicillin–streptomycin and at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Cytotoxicity assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed to determine the cell viability. Briefly, cells were seeded onto 96-well plate at a density of 5000 cells per each well. Then, the cells were treated with anethole at various concentrations (0, 25, 50, 100, and 200 μM). After 24 h, 5 mg/ml MTT was added onto each wells, then incubated until formazan was constituted. After medium was removed, formazan was dissolved with MTT lysis solution (20% SDS, 50% Dimethylfermamide) and then measured using microplate reader (TECAN, Austria) at 450 nm. Cell viability was calculated as a percentage of viable cells in anethole treated group versus untreated control by following equation. Cell viability (%) = [OD (EP) − OD (Blank)/OD (Control) − OD (Blank)] × 100. Each experiment was repeated three times.

2.4. Western blotting

Cells (1×10^6) treated with 100 μM anethole were collected and washed with cold PBS. Whole cell pellets were lysed in 100 μl of lysis buffer (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 mM iodoacetamide, 2 mM PMSF) for 30 min on ice. Lysates were centrifuged at 12,000*g* for 20 min at 4 °C and quantified using a Bio-Rad DC protein assay kit II. 30 μg of total protein were separated on SDS–PAGE and electrotransferred onto a Hybond ECL membrane with transfer buffer (25 mM Tris, 250 mM glycine, 20% methanol). The membranes were blocked with 3% BSA in TBST and immunoblotted with CXCR4, PTEN, p-PI3K (Tyr 199 and Tyr 458), p-AKT (Ser 473), and AKT in blocking buffer. After washing with TBST, the membranes were incubated with HRP conjugated secondary antibody in TBST and developed using an ECL detection kit. Protein contents were normalized by probing the same membrane with anti-β-actin antibody.

2.5. RNA isolation and reverse transcription-PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). 1 μg of total RNA was converted to cDNA by Superscript reverse transcriptase and then amplified by Platinum Taq polymerase using Superscript One-Step reverse transcription-PCR (RT-PCR) kit (Invitrogen). The relative expression of CXCR4 was analyzed by quantitative RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The following pairs of forward and reverse primer of CXCR4 sets were used: 5′-GAAGCTGTTGGCTGAAAAGG-3′ and 5′-GAGTCGATGCTGATCCCAAT-3′ (product size, 345 bp; GenBank Accession No. NM_003467).

2.6. Invasion assay with Boyden chamber

The invasive ability of DU145 cells was measured by the Boyden chamber invasion assay. Matrigel (BD Biosciences, Bedford, MA) was diluted with cold PBS and applied to the top side of the 8-μm pore polycarbonate filter. At the lower chamber, serum-free medium, in which DU145 cells were cultured, was applied. On the upper chamber, DU145 cells were seeded at a density of 1×10^5 cells/well in serum-free medium with 50, 100 μM of anethole. After incubation for 8 h, the cells in the upper surface of the membrane were carefully removed with a cotton swab and cells that invaded across the matrigel to the lower surface of the membrane were fixed with methanol and stained with Diff-quick solution (Sysmax Corporation, Kobe, Japan). The invasive cells on the lower surface of the membrane filter were counted with a light microscope. The data are presented as the average number of cells attached to the bottom surface from randomly chosen fields. Each experiment was carried out in triplicate.

2.7. Analysis of MMP-2, MMP-9 by Zymography

The activities of MMP-2 and MMP-9 were performed by gelatin zymography. Briefly, cultured media of DU145 was collected and concentrated with microcon (Millipore, Bedford, MA). Samples electrophoresed on 10% SDS–polyacrylamide gel containing 0.1% gelatin. Gel was washed in 2.5% Triton X-100 in H_2O incubated at 37 °C for 12 h in reaction buffer (40 mM Tris–HCl (pH 8.0), 10 mM $CaCl_2$, 0.02% NaN_3). Gel was stained with 0.125% Comassie blue R-250, 20% methanol, 10% acetic acid, and destained with 20% methanol, 10% acetic acid, 70% H_2O. The destained bands representing the levels of the latent form of MMP-2 and MMP-9 were quantified by densitometer measurement using a digital imaging analysis system (Image station, Kodak, Rochester, NY).

2.8. PTEN siRNA transfection

siRNA PTEN was purchased from SantaCruz (SantaCruz, CA) and transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommended protocol. PTEN silencing was detected by Western blot analysis against PTEN.

2.9. CXCR4 degradation by anethole

Cells were treated with the 50 μM lactacystin or 200 μM chloroquine for 1 h at 37 °C, and followed with 100 μM anethole for 24 h. Whole lysates were prepared and analyzed by Western blot analysis against CXCR4.

2.10. Statistical analysis

All data were expressed as means ± SD. The statistically significant differences compared with untreated group were calculated by Student's *t*-test.

3. Results

3.1. Anethole had no effect on cell viability on DU145 prostate cancer cell

To investigate whether anethole had an effect to the cell survival, the cytotoxicity assay was performed with various concentrations in 24 h. As shown in Fig 1B, anethole had no effect on cell viability up to 200 μM in DU145 cells.

3.2. Anethole suppresses the expression both of protein or mRNA levels of CXCR4 in prostate cancer cells

CXCR4 over-expression has been linked with bone metastasis of prostate cancer. As shown in Fig. 1C, both of the mRNA level and the protein level of CXCR4 was decreased with anethole dose-dependently in DU145 prostate cancer cell. Whether anethole suppressed the expression of CXCR4 in other prostate cancer cell, we also investigated the effect of anethole in PC3 and LNCaP. The protein expression of CXCR4 was reduced PC3 and DU145, whereas CXCR4 was not detected in LNCaP (Fig. 2A).

3.3. Anethole augmented the PTEN expression and down-regulated the CXCR4 related proteins

Since CXCR4 was known to increase the motility of tumor cells and to be down-regulated by PTEN, we checked the expression of PTEN with anethole. As shown in Fig. 2B, anethole augmented the PTEN expression in DU145 and LNCaP. However, PTEN was not detected in PC3 cells. Anethole affects the expression of other phosphorylation of PI3K (Tyr 458, Tyr 199) and AKT (Ser473) in DU145 cancer cells. PTEN has been well established as a negative regulator of PI3K and AKT phosphorylation [12].

3.4. Anethole reduced the invasion and MMP levels from DU145 cells

CXCR4 is related the cell motility, invasion and chemotaxis [12]. Whether anethole inhibits tumor invasion, Boyden chamber invasion assay performed. As shown in Fig. 3A, DU145 cells with anethole lost their invasion ability in a dose-dependent manner. Besides, anethole inhibited the secretion of MMP-2 and MMP-9 gelatinolytic abilities. As shown in Fig. 3B, MMP-2 and -9 were clearly inhibited after anethole treatment in a dose-dependent

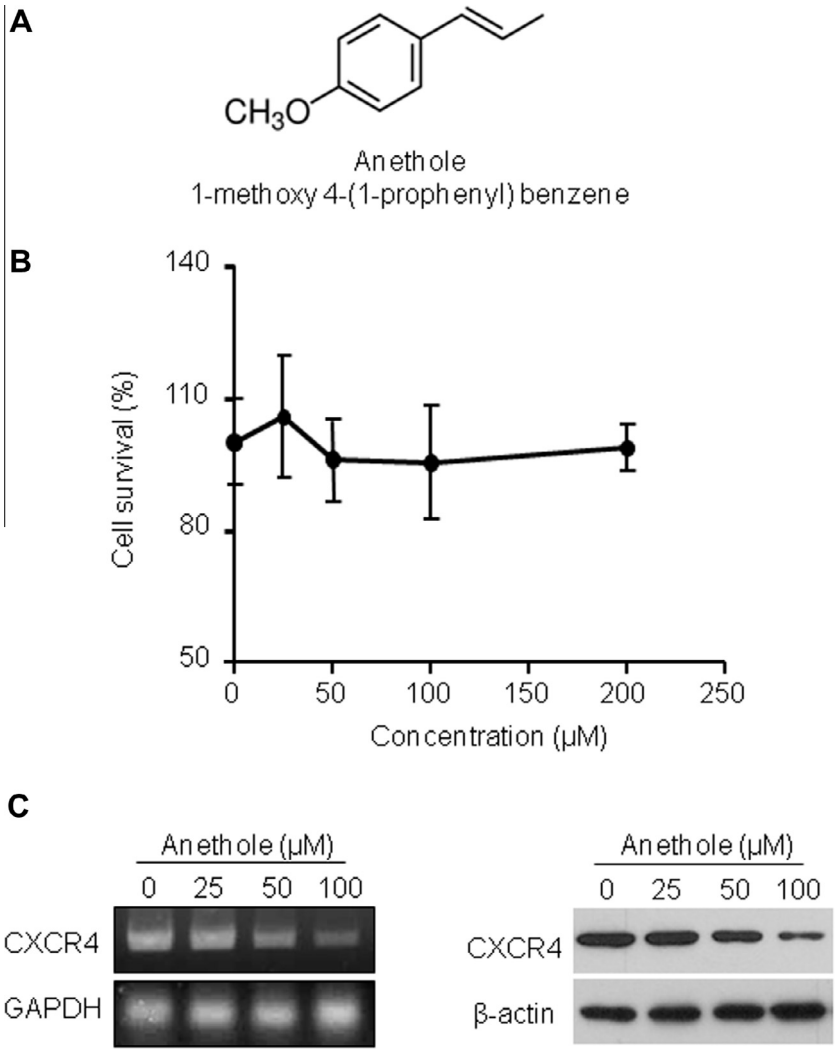

Fig. 1. (A) The chemical structure of anethole; (B) The cytotoxicity of anethole on DU145 prostate cells. The cytotoxicity and proliferation rate were calculated as a percentage of viable cells in anethole treated group versus untreated control (*$p < 0.01$); (C) The mRNA level of CXCR4 by RT-PCR and the protein level of CXCR4 by Western blot. Cells were prepared each of RNA and total protein, and performed each of reverse transcriptional PCR assay (left) and Western blotting against anti-CXCR4 (right). Protein contents were normalized by probing the same membrane with anti-β-actin antibody.

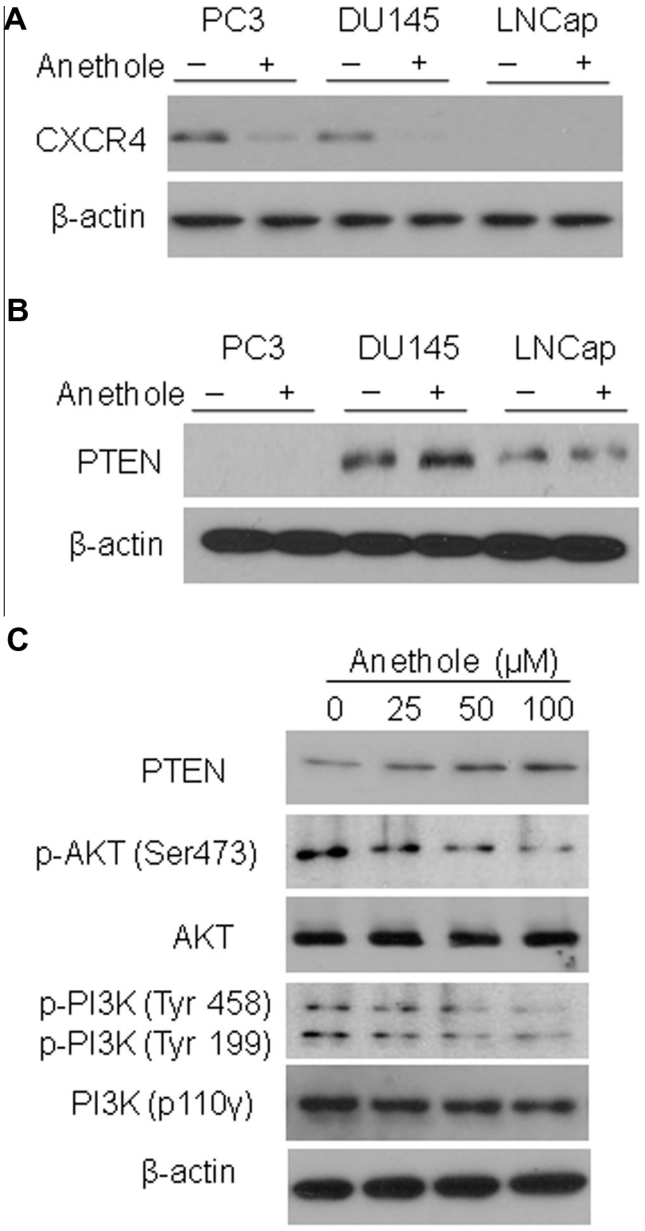

Fig. 2. (A) The protein level of CXCR4 by Western blot in prostate cell line. Each cell was collected and Western blotting was performed with anti-CXCR4; (B) The protein level of PTEN by Western blot in prostate cell line. Each cell was collected and Western blotting was performed with anti-PTEN; (C) The phsophsrylation of PI3K/AKT in DU145 cells. Cells were collected and Western blotting was performed with anti-p-AKT (Ser473), anti-AKT, anti-p-PI3K (Tyr458 and Tyr199), and anti-PI3K (p110γ). Protein contents were normalized by probing the same membrane with anti-β-actin antibody.

manner in DU145 prostate cancer cells. Taken together, anethole effectively abrogated the invasion and migration of DU145 cells via reducing the expression of CXCR4 and MMPs.

3.5. The loss of PTEN restored the phosphorylation of PI3K and AKT but not CXCR4 in DU145 prostate cancer cells

Whether loss of PTEN can restore the expression of CXCR4 and related proteins, we investigated the phosphorylation of PI3K and AKT. First, we confirmed the loss of PTEN in DU145 with PTEN siRNA transfection (Fig. 4A). After the loss of PTEN confirmation, we observed the phosphorylation of PI3K, AKT and CXCR4 with anethole. Anethole reduced the expression of CXCR4 without loss of PTEN in DU145 prostate cancer cells. However, phosphorylation of PI3K and AKT was restored though anethole treatment (Fig. 4B). These finding suggested that anethole could down-regulate the CXCR4 expression and augment PTEN expression immediately, though anethole could down-regulate the phosphorylation and PI3K and AKT thorough PTEN signal cascade.

3.6. Down-regulation of CXCR4 by anethole is not mediated through its degradation

In case of down-regulation of CXCR4 expression by enhancing its degradation causing to undergo ubiquitination at its lysine residue followed by degradation [2,13], the possibility of CXCR4 degradation by anethole through the activation of proteasomes or lysosome was determined. To determine the ability of lactacystin, the proteasome inhibitor, and chloroquine, the lysosomal inhibitor, DU145 prostate cancer cells were pretreated with 50 μM lactacystin or 200 μM chloroquine for 1 h before being exposed to anethole. As shown in Fig. 4C, both of lactacystin and chloroquine had no effect on anethole-induced degradation of CXCR4 in DU145 prostate cancer cells.

4. Discussion

Metastasis is the major cause of morbidity and mortality in the cancers. Many evidences have accumulated that cancer cells employ several mechanisms that regulate the trafficking of normal cells for metastasis. Chemokines are superfamily of cytokines known to play an important role on regulating the metastatic behavior for tumor cells. Particularly, CXCR4 has been shown to be a key receptor in mediating the metastasis of multiple types of tumors. Binding of CXCL12 (SDF-1) to CXCR4 induces the activation of Src, PI3K/Akt, ERK, and JNK pathways, contributing to protease production and cellular migration and invasion [6]. Thus, selective inhibition of tumor-associated receptors may be possible by linking inhibitors with specific surface antigens on tumor cells.

In the previous study, we demonstrated that anethole inhibited cell migration and invasion in human fibrosarcoma [5]. However, the further studies of mechanism causing cell motility were no progressed. In present study, we demonstrate that anethole, a component of funnel or anise oil, has anti-metastatic activity in brain-derived metastatic DU145 prostate cancer cells. Anethole significantly inhibited both mRNA level and protein level of CXCR4 in a dose-dependent manner without cytotoxicity in DU 145 prostate cancer cells (Fig. 1). We observed the expression of CXCR4 in other prostate cancer cells, and found CXCR4 was expressed in PC3 and DU145 not in LNCaP cells (Fig. 2A). Engle et al. reported that PC3 and DU145 cells were found to be highly aggressive, whereas LNCaP represented the low aggressive and such as CXCL-1, -3, -5, -6 were not detected in LNCaP [8]. Furthermore, anethole augmented the PTEN expression in DU145 and LNCaP not in PC3 (Fig. 2B). Many researchers reported that PTEN deletions and/or mutations are found in up to 30% of primary prostate cancers and 60–63% of metastatic prostate cancers such as PC-3 cells [4,14,18]. Thus, we chose the DU145 prostate cancer cells to observe a correlation between PTEN and CXCR4 based on our *in vitro* data which both of PTEN and CXCR4 expression were detected in DU145.

The phosphorylation of PI3K (Tyr 458 and Tyr 199) and AKT (Ser 473) were reduced with anethole (Fig. 2C). PTEN functions as a suppressor that inhibits cell proliferation, survival, and dephosphorylation of phosphatidylinositol 3,4,5-triphosphate (PIP3), blocking PI3K and AKT-mediated signaling cascade. These PTEN

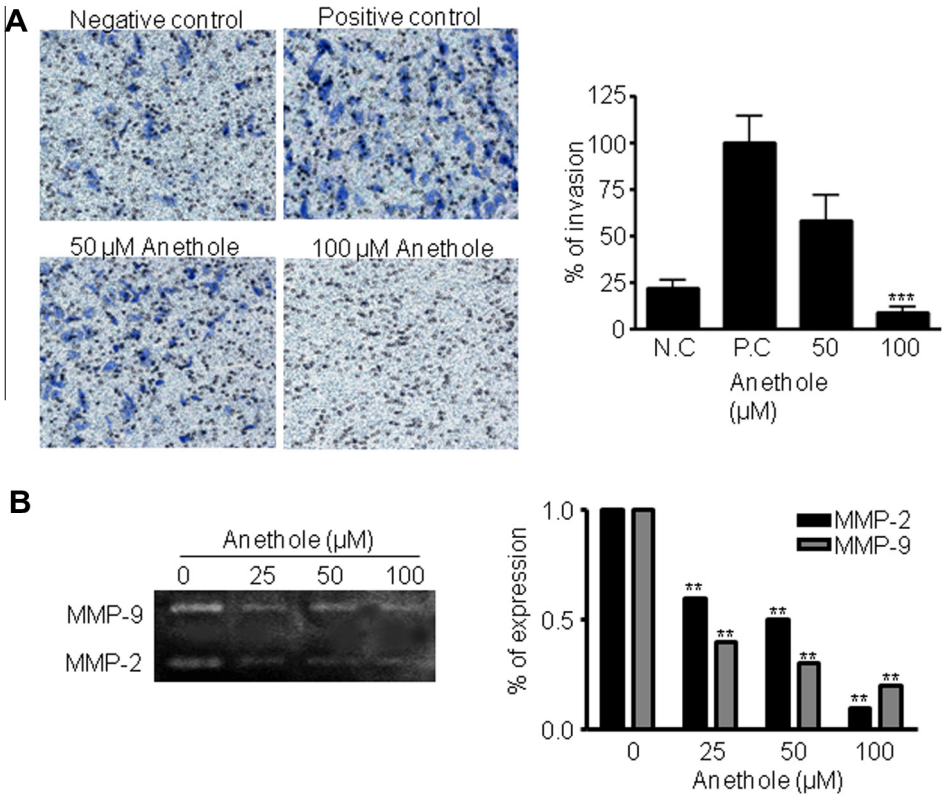

Fig. 3. (A) The invasion of anethole on DU145 prostate cells. The invasion assay was performed with Boyden chamber; (B) The analysis of MMP-2 and -9 by zymography, The distained bands representing the levels of the latent form of MMP-2 and MMP-9 were quantified by densitometer measurement using a digital imaging analysis system (**$p < 0.005$).

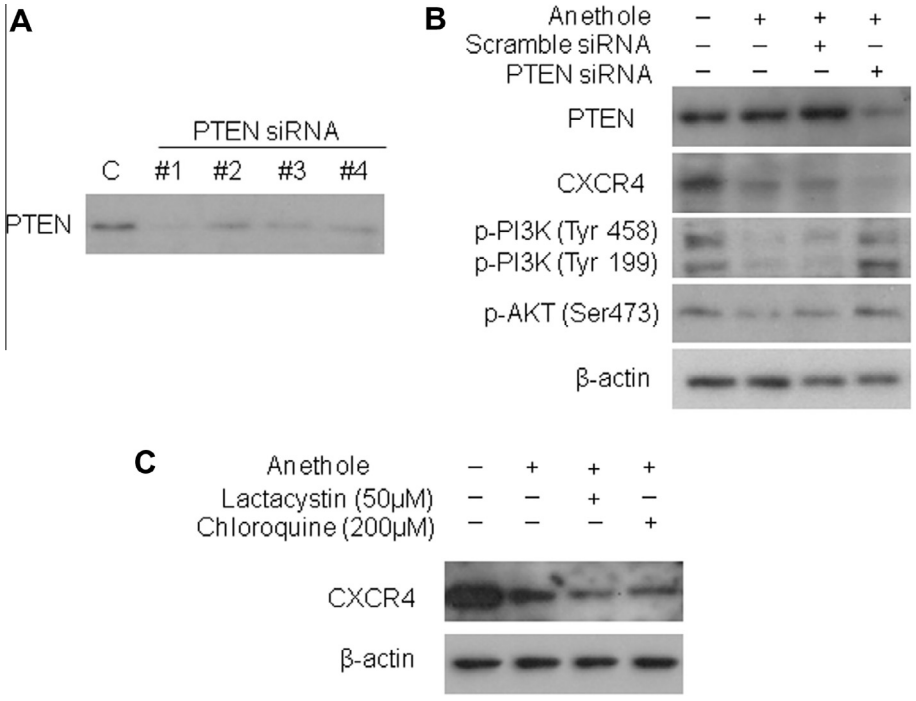

Fig. 4. (A) siRNA PTEN was transfected into DU145 cells and PTEN silencing was detected by Western blot analysis; (B) The transfected cells were collected and Western blotting was performed with anti-PTEN, anti-CXCR4, anti-p-PI3K (Tyr458 and Tyr199), and anti-p-AKT (Ser473). Protein contents were normalized by probing the same membrane with anti-β-actin antibody; (C) The suppression of CXCR4 through lysosomal and proteosomal degradation: Cells were treated with lactacystin or chloroquine for 1 h at 37 °C, and then anethole treatment followed. The CXCR4 expression was analyzed by Western blot analysis. Protein contents were normalized by probing the same membrane with anti-β-actin antibody.

negative regulation of PI3/AKT results on inhibition of invasion, metastasis and angiogenesis [4,6].

Morphologically, the invasion of DU145 prostate cancer cells to extracellular membrane (ECM) was inhibited by anethole in a dose-dependent manner. Gelatin zymography also revealed that anethole significantly reduced the activities of matrix metalloproteinase (MMP)-2 and -9. The MMPs are considered as essential proteases in the ECM degradation and remodeling. Many studies showed that activated CXCR4 regulated the activity of MMPs leading to ECM degradation and cell motility [10]. Therefore, it is possible that CXCR4 abrogation by anethole inhibit metastasis events through down-regulation of PI3K-AKT activation.

PTEN is commonly found in androgen-insensitive prostate tumors and reported as a suppressor to uncontrolled cell proliferation, invasion, and metastasis [6]. PTEN functions as inhibitor of AKT activation and negative regulator of chemotaxis [9]. Our result described that PTEN loss by siRNA transfection restored the phosphorylation of PI3K and AKT in DU145 prostate cancer cells. However, CXCR4 down-regulating by anethole was not reversed though PTEN siRNA transfection (Fig. 4B) in DU145 prostate cancer cells. These results suggested that CXCR4 down-regulating by anethole did not concerned with PTEN signal cascade. Therefore, anethole affected anti-metastasis with two ways to inhibit the CXCR4 expression and to augment the PTEN activity. Besides, dephosphorylation of PI3K and AKT was outcome from down-regulation of CXCR4 and up-regulation of PTEN by anethole.

Meanwhile, the possibility of CXCR4 degradation by anethole through the activation of proteasomes or lysosome was determined. The ligand-dependent down-regulation of the CXCR4 receptor by lysosomal degradation is well documented [16,13]. Bhandari et al. also suggested that atrophin-interacting protein-4 involved ubiquitination and degradation [2]. In this study, we used lactacystin as a proteasome inhibitor, and chloroquine as a lysosomal inhibitor. As shown in Fig. 4C, both of lactacystin and chloroquine had no effect on anethole-induced degradation of CXCR4, which suggested that the CXCR4 down-regulation with anethole is not to undergo on proteasome or ligand-dependent lysosomal degradation in DU145 prostate cancer cells.

Taken together, our study suggests that the anethole regulated the invasion, and migration of metastatic prostate cancers through CXCR4 suppression, and PTEN augmentation which affect to phosphorylation of PI3K-AKT negatively.

Acknowledgements

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