Pinosylvin inhibits migration and invasion of nasopharyngeal carcinoma cancer cells via regulation of epithelial-mesenchymal transition and inhibition of MMP-2

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Abstract. Nasopharyngeal carcinoma (NPC) is a tumor located in the nasopharynx with highly invasive and metastatic properties. Metastasis is a primary cause of mortality in patients with NPC. The terpenoid polyphenol pinosylvin is a known functional compound of the *Pinus* species that exhibits anti-inflammatory effects; however, the effect of pinosylvin on human NPC cell migration and invasion is unclear. The present study aimed to investigate the functional role of pinosylvin in NPC cells (NPC-039, NPC-BM and RPMI 2650). Gap closure and Transwell assay indicated that pinosylvin at increasing concentrations inhibited migration and invasion of NPC-039 and NPC-BM cells. In addition to inhibiting the enzyme activity of MMP-2, pinosylvin also decreased the protein expression levels of MMP-2 and MMP-9. Pinosylvin decreased the expression of vimentin and N-cadherin and significantly increased the expression of zonula occludens-1 and E-cadherin in NPC cells. Additionally, pinosylvin suppressed the invasion and migration ability of NPC-039 and NPC-BM cells by mediating the p38, ERK1/2 and JNK1/2 pathways. The present results revealed that pinosylvin inhibited migration and invasion in NPC cells.

Introduction

The terpenoid polyphenol pinosylvin (trans-3,5-dihydroxystilbene) is a stilbene present in the heartwood of coniferous trees of the genus *Pinus* (1). Many studies have demonstrated that biological characteristics of pinosylvin include antibacterial and antifungal activity (2) and protection against oxidative stress in human cells (3). Pinosylvin regulates Src/ERK and GSK-3/β-catenin signaling to inhibit tumor cell growth (4). Pinosylvin has been shown to inhibit the expression of MMP-2 and MMP-9 in human fibrosarcoma HT1080 cells (5).

Nasopharyngeal carcinoma (NPC) is a tumor located in the nasopharynx and is caused by epithelial cells covering the nasopharyngeal surface. Unlike other head and neck epithelial cancers, NPC is highly invasive and metastatic (6). NPC is particularly prevalent in Southern China, Southeast Asia, North Africa and the Arctic region, which is a unique geographical distribution (7). Four primary causes of nasopharyngeal carcinoma have been identified, including Epstein-Barr and human papillomavirus infection, genetic susceptibility and consumption of salted fish (8). NPC occurs adjacent to cervical lymph nodes, which increases the risk of metastasis in other parts of the body, thereby causing difficulties in surgical treatment (8). Currently, chemotherapy and radiotherapy can improve the survival rate of patients with advanced NPC (9). Preventing distant metastasis is key to treatment, and more effective systemic drugs should be investigated (10).

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Abbreviation: NPC, nasopharyngeal carcinoma

The metastasis of NPC occurs in two stages: Translocation to distant tissue and colonization (11). The initial step degrades and penetrates the extracellular matrix of surrounding tissue (12). Among the involved proteolytic enzymes, zinc-dependent MMPs contribute substantially to proteolytic degradation and intercellular interaction damage (13). Research has indicated that MMP-2 and MMP-9 are key treatment targets for regulation of tumor metastasis in NPC (14), cervical cancer (15) and retinoblastoma (16). Lyu *et al* (17) reported that liposome-containing thermosensitive liposomes can deliver MMP inhibitors, decreasing the activity of MMP-2 and MMP-9 by 50 and 43%, respectively, to inhibit metastasis and angiogenesis. Huang *et al* (18) demonstrated that exosomes with low expression levels of microRNA-34c-3p affect expression of integrin $\alpha2\beta1$ and promote the invasion and migration of non-small cell lung cancer cells.

Epithelial-mesenchymal transition (EMT) is a key process involved in tumor metastasis and recurrence (19,20). Research has indicated that the expression of mesenchymal markers, such as vimentin and N-cadherin, increases during EMT, whereas epithelial marker E-cadherin, a powerful tumor cell invasion inhibitor, is downregulated (21,22). The MAPK pathway is an important intracellular signal transduction pathway that serves a key role in regulating tumor metastasis, as well as regulating cell proliferation, differentiation, apoptosis and angiogenesis (23). The ERK subfamily (typical ERK 1/2/5 and atypical ERK 3/4/7/8) of proteins is known for its contributions to EMT (23,24). PI3K/AKT and MAPK pathways contribute to TGF-β2-induced upregulation of Jagged-1, which mimics TGF-β2-induced EMT in retinal pigment epithelium cells (25). TGF-β, in addition to its role in cell differentiation, migration and adhesion, also induces EMT via both Smad and MAPK pathways (26). A previous study indicated that pinosylvin exerts antimetastatic effects on human oral cancer cells (27). However, the antimigratory effect of pinosylvin on NPC cells remains unknown. Therefore, the present study investigated the effect of pinosylvin on NPC cell metastasis and regulation of its signaling.

Materials and methods

Chemicals. Pinosylvin ($\geq$97% purity) was purchased from ChemFaces. DMSO was used to prepare 100 mM storage solution of pinosylvin, which was stored at -20˚C. The maximum concentration of DMSO used for treatment in medium was <0.2%. MTT, ERK1/2, p38 and JNK1/2 specific inhibitors (U0126, SB203580 and SP600125) were obtained from Sigma-Aldrich (Merck KGaA).

Cell culture. Nasal cavity cancer cells (RPMI 2650) were obtained from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Human nasopharyngeal cancer cell lines (NPC-039 and NPC-BM) were provided by Dr Jen-Tsun Lin, Department of Hematology and Oncology, Changhua Christian Hospital (Changhua, Taiwan). RPMI-2650 cells were cultured in Eagle's Minimum Essential Medium (Gibco; Thermo Fisher Scientific, Inc.); NPC cell lines were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.). All culture media were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.),

1 mM glutamine, 1% penicillin/streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin), 1.5 g/l sodium bicarbonate and 1 mM sodium pyruvate. All cell cultures were maintained at 37˚C in a humidified atmosphere of 5% CO_2.

In vitro cytotoxicity assay (MTT assay). Cytotoxicity was assessed via MTT (0.1%,) assay. All cells were cultured in 96-well plates ($1x10^4$/well) at 37˚C in 5% CO_2 overnight. Subsequently, supernatant was removed and cultures were treated with different concentrations of pinosylvin (0, 20, 40 and 80 μM) at 37˚C for 24 h. Following treatment, the medium containing pinosylvin was removed and MTT reagent (1 mg/ml) was added to each well at 37˚C in 5% CO_2. After 4 h, the supernatant containing MTT reagent was removed and DMSO was added to dissolve the formed blue formazan crystals. Absorbance was measured at 595 nm using spectrophotometry. A total of three independent experimental replicates was performed.

Gap closure assay. Gap closure assay was used to measure migration of NPC-039 and NPC-BM cells over a certain distance. NPC-039 and NPC-BM cells ($3x10^4$) were grown onto each side of a culture insert (Ibidi GmbH) at 37˚C overnight. After reaching 90% confluence, culture inserts were removed and gap closure assay was performed. Cultures were treated with pinosylvin (0, 20, 40 and 80 μM) in serum-free RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C for 24 h. The cell migration distance was observed and photographed after 0, 3 and 6 h. Migration was measured using ImageJ 1.47 version software (National Institutes of Health) and expressed as a percentage using the following formula: (Initial gap width of the experimental group-remaining width of the experimental group)/(initial gap width of the control group-remaining width of the untreated control group) x100. Images were captured under a light microscope (Lecia GmbH). The entire procedure was repeated three times and the values are indicated as mean ± SD.

Cell migration and invasion assay. NPC-039 and NPC-BM cell migration and invasion assays were performed as described by Yang *et al* (28). Briefly, NPC cells ($3x10^4$) were placed on the upper well of a Transwell insert (Greiner Bio-One International GmbH) with serum-free medium (RPMI-1640) and 10% FBS-containing medium (RPMI-1640 medium) (600 μl) was added to the lower chamber for 24 h at 37˚C. For the invasion assay, Matrigel (25 mg/50 ml; 60 μl; BD Biosciences) was coated on the upper Transwell at 37˚C, overnight. Migrated or invaded cells were fixed with 99% methanol at room temperature for 15 min and stained with Giemsa (1X) at room temperature for 2 h. Images were captured and number of cells was counted under an optical light microscope (Lecia Germany) at 100x magnification using ImageJ 1.47 version cell count software (National Institutes of Health). A total three fields of view was randomly selected for each concentration. Data are presented as the mean ± SD (n=3).

Gelatin zymography. Enzyme activity of MMP-2 was analyzed via gelatin zymography. Briefly, after plating NPC-039 and NPC-BM cells ($5x10^4$ cells/well) in 24-well plates at 37˚C for 16 h, cells were treated with different concentrations
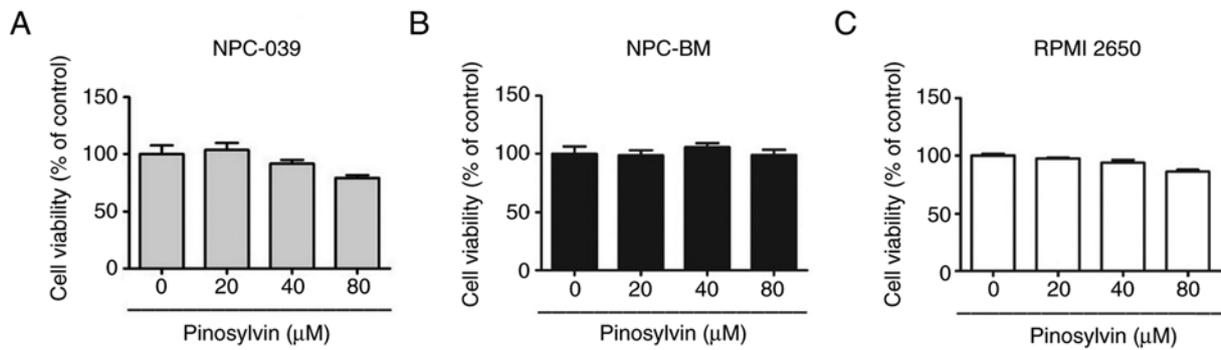
Figure 1. Pinosylvin does not inhibit cell proliferation in three cell lines. (A) NPC-039, (B) NPC-BM and (C) RPMI 2650 cells were treated with pinosylvin (0, 20, 40 and 80 μM) for 24 h. Cytotoxicity was measured using an MTT assay. Data are presented as the mean ± SD (n=3). NPC, nasopharyngeal carcinoma.

(0, 20, 40 and 80 μM) of pinosylvin at 37°C for 24 h. Culture medium was collected and subjected to 8% SDS-PAGE with 0.1% gelatin as described previously (29).

Western blot analysis. Following treatment with different concentrations of pinosylvin, cells were lysed with 1X RIPA buffer (EMD Millipore) containing protease and phosphatase inhibitor cocktails and subjected to BCA (Thermo Fisher Scientific, Inc.) protein concentration assay. All samples were separated using 10.0 or 12.5% SDS-PAGE and proteins were transferred onto a PVDF membrane (EMD Millipore). Membranes were blocked with 5% non-fat milk in TBST (0.05% Tween-20) at room temperature for 1 h. Detection was performed with a primary antibody overnight at 4°C followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (Anti-rabbit IgG, #7074, 1:3,000; Anti-mouse IgG, #7076, 1:3,000, Cell Signaling Technology, Inc.) at room temperature for 1 h. The following antibodies (all 1:1,000; all Cell Signaling Technology, Inc. unless otherwise indicated) were used: Anti-ERK1/2 (cat. no. #4695; 42, 44 kDa), anti-JNK1/2 (cat. no. #9252; 46, 54 kDa), anti-p38 (cat. no. #8690; 40 kDa), anti-phosphorylated (phospho-) ERK1/2 (cat. no. #4370; 42, 44 kDa), anti-phospho-JNK1/2 (cat. no. #4668; 46, 54 kDa), anti-phospho-p38 (cat. no. #4511; 43 kDa), anti-MMP-2 (cat. no. #87809; 64 kDa), anti-N-cadherin (cat. no. #13116; 140 kDa), anti-E-cadherin (cat. no. #3195; 135 kDa), anti-zonula occludens (ZO)-1 (cat. no. #8193; 220 kDa), anti-vimentin (cat. no. #5741; 57 kDa), anti-MMP-9 (cat. no. #AB19016; 92 kDa; EMD Millipore) and anti-b-actin (1:5,000; cat. no. NB600-501; 42 kDa; Novus Biologicals). Immunoblotting was observed using HRP chemiluminescent substrates (EMD Millipore). Images were captured using ImageQuant LAS 4000 mini (GE Healthcare) and relative density was quantitated by ImageJ 1.47 version software (National Institutes of Health).

Proteome profiler human protease array. The Proteome Profiler Human Protease Array kit (cat. no. ARY021B; R&D Systems, Inc.) was used according to the manufacturer's instructions. Array buffer 6 was added into each well of the 4-well Multi-dish and incubated at room temperature for 1 h. Then, 15 μl reconstituted protease detection antibody cocktail was added at room temperature for 1 h. Sample mixtures were incubated with membrane overnight at 4°C. Each membrane

was washed with wash buffer for 10 min. Streptavidin-HRP was added into each well and incubated for 30 min at room temperature. Immunoblotting was observed using HRP chemiluminescent substrate (EMD Millipore). Images were captured using ImageQuant LAS 4000 mini (GE Healthcare) and relative density was quantitated by ImageJ 1.47 version software (National Institutes of Health).

Statistical analysis. The experimental data are expressed as the mean ± SD (n≥3). Comparisons between >2 groups were analyzed by one-way ANOVA followed by post hoc Tukey's test. Paired student's t-test was used to analyze differences between two groups. All statistical analyses were performed using GraphPad Prism Software Version 5.0 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Pinosylvin does not induce cytotoxicity in three cell lines. The cytotoxic effects of various concentrations of pinosylvin (0, 20, 40 and 80 μM) on cell lines were assessed using MTT assay for 24 h (Fig. 1A-C). Pinosylvin did not exert significant cytotoxic effects on the viability of NPC-039, NPC-BM and RPMI-2650 cell lines. All subsequent experiments examined antimetastatic properties of pinosylvin at non-cytotoxic concentrations.

Pinosylvin inhibits migration and invasion in NPC cell lines. Gap closure assay was performed to assess the effect of pinosylvin on the mobility of NPC cells treated with 0-80 μM pinosylvin for 0, 3 and 6 h (Fig. 2). Compared with the control group, the migrated distance of the cell monolayers was significantly decreased at high concentrations (80 μM) of pinosylvin. In addition, the effect of pinosylvin on the migration and invasion ability in NPC cells was assessed by Transwell assay (Fig. 3A-D); pinosylvin significantly decreased the migration and invasion abilities of two NPC cell lines.

Pinosylvin changes migration of NPC cell line and inhibits MMP-2 activity. According to the results of Proteome Profiler Human Protease Array (Fig. S1), to lack of observed differences. MMPs regulate cancer cell migration and invasion (13). In order to determine whether MMP-2 and MMP-9
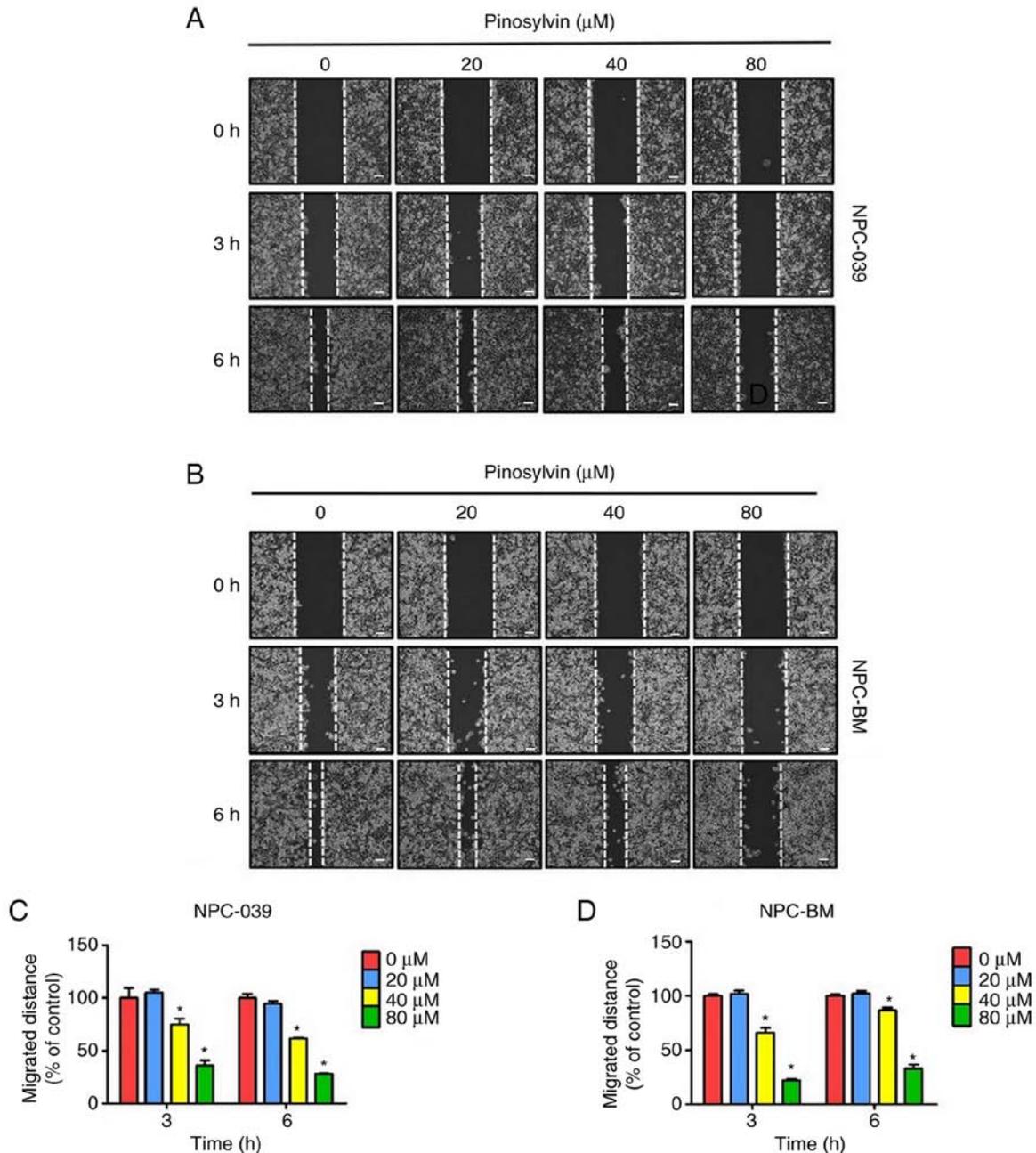
Figure 2. Effect of pinosylvin on gap closure. Cell motility was determined by gap closure assay from 0 and 6 h in (A) NPC-039 and (B) NPC-BM. Migration of (C) NPC-039 and (D) NPC-BM cells was quantified. Data are presented as the mean ± SD (n=3). *P<0.05 vs. control. Scale bar, 100 μm. NPC, nasopharyngeal carcinoma.

are regulated by pinosylvin in two NPC cell lines, zymography and western blotting were performed to analyze enzyme activity and protein concentration. Pinosylvin at the highest concentration significantly decreased enzymatic activity of MMP-2 in two NPC cell lines (Fig. 4A-D). Following 24 h treatment, a high pinosylvin concentration (80 μM) decreased expression levels of MMP-2 and MMP-9 to 54 and 66 in NPC-039 and 52 and 41% in NPC-BM cells, respectively (Fig. 4E-H).

Pinosylvin affects EMT-associated protein expression in NPC cell lines. When wound healing occurs, organ fibrosis and the initiation of metastasis in cancer progression prompt EMT (30). Analysis of expression levels of EMT-specific proteins (Fig. 5A-D) demonstrated that pinosylvin at a

high concentration significantly decreased expression of vimentin and N-cadherin to 58.5 and 62.5 in NPC-039 and 55.0 and 58.5% in NPC-BM, respectively, and significantly increased expression of ZO-1 and E-cadherin to 80.0 and 101.5 in NPC-039 and 90.0 and 123.0% in NPC-BM, respectively.

Pinosylvin decreases invasion and migration ability of NPC cell lines via MAPK pathways. Western blotting was performed to detect changes in the molecular mechanisms of MAPK pathways in response to treatment with pinosylvin (Fig. 6A-D). As the concentration of pinosylvin increased, phosphorylation of ERK1/2 and p38 decreased significantly. According to ImageJ analysis of blots, treatment with 80 μM
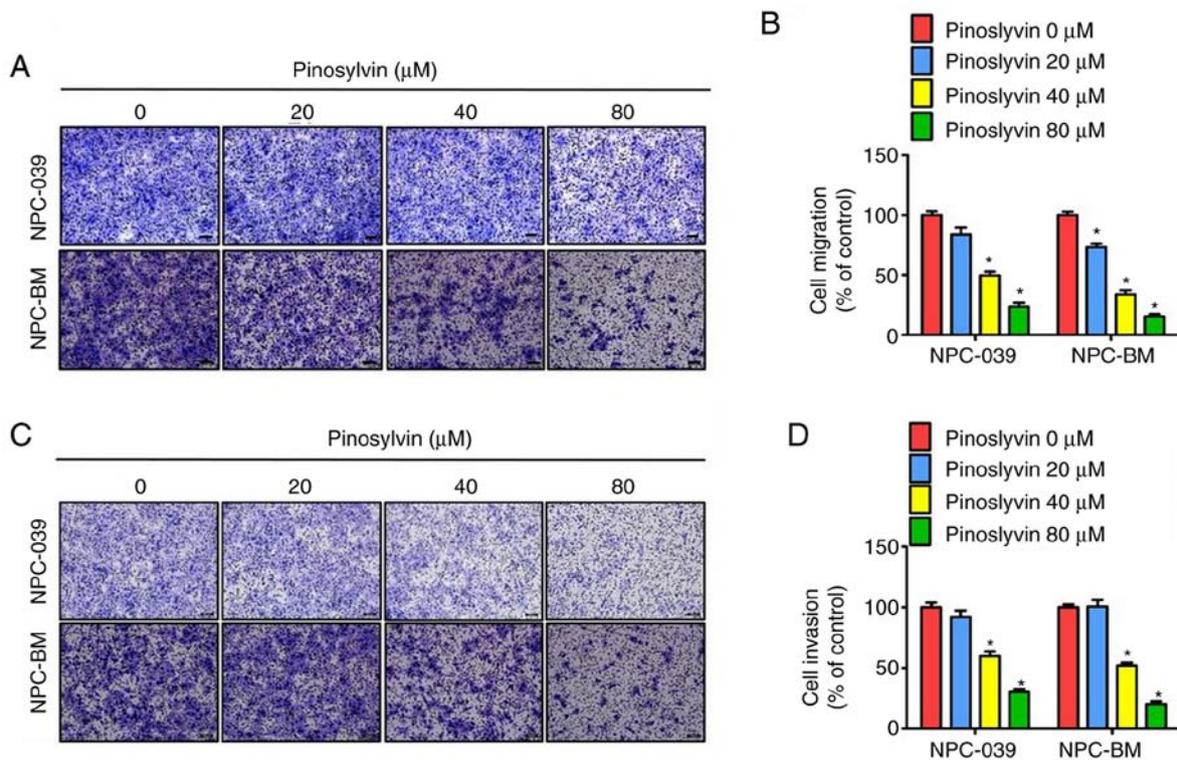
Figure 3. Pinosylvin inhibits migration and invasion in NPC cells. Transwell assay was performed to assess cell (A and B) migration and (C and D) invasion ability, which demonstrated a dose-dependent effect of pinosylvin on NPC cell migration and invasion ability. Data are presented as the mean ± SD (n=3). *P<0.05 vs. control. Scale bar, 100 μm. NPC, nasopharyngeal carcinoma.

pinosylvin decreased the phosphorylation of ERK1/2 and p38 to 63 and 51 in NPC-039 cells and 59 and 46% in NPC-BM cells, respectively, at 24 h compared with untreated controls. By contrast, phosphorylation of JNK1/2 was significantly increased in the two NPC cell lines. In order to confirm the molecular mechanism underlying pinosylvin-induced inhibition of NPC cell migration, cells were pre-treated with specific inhibitors of ERK1/2, p38 and JNK1/2; following pre-treatment with specific inhibitors, pinosylvin-inhibited cell migration and invasion ability were significantly improved (Fig. 7A-D). Taken together, these findings indicate that pinosylvin exerted anti-metastatic effects via p38, ERK1/2 and JNK1/2 signaling pathways in human NPC cells.

Discussion

In an analysis of metastasis patterns of 629 patients with NPC, Huang *et al* (31) found that 95% of distant metastases occurred <3 years after completion of radiotherapy. Hence, determining effective methods of suppressing distant metastasis is important in the treatment of NPC. Plant polyphenols are important plant secondary metabolites with biological functions (such as countering infection by pathogens or mitigating environmental stresses), as well as antioxidant, anticancer and anti-inflammatory properties (1,3,5,7). Studying compounds with such biochemical activity is beneficial for drug development in the pharmaceutical industry (32). Pinosylvin and resveratrol are terpenoid polyphenols with similar structures (2). Research has indicated that pinosylvin inhibits growth of human colorectal cancer cells (4), suppresses MMP-2 and MMP-9 activity in HT1080

cells (5) and suppresses migration and invasion in SCC-9, SAS and HSC-3 cell lines (27). In the present study, pinosylvin did not decrease the viability of the two NPC cell lines or a nasal cavity cancer cell line (RPMI 2650), however, high concentrations of pinosylvin inhibited the migration and invasion of NPC-039 and NPC-BM cells. Furthermore, the present results indicated that pinosylvin inhibited NPC cell metastatic effects by downregulating MMP-2/MMP-9 expression levels and modifying the regulation of EMT markers.

MMPs serve important roles in mediating cancer cell growth, differentiation, apoptosis, migration, invasion and angiogenesis (33). Di Carlo *et al* (34) performed zymography analysis and demonstrated that the ratio of MMP-9/MMP-2 in patients with cancer was increased compared with that in patients with benign disease and healthy individuals. High expression of MMP-2 and MMP-9 is significantly correlated with local and distant metastatic tumor recurrence and poor prognosis in head and neck squamous cell carcinoma (35-37). In the present study, gelatin zymography and western blotting were performed to analyze the effects of pinosylvin on MMPs in two NPC cell lines; pinosylvin significantly inhibited expression of MMP-2 and MMP-9 as well as MMP2 activity. Tissue inhibitors of metalloproteinase (TIMPs) control proteolytic activity and are a specific endogenous inhibitor of MMPs (38). Western blotting here showed that pinosylvin did not increase TIMP-1 or -2 protein levels in the two NPC cell lines (data not shown). This indicated that pinosylvin decreased MMP-2 protein expression levels and activity, via regulated the activation of zymogen at the post-transcriptional level.
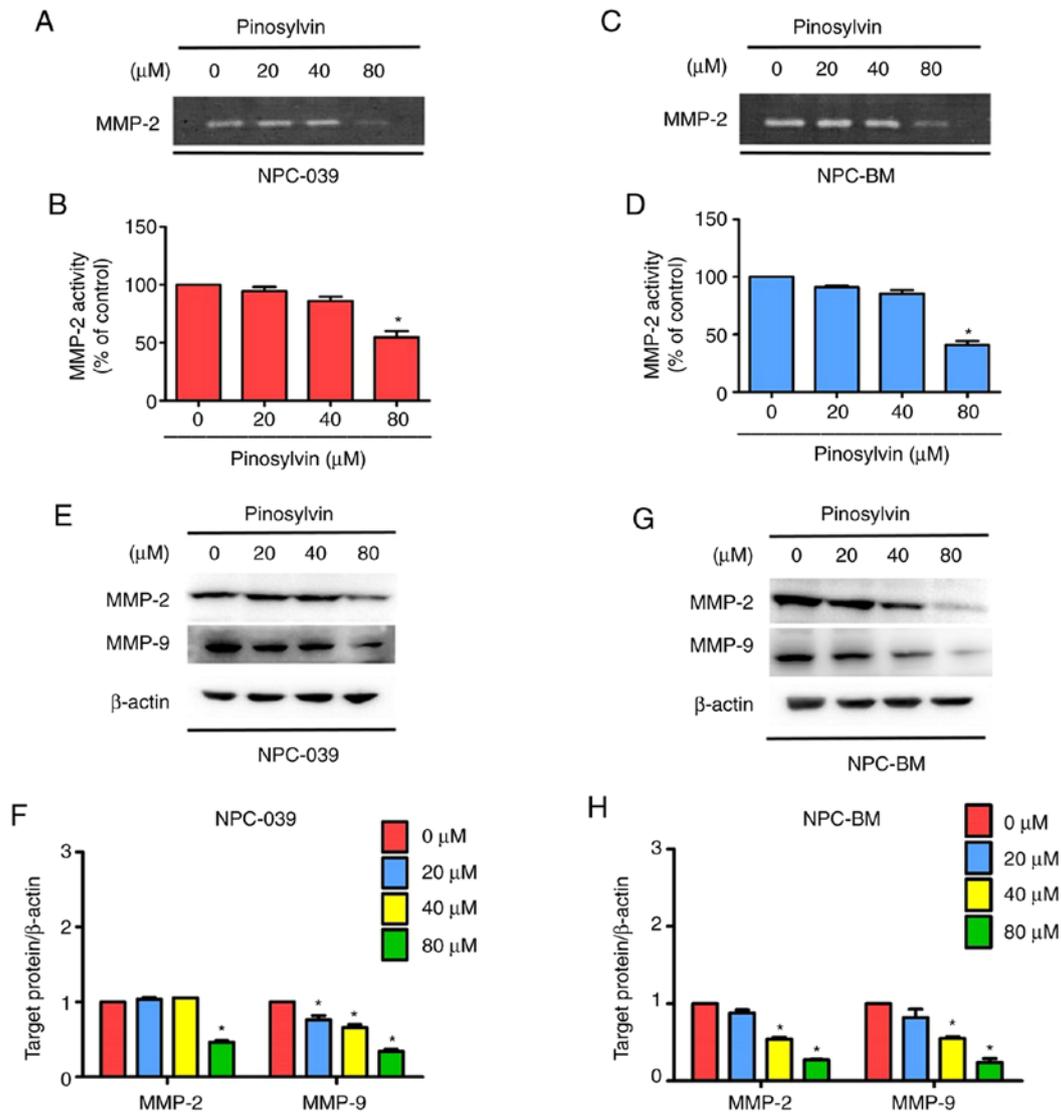
Figure 4. Effects of pinosylvin on activity of MMP enzymes and protein expression in NPC cells. Determination of MMP-2 enzyme activity using gelatin zymography in (A and B) NPC-039 and (C and D) NPC-BM cells. Western blotting was used to measure the expression of MMP-2 and MMP-9 protein after 24 h pinosylvin treatment in (E and F) NPC-039 and (G and H) NPC-BM cell lines. ImageJ software was used for quantitative analysis of protein. Data are presented as the mean ± SD (n=3). *P<0.05 vs. control. NPC, nasopharyngeal carcinoma.

EMT is a key step in tumor cell migration and invasion in various types of human cancer (39-41). Upregulation of N-cadherin induces EMT (40); another regulator of EMT is E-cadherin, which inhibits the occurrence of EMT and serves as a tumor suppressor (41). Vimentin is the primary cytoskeletal component of mesenchymal cells (42). ZO-1 and ZO-2 are required for tight junction formation and function (43,44); mutations in ZO-1 and claudin-1 induce EMT (45). In the present study, pinosylvin-treated NPC-BM and NPC-039 cells exhibited significantly induced E-cadherin and ZO-1 expression, but decreased expression of N-cadherin and vimentin. These findings suggest that pinosylvin inhibited EMT at the initiation step of tumor metastasis.

Compared with other intracellular signal transduction pathways (23), the MAPK pathway serves a more important role in cell proliferation, differentiation, apoptosis, angiogenesis and tumor metastasis (23,24). A study indicated that TBL-12, a sea cucumber extract, inhibits migration and invasion of human PCa cells by inhibiting MMP-2 and MMP-9 via decreased phosphorylation of p38 (46). Additionally, 18β-glycyrrhetinic acid inhibits migration and invasion of gastric cancer cells via the reactive oxygen species/protein kinase C-α/ERK signaling pathway (47). Therefore, the present study investigated whether the MAPK pathway is altered by pinosylvin treatment. Western blot analysis revealed that pinosylvin suppressed ERK1/2 and p38 protein phosphorylation but induced JNK protein phosphorylation in both NPC cell lines. This result is consistent with previously reported inhibition of Huh7 cell proliferation and metastasis by cucurbitacin E via suppression of MAPKs (48). A previous study showed that pinosylvin inhibits the growth of human colorectal cancer cells via suppression of Src/ERK and GSK-3/β-catenin signaling (4). In our previous research, pinosylvin inhibited migration and invasion of oral cancer cells by suppressing the expression and activity of MMP-2 and ERK1/2 signaling (27). The present results suggest that pinosylvin was involved in MMP-2/MMP-9 regulation in NPC cells and that the MAPK pathway may serve a key role.
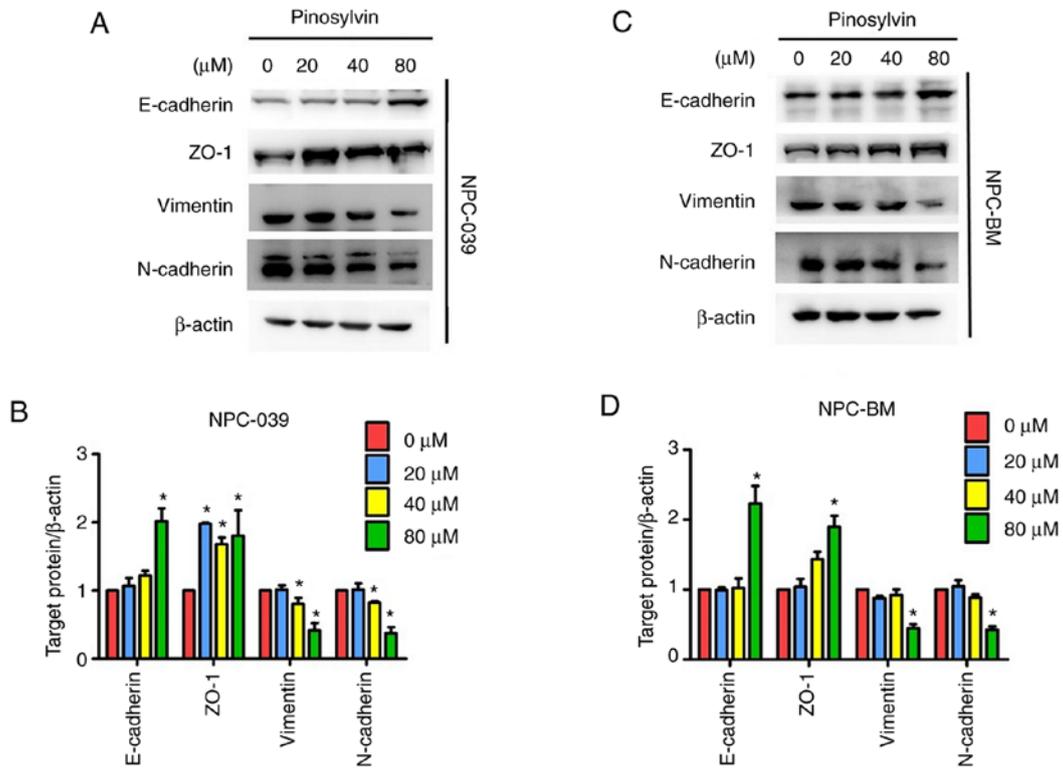
Figure 5. Pinosylvin affects mesenchymal marker protein expression in NPC cell lines. Western blotting was used to measure the expression of mesenchymal marker proteins following 24 h pinosylvin treatment in (A and B) NPC-039 and (C and D) NPC-BM cell lines. ImageJ software was used for quantitative analysis of protein. Data are presented as the mean ± SD (n=3). *P<0.05 vs. control. NPC, nasopharyngeal carcinoma; ZO-1, zonula occludens-1.
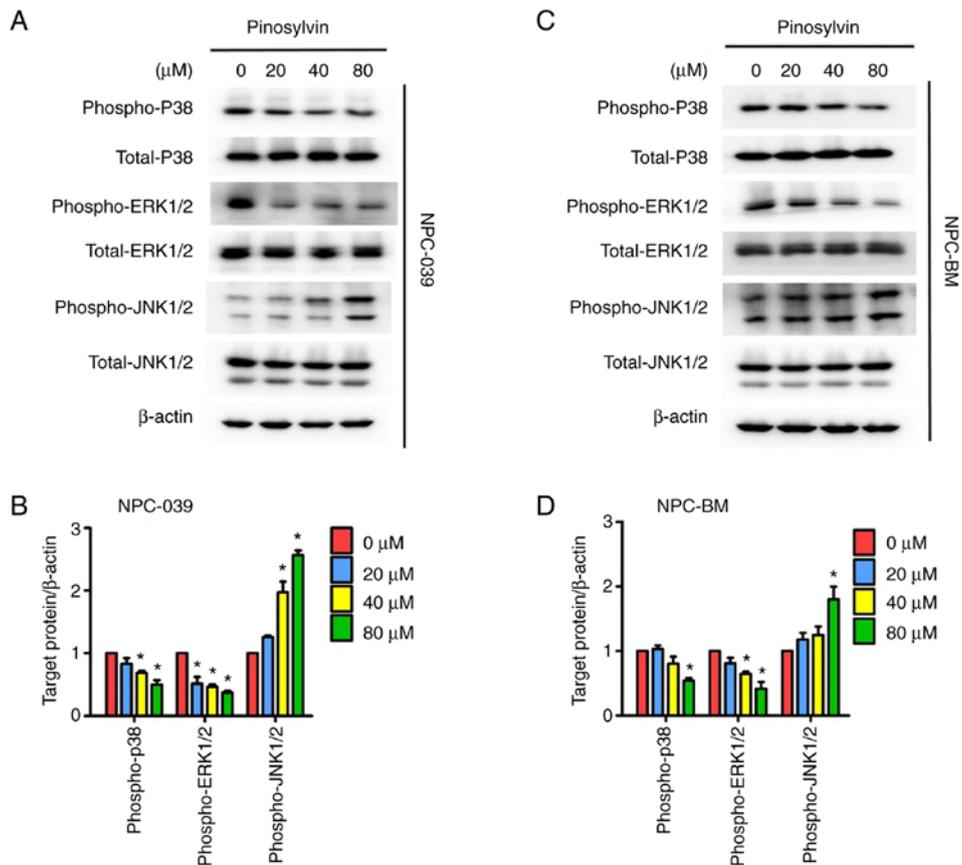
Figure 6. Pinosylvin affects MAPK pathways in NPC cell lines. Western blotting was used to measure expression levels changes in phospho-p38, -ERK1/2 and -JNK1/2 following 24 h pinosylvin treatment in (A and B) NPC-039 and (C and D) NPC-BM cell lines. Data are presented as the mean ± SD (n=3). *P<0.05 vs. control. NPC, nasopharyngeal carcinoma; phospho-, phosphorylated.
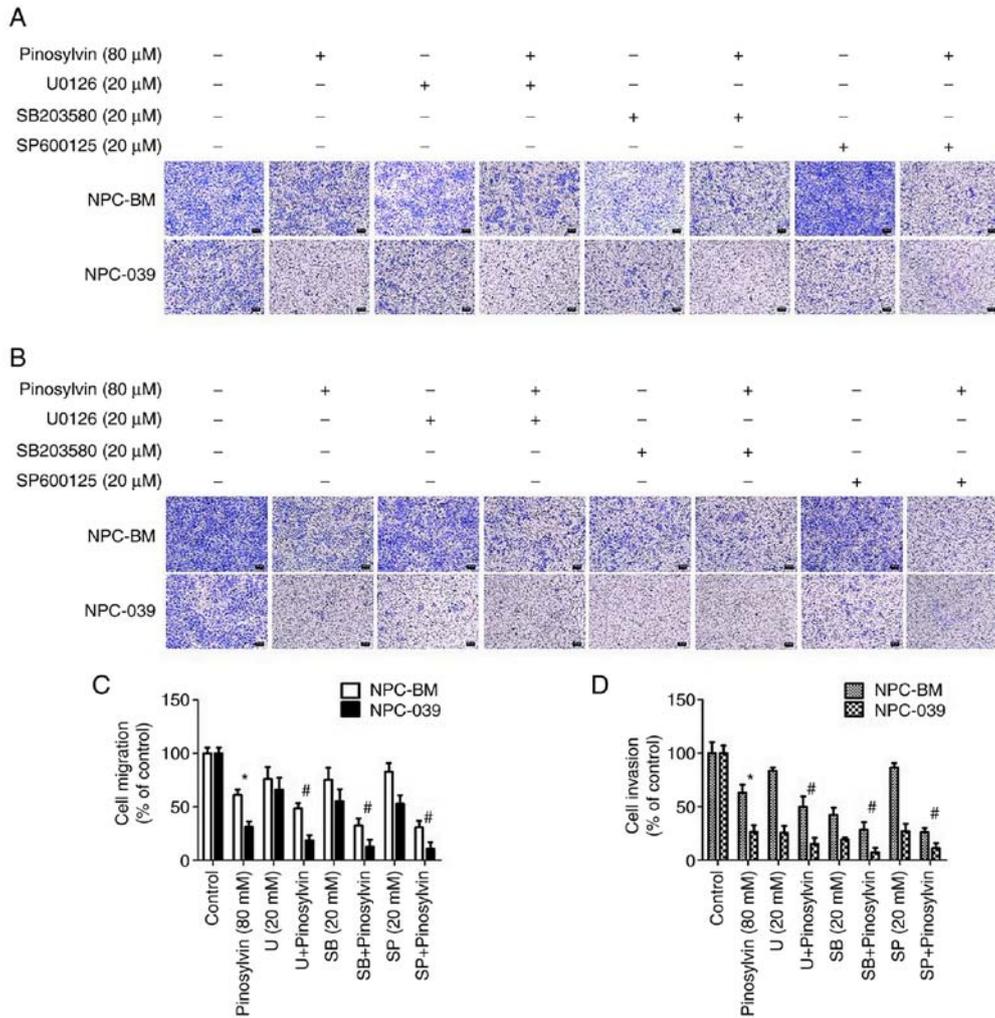
Figure 7. Pinosylvin inhibits migration and invasion by affecting the MAPK pathway in NPC cell lines. Cell lines were pre-treated with U, SB or SP for 1 h, then with treated pinosylvin for 24 h. Cell (A) migration and (B) invasion were determined via Transwell assay. Scale bar, 100 μm. Quantification of cell (C) migration and (D) invasion. Data are presented as the mean ± SD (n=3). *P<0.05 vs. control; #P<0.01 vs. pinosylvin-alone. NPC, nasopharyngeal carcinoma; U, U0126; SB, SB20035820; SP, SP600125.

Identifying effective methods for treating distant metastases resulting from NPC is crucial. In summary, the present results demonstrated that pinosylvin decreased activity of MMP-2 and expression of MMP-2/MMP-9 in both NPC-BM and NPC-039 cell lines. Pinosylvin significantly inhibited both cell migration and invasion. The expression levels of epithelial markers increased, while those of mesenchymal markers decreased following treatment with pinosylvin. Following pre-treatment with specific inhibitors of ERK1/2, p38 and JNK1/2, pinosylvin-inhibited cell migration and invasion significantly improved. However, the lack of activator experiments is a potential limitation to the present study. A recent study suggested that pinosylvin is mostly metabolized *in vivo* and may provide a material basis for studying the pharmacological action of pinosylvin, thus providing information for the clinical treatment of chronic gastritis and gastric ulcers using Radix Linderae Reflexae (49). The short half-life and limited systemic exposure of pinosylvin prompt caution in its therapeutic application (50). However, the lack of *in vivo* experiments is a potential limitation to the present study. The present results suggested that pinosylvin may be useful in the development of drugs for treating NPC and preventing migration and invasion of NPC cells.

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Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MCH, MJH and JTL conceptualized and designed the study. CCL, YCC, YSL and HYH. acquired, analyzed and interpreted data. MCH, YCC and MJH drafted and revised the manuscript.

MJH and JTL had overall responsibility for the published work. MJH and CCL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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