

Anti-metastatic effects of isolinderalactone via the inhibition of MMP-2 and up regulation of NM23-H1 expression in human lung cancer A549 cells

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Abstract. Metastatic lung cancer is a leading cause of mortality and has a mortality rate of $\geq 90\%$. Isolinderalactone (ILL) is a sesquiterpene lactone compound that has been used in traditional Chinese medicine. Research has demonstrated that ILL has anti-inflammatory and anti-proliferative properties; however, to the best of our knowledge, studies investigating whether ILL can inhibit lung cancer cell metastasis have not been conducted. In the present study, 1-10 μM ILL was applied in the culturing of the A549 lung cancer cell line to investigate the effects of ILL on the invasion and migration of lung cancer cells, including whether the possible mechanisms of ILL are associated with the expression of matrix metalloproteinase (MMP)-2 and NME/NM23 nucleoside diphosphate kinase 1 (NM23-H1) genes. The results of the present study indicated that ILL inhibited the invasion and migration of the A549 cancer cells and exhibited a dose-response association. ILL also significantly inhibited the protein expression and activity of MMP-2 ($P < 0.05$), exhibiting a trend similar to that of its invasion- and migration-associated properties. Further research revealed that ILL significantly increased the expression of NM23-H1 protein and inhibited the expression of β -catenin protein ($P < 0.05$). The results of the present study is, to the best of our knowledge, the first to confirm that ILL can inhibit the invasion and migration of A549 cancer cells, with

the possible mechanisms potentially involving the inhibition of MMP-2 and β -catenin protein expression resulting from the up regulation of NM23-H1 expression.

Introduction

Cancer is a leading cause of mortality; ~ 8.2 million people worldwide succumbed to cancer-associated mortality in 2012, with lung cancer accounting for ~ 1.59 million cases of mortality, or $\sim 20\%$ of the total (1). Lung cancer cell metastasis is a primary cause of mortality and those that experience it have a mortality rate of $\geq 90\%$ (2). When it metastasizes, lung cancer has a 95% chance of affecting local lymph nodes and an 80% chance of affecting other organs (3,4). Thus, in clinical terms, the overall 5-year survival rate from the time of diagnosis to mortality for patients with lung cancer undergoing treatment is only 10-15% (5). Cancer cell metastasis is a complex multistep process involving invasion and migration (6-8). Matrix metalloproteinases (MMPs) are zinc/calcium-dependent endopeptidases involved in the degradation of the extracellular matrix, two examples of which are MMP-2 and -9 (7,9). MMP-2 is a notable factor in the development of tumor metastasis (7,10). MMP-2 is highly expressed in malignant tumors and serves a key role in cancer invasion and angiogenesis (7,11). The inhibition of MMP-2 is, therefore, an effective strategy for preventing tumor cell metastasis (11,12). The NME/NM23 nucleoside diphosphate kinase 1 (NM23) gene was first identified in the murine melanoma cell line exhibiting high metastatic activity; increasing the expression of this gene may also reduce the metastatic activity of tumor cells (13). When transfected with NM23 genes, a variety of tumor cell types were reported to exhibit the inhibition of metastatic properties, including migration, invasion, and colonization (14-18). The NM23-H1 (NME1) gene was reported to be the one closely associated with the metastasis of cancer cells, including breast, lung and liver cancer (19-21).

Lindera aggregata (Sims) Kosterm is a traditional Chinese herbal medicine often used in Asia. It has been used to treat chest and abdominal pain, indigestion, regurgitation, colds, hernia and frequent urination (22). Studies have demonstrated that *L. aggregata* extract has antioxidant properties, and can inhibit tumor cell growth and induce apoptosis (23-26). For

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Abbreviations: MMP-2, matrix metalloproteinase-2; ILL, isolinderalactone; FOXO3, transcriptional factor fork head box O3; STAT3, signal transducer and activator of transcription 3; CUM, curcumin; DMSO, dimethyl sulfoxide; Nm23-H1, NME/NM23 nucleoside diphosphate kinase 1

Key words: isolinderalactone, matrix metalloproteinase-2, invasion, migration, NME/NM23 nucleoside diphosphate kinase 1, lung cancer cells

instance, Li *et al* (23) identified that *Lindera strychnifolia* extract was able to inhibit the growth of lung cancer A549 and SBC-3 cell lines, and induce cell apoptosis. Allografts also produced similar results; *L. strychnifolia* extract was able to inhibit the growth of Lewis lung, A549 and SBC-3 cancer cells, and induce cancer cell apoptosis (23). Isolinderalactone (ILL) is a type of sesquiterpene compound obtained from the root tuber of *L. aggregata*. Yen *et al* (27) verified that ILL could induce apoptosis in the human breast cancer MDA-MB-231 cell line, possibly via the inhibition of microRNA hsa-miR-30c expression and increasing the expression of suppressor of cytokine signaling 3 (SOCS3). This in turn inhibits the phosphorylation of signal transducer and activator of transcription 3 (STAT3) and regulates the downstream processing of STAT3 pathways, increasing B-cell lymphoma-2 (Bcl-2) and Bcl-extra large protein expression, and inhibiting X-linked inhibitor of apoptosis expression. A previous study also revealed that sesquiterpene lactone compounds, including ILL, linderalactone and linderane, were able to inhibit the proliferation of the A549 cancer cells, with ILL exhibiting the best inhibitory properties (28). However, it remains unclear whether ILL can inhibit lung cancer cell metastasis and the associated mechanisms require further investigation.

The present study aimed to investigate the effects of ILL on lung cancer A549 cell invasion and migration, as well as the association between potential mechanisms, and the expression of MMP-2 and NM23-H1 genes.

Materials and methods

Chemicals and reagents. ILL was purchased from Wuhan Chem Faces Biochemical Co., Ltd. (Wuhan, China). RPMI-1640, minimum essential medium-non-essential amino acids, Gluta MAX, trypsin, penicillin, streptomycin, and sodium pyruvate were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Dimethyl sulfoxide (DMSO) was purchased from Merck KGaA (Darmstadt, Germany). Anti- β -catenin monoclonal antibody (mAb; cat. no. NBP1-54467), anti-NM23 mAb (cat. no. NBP1-47398) and anti-E-cadherin mAb (cat. no. NBP2-19051) were purchased from Novus Biologicals, LLC. (Littleton, CO, USA). Anti-MMP-2 mAb (cat. no. 031129) and anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) antibodies (cat. no. 140769-HRP) were purchased from United States Biological (Salem, MA, USA). Anti-tissue inhibitor metalloproteinase-2 (TIMP-2; cat. no. 5738) mAb was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Transwell inserts were acquired from Costar; Corning Incorporated (Corning, NY, USA). Matrigel was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Curcumin (CUM) and protease inhibitor cocktail (cat. no. S8820) were obtained from Sigma-Aldrich; Merck KGaA. All chemicals used were of reagent grade or higher.

Cell culture. Human A549 lung cancer cells were obtained from the Bioresource Collection and Research Center, Institute of Biological Resources Conservation and Research (Hsinchu, Taiwan) and were cultured in RPMI-1640 medium containing 10% (v/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 0.37% (w/v) NaHCO₃, penicillin (100 U/ml),

and streptomycin (100 μ g/ml) at 37°C in a humidified incubator under 5% CO₂ and 95% air. An equal number (1x10⁴/ml) of cells were incubated for 24 h prior to the various treatments. Prior to experimentation, the medium was removed, and the cells were washed twice with PBS. Next, fresh RPMI-1640 medium (with 10% FBS) containing various concentrations (1, 5, 10, and 20 μ M) of ILL were added and the samples were incubated for 24 h. In addition, the effects of CUM at a concentration of 10 μ M were also evaluated and used as a positive control, as CUM has been reported to inhibit the migration and invasion of tumor cells by decreasing protein expression and the activity of MMP-2 in tumor cells (29,30). Stock solutions of ILL and CUM were dissolved in DMSO. Prior to use, the compounds were diluted in 10% FBS in RPMI-1640 medium to the desired concentrations at the time of addition. The highest concentration of DMSO used did not exceed 0.1% (v/v) of the total assay volume, which did not affect cell viability.

Cell growth analysis. Cell growth was assayed as described previously by Yeh *et al* (31). A549 cells were seeded in 6-well plates at a density of 1x10⁵ cells/well and cultured at 37°C for 24 and 48 h. Various concentrations of ILL were then added to the cells to reach final concentrations of 1, 5, 10, and 20 μ M in the presence of FBS. The control group contained 10% FBS. The cells were then cultured at 37°C, 5% CO₂, and 95% air for 24 and 48 h, and the trypan blue exclusion protocol was used to determine cell viability, as described previously (32,33). Briefly, an amount of 0.4% trypan blue dye solution equal to the sample volume was added. After gentle mixing, samples were loaded into a hemocytometer (cat. no. 065003, Marienfeld-Superior, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) for counting under a microscope at x100 magnification (33).

Cell migration assay. Tumor cell migration was assayed in transwell chambers according to the methods reported by Repesh (34), with certain modifications as previously described by Chuang *et al* (35). Briefly, transwell chambers with 6.5-mm polycarbonate filters of 8- μ m pore size were used. Following preincubation with the tested compounds for \geq 24 h, A549 cells (2.5x10⁵/ml) were suspended in 100 μ l serum-free RPMI-1640 medium and placed in the upper transwell chamber. RPMI-1640 medium containing 10% FBS (300 μ l) was placed in the lower transwell chamber to serve as the source of chemoattractant. The transwell was incubated for 12 h at 37°C. Subsequently, the cells on the upper surface of the filter were completely wiped away with a cotton swab. The cells on the lower surface of the filter were fixed in methanol at 25°C for 10 min, stained with Giemsa at 25°C for 1 h, and counted under a microscope (Inverted Microscope; ECLIPSE/TS100; Nikon Corporation, Tokyo, Japan; magnification, x100). For each replicate, the tumor cells in 5 randomly selected fields were determined, and the counts were averaged.

Cell invasion assay. Cell invasion was also assessed using transwell chambers as described in the migration assay; however, each filter was first coated with 100 μ l of 1:20 diluted Matrigel in cold RPMI-1640 medium (without chemoattractant) to form a thin continuous film on the top of the filter.

An aliquot (100 μ l) of serum-free RPMI-1640 containing 5×10^4 cells was added to each of the triplicate wells in RPMI-1640 medium containing 10% FBS, which served as a chemoattractant in the assay. Following incubation at 37°C for 24 h, cells were stained and counted as aforementioned. The number of cells invading the lower side of the filter was measured as representative of invasion activity.

Western blotting. Expression levels of NM23-H1, MMP-2, E-cadherin, β -catenin and, TIMP-2 proteins were determined by western blotting. Western blot analysis was performed as described previously (35). Briefly, the medium was removed and cells were lysed with protein lysis buffer (50 mM Tris-HCl buffer pH 7.4, 1 mM EDTA, 5% SDS, 1 mM phenylmethylsulfonyl fluoride, 1X protease inhibitor cocktail). The lysate was sonicated (frequency, 20 kHz), for 30 sec at 4°C followed by centrifugation at 12,000 x g for 30 min at 4°C. An amount of protein (40 μ g per lane) from the supernatant was resolved by 0.1% SDS-PAGE and transferred onto a nitrocellulose membrane. Following blocking with Tris-buffered saline buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 5% non-fat milk for 1 h at 25°C, the membrane was incubated at 4°C for 12 h with anti-NM23-H1 mAb (1:500 dilution), anti-MMP-2 mAb (1:500 dilution), anti-E-cadherin mAb (1:500 dilution), anti- β -catenin mAb (1:1,000 dilution) and, anti-TIMP-2 mAb (1:1,000 dilution) followed by HRP-conjugated anti-mouse IgG (incubated at 25°C for 2 h; 1:5,000 dilution) and then visualized using an ECL Chemiluminescent Detection kit (EMD Millipore, Billerica, MA, USA). The relative expression levels of NM23-H1, MMP-2, E-cadherin, β -catenin and TIMP-2 proteins were quantitated using Matrox Inspector version 2.1 software (Matrox Electronic Systems Ltd. Dorval, QC, Canada).

Gelatin zymography assay. MMP-2 activity was assayed using gelatin zymography according to the method reported by Hwang *et al* (36), with certain modifications, as previously described in a study by Chuang *et al* (35). The cells (5×10^4 cells/ml) were pretreated with ILL or CUM in RPMI-1640 medium containing 10% FBS for 24 h. Following two washes with PBS, the cells were incubated in serum-free RPMI-1640 medium for 24 h. Subsequently, the culture medium was harvested and stored at -20°C until use. For the gelatin zymography assay, the culture medium was separated on a 10% SDS-PAGE gel containing 0.1% (w/v) gelatin. Following electrophoresis, the gel was washed for 30 min at room temperature in a solution containing 2.5% (v/v) Triton X-100, with two changes, and then transferred to a reaction buffer for enzymatic reaction containing 1% sodium azide (NaN₃), 10 mM calcium chloride (CaCl₂), and 40 mM Tris-hydrochloride, pH 8.0, at 37°C with agitation overnight (for 15 h). Finally, the gel was stained at 25°C for 30 min with 0.25% (w/v) Coomassie blue in 10% acetic acid and 50% methanol and de-stained with 10% acetic acid and 50% methanol at 25°C for 2 h. The relative MMP-2 activities were compared with control group and quantitated using Matrox Inspector version 2.1 software.

Scavenging of DPPH radicals. The effect of ILL on DPPH radicals was studied by employing the modified method

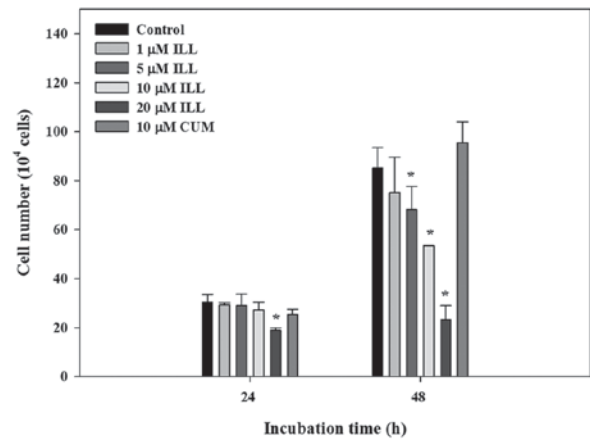


Figure 1. Effects of ILL and CUM on cell growth in human A549 lung cancer cells. Cells were treated with ILL (1-20 μ M) or CUM (10 μ M as the positive control) for 24 and 48 h. Cell numbers were counted using a hemocytometer. Bars represent mean \pm standard deviation (n=3). P<0.05 was considered to indicate a statistically significant difference (significance compared with control, *P<0.05). CUM, curcumin; ILL, isolinderalactone.

described by Blois (37) with some modifications as previously described in a study by Lin *et al* (38). Briefly, 0.1 ml 1 mM methanol solution of DPPH (cat. no. D9132, Sigma-Aldrich; Merck KGaA) was incubated with varying concentrations (1, 5, 10 and 100 μ M) of ILL. After a 30 min incubation period at room temperature, the absorbance of the resulting solution was read at 517 nm against a blank. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation: Scavenging effect (%)=[(1-absorbance_{sample})/absorbance_{control}] x 100.

Statistical analysis. Data were expressed as the mean \pm standard deviation and analyzed using one-way analysis of variance followed by Duncan's multiple range test for comparisons of group means. The correlation analysis was conducted using simple linear regression analysis. Statistical analysis was performed using SPSS version 10 (SPSS, Inc. Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of ILL on cell viability. To examine the effect of ILL on cell growth, the numbers of treated A549 cells were counted. As presented in Fig. 1, incubation of A549 cells with 1, 5 and 10 μ M ILL for 24 h did not decrease cell viability. However, cell viability significantly decreased at 48 h of incubation with 5, 10 and 20 μ M ILL. Neither ILL (1-10 μ M) nor CUM (10 μ M) inhibited the proliferation of A549 cancer cells at 24 h of incubation; thus, the following experiments were conducted after a 24 h incubation.

Effects of ILL on invasion and migration of A549 cancer cells. The anti-invasive and anti-migratory activity of ILL was investigated using transwell inserts, coated with Matrigel in the case of the invasion assay. As presented in Fig. 2, ILL significantly inhibited the invasion of A549 cancer cells in a dose-dependent manner, with a maximum inhibition rate of

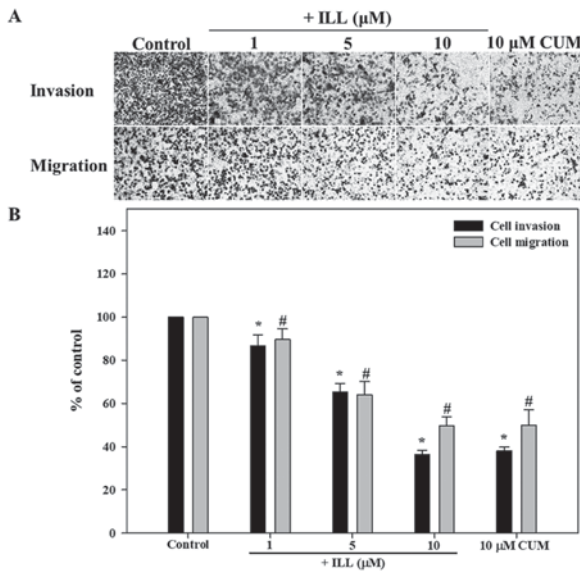


Figure 2. Effect of ILL and CUM on cell invasion and migration of A549 human lung cancer cells. The cells were incubated with ILL (1, 5 and 10 μM) and CUM (10 μM ; as the positive control) for 24 h. (A) Representative phase-contrast photomicrographs (magnification, $\times 100$) were captured at 24 h for the invasion assay and at 12 h for migration assay. (B) Densitometric analysis of (A). Bars represent mean \pm standard deviation ($n=3$). $P<0.05$ was considered to indicate a statistically significant difference (significance compared with control, * $P<0.05$ or # $P<0.05$). CUM, curcumin; ILL, isolinderalactone.

64% at 10 μM of ILL, compared with the control ($P<0.05$). As a positive control, the addition of 10 μM of CUM significantly inhibited the invasion of A549 cancer cells by 62% ($P<0.05$). Similar to the invasion assay results, ILL inhibited the migration of A549 cells in a dose-dependent manner, with a maximum inhibition of 50% at 10 μM of ILL, compared with the control ($P<0.05$).

Effects of ILL on MMP-2 activity and protein expression in A549 cells. The activity of MMP-2 was assayed using gelatin zymography. As presented in Fig. 3, ILL significantly inhibited MMP-2 activity in a dose-dependent manner, with a maximum inhibition of 32% at 10 μM ILL, compared with the control ($P<0.05$). The addition of 10 μM CUM significantly inhibited MMP-2 activity by 31% ($P<0.05$). As presented in Fig. 3, the results demonstrated that ILL significantly decreased MMP-2 protein expression levels in a dose-dependent manner ($P<0.05$). The addition of ILL at 5 and 10 μM significantly inhibited MMP-2 protein expression by 26 and 58%, respectively ($P<0.05$). In addition, CUM inhibited MMP-2 protein expression levels by 46% at 10 μM . These results were in accordance with those of the MMP-2 activity assay. Collectively, the data of the present study implied that inhibition of MMP-2 protein expression may inhibit the progression of the metastasis of A549 cancer cells.

Effects of ILL on NM23-H1 protein expression in A549 cells. As presented in Fig. 4, ILL significantly up regulated the expression of NM23-H1 protein in a concentration-dependent manner. The addition of ILL at 5 and 10 μM significantly increased the protein expression of NM23-H1 by 32 and 61%, respectively ($P<0.05$). In addition, CUM increased the expression of NM23-H1 by 35% ($P<0.05$). However, the level

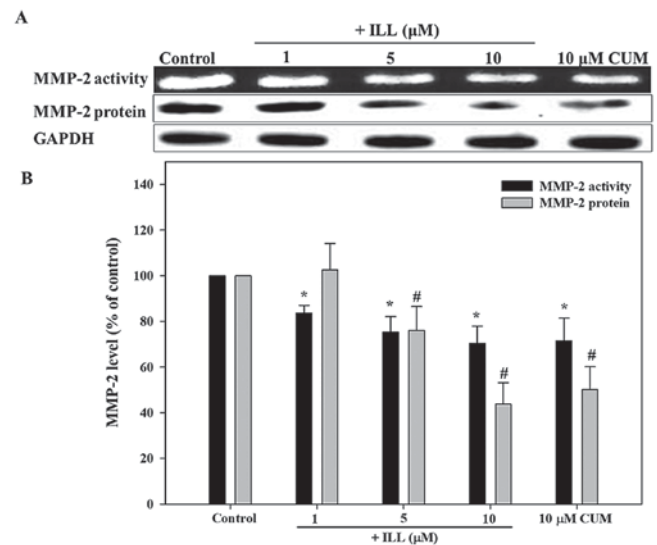


Figure 3. Effect of ILL and CUM on MMP-2 activity and protein expression in A549 human lung cancer cells. The cells were incubated with ILL (1, 5, 10 μM) and CUM (10 μM ; as the positive control) for 24 h. (A) Genolytic activity of MMP-2 (as determined by zymographic assay) and western blots of MMP-2 and GAPDH. (B) Relative MMP-2 activity and protein expression (using control as 100%). Bars represent mean \pm standard deviation ($n=3$). $P<0.05$ was considered to indicate a statistically significant difference (significance compared with control, * $P<0.05$ or # $P<0.05$). CUM, curcumin; ILL, isolinderalactone; MMP-2, metalloproteinase-2.

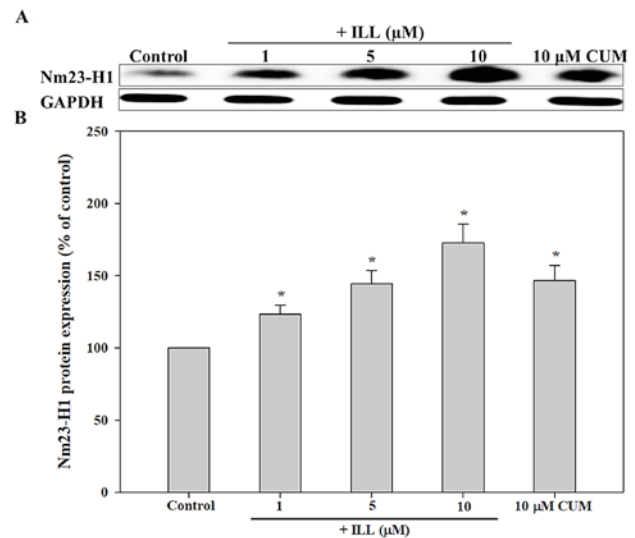


Figure 4. Effect of ILL and CUM on NM23-H1 expression in A549 human lung cancer cells. Cells were incubated with ILL (1, 5 and 10 μM) and CUM (10 μM ; as the positive control) for 24 h. (A) Western blot analysis of NM23-H1 protein and GAPDH. (B) Densitometric analysis of (A) (using control as 100%). Bars represent mean \pm standard deviation ($n=3$). $P<0.05$ was considered to indicate a statistically significant difference (significance compared with control, * $P<0.05$). CUM, curcumin; ILL, isolinderalactone; NM23, NME/NM23 nucleoside diphosphate kinase 1.

of TIMP-2 protein in A549 cancer cells was not significantly different among the groups ($P>0.05$; data not shown).

Effects of ILL on E-cadherin and β -catenin protein expression in A549 cells. As presented in Fig. 5, ILL

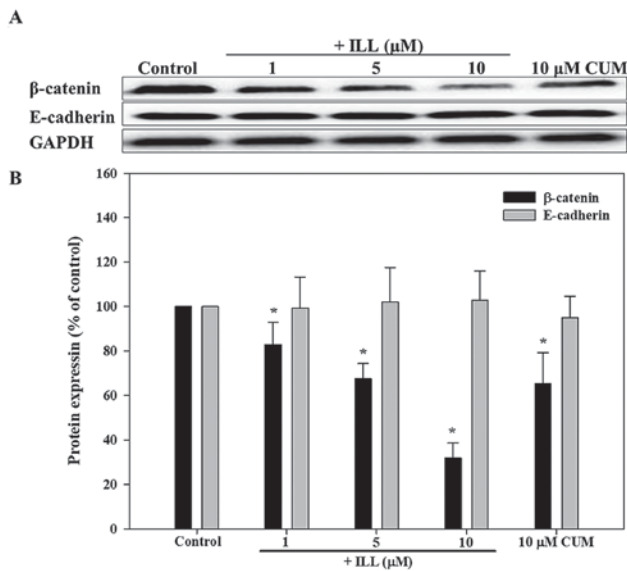


Figure 5. Effect of ILL and CUM on E-cadherin and β -catenin protein expression in A549 human lung cancer cells. The cells were incubated with ILL (1, 5 and 10 μ M) and CUM (10 μ M; as the positive control) for 24 h. (A) Western blots of E-cadherin protein, β -catenin protein and GAPDH. (B) Densitometric analysis of (A) (using control as 100%). Bars represent mean \pm standard deviation (n=3). P<0.05 was considered to indicate a statistically significant difference (significance compared with control, *P<0.05). CUM, curcumin; ILL, isolinderalactone.

significantly decreased β -catenin protein expression levels in a dose-dependent manner (P<0.05). The addition of 5 and 10 μ M ILL significantly decreased the protein expression levels of β -catenin by 32.5 and 68.1%, respectively (P<0.05). In addition, CUM decreased the protein expression of β -catenin by 34.7% (P<0.05). However, the levels of E-cadherin protein in A549 cancer cells were not significantly different between groups.

Antioxidant activity of ILL. DPPH is widely used as a reagent to evaluate free radical scavenging activity of antioxidants (39). The present study indicated that, at a concentration of 1-10 μ M, ILL did not significantly halt the activity of DPPH radicals (data not shown).

Correlation of NM23-H1 protein with MMP-2 activity and MMP-2 protein expression. NM23-H1 protein expression was negatively correlated with MMP-2 activity ($R^2=0.69$, P<0.001; Fig. 6A) and MMP-2 protein expression ($R^2=0.74$, P<0.001; Fig. 6B) in A549 cancer cells.

Discussion

Tumor cell metastasis is the leading cause of mortality in patients with lung cancer (2,4). The present study reported that ILL was able to inhibit the invasion and migration of A549 cancer cells, exhibiting a dose-response association. Numerous studies have reported that MMP-2 serves an important role in the invasion and migration of tumor cells and angiogenesis (6-8). Qian *et al* (12) reported that the expression level of MMP-2 was an independent prognostic factor for patients with non-small cell lung cancer, and was closely associated with

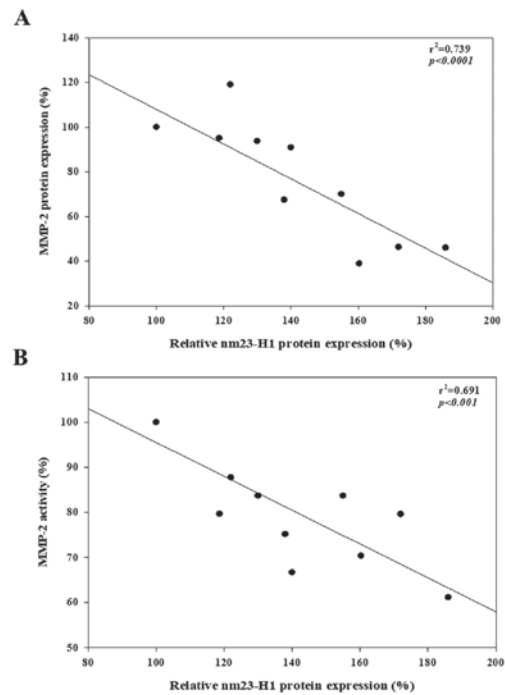


Figure 6. Correlation of NM23-H1 expression (%) with (A) MMP-2 activity and (B) MMP-2 protein expression (%). MMP-2, matrix metalloproteinase-2; NM23, NME/NM23 nucleoside diphosphate kinase 1.

pathological grade, clinical stage and lymphatic metastasis. Further analysis of MMP-2 expression and activity revealed that ILL was able to significantly inhibit MMP-2 expression and activity, again exhibiting a dose-response association. The inhibition trend of MMP-2 was similar to that of its invasion and migration-associated properties. The results of the present study indicated that the ability of ILL to inhibit the invasion and migration of A549 cells might be associated with reduced protein expression and secretion of MMP-2 in lung cancer cells. TIMP-2 is the endogenous inhibitor of MMP-2, and can combine extra cellularly with MMP-2 to inhibit MMP-2 activity (40,41). However, the present study did not observe any significant increase in TIMP-2 protein expression brought about by ILL (data not shown). It is thus speculated that the inhibition of MMP-2 by ILL was not associated with any mechanisms involving TIMP-2 expression.

The gene encoding NM23-H1 is a suppressor of tumor metastasis; research has revealed that it may reduce MMP-2 and -9 expression in various types of cancer cells (18-21,35,42,43). Boissan *et al* (19) previously reported that NM23-H1 silencing promotes the extracellular matrix invasion of HepG2 cells by up regulating numerous MMPs, including MMP-2. The results of the present study demonstrated that ILL was able to significantly increase NM23-H1 expression in a dose-dependent manner. Additionally, NM23-H1 expression does not affect the status of MMP-2 and -9 for certain tumor cells: Examples include oral cancer cell lines (44), cervical cancer (45) and gastric cancer (46). It is speculated that this may be associated with tumor cell type and the extent of oncogenesis. Thus, the ILL treatment group exhibited a negative correlation with respect to NM23-H1 protein expression ($R^2=0.74$, P<0.001) and MMP-2 protein expression ($R^2=0.69$, P<0.001) and

activity; however, it remains unclear whether the alterations in MMP-2 were correlated with the alterations in NM23-H1.

Numerous studies have reported that NM23-H1 is critical for control of cell-cell adhesion and cell migration during the early stages of the invasive program in tumor cells (19,43,47). Che *et al* (43) transfected NM23-H1 cDNA into the human non-small cell lung cancer L9981 cell line, which is negative NM23 expression, and reported that, via increasing β -catenin and E-cadherin protein expression, NM23-H1 may simultaneously inhibit MMP-2 and vascular endothelial growth factor protein expression, inhibiting cell mobility and metastasis. In another previous study, it was reported that NM23-H1 silencing may disrupt the cell-cell adhesion mediated by E-cadherin, resulting in β -catenin nuclear translocation in HepG2 cells (19). β -catenin is an important component of cell-cell adhesion (19,43,48). However, the loss of cell-cell adhesion due to the activation of β -catenin can increase the migratory potential of tumor cells (48). In the present study, ILL treatment significantly decreased β -catenin protein expression in a dose-dependent manner. We therefore hypothesize that the mechanisms of action of ILL, with respect to the reduction of A549 cancer cell migration, may be associated with the inhibition of β -catenin by up regulating the expression of NM23-H1 genes.

Recent evidence has also indicated that the transcriptional factor forkhead box O3 (FOXO3) in A549 cancer cells will reduce the expression of NM23-H1 (49). Additionally, Gong *et al* (50) also reported that STAT3 activation was inhibited by NM23-H1. STAT3 may bind directly to the STAT3 binding site on the NM23-H1 promoter and activate NM23-H1 expression, whereas its own expression may be inhibited, thereby reducing the metastasis of A549 cancer cells. Yen *et al* (27) also demonstrated that ILL is able to reduce microRNA hsa-miR-30c expression, increase SOCS3 activation and inhibit STAT3 phosphorylation. In this manner, cell apoptosis was induced in human breast cancer cells. In light of these findings, further research is required to determine whether the inhibition of A549 cancer cell metastasis by ILL is associated with the regulation of STAT3 or FOXO3 activity by NM23-H1.

The production of reactive oxygen species (ROS) in tumor cells may promote the invasion and migration of tumor cells (51). Therefore, the inhibition of cancer cell metastasis could be partially linked to the inhibition of ROS production. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) analysis is widely used to assess the effects of various antioxidants on the efficiency of free radical scavenging (52). In this study, 1-10 μ M of ILL did not significantly reduce the activity of DPPH free radicals (data not shown), and it is thus speculated that the inhibition of A549 lung cancer cell metastasis may be not be associated with the clearance of ROS.

On the basis of the aforementioned results and to the best of our knowledge, the present study is the first to confirm that ILL can significantly inhibit the invasion and migration of A549 lung cancer cells; the possible molecular mechanisms may constitute increases in NM23-H1 expression, and the inhibition of MMP-2 activity and protein expression, as well as the inhibition of β -catenin protein expression. However, further investigation into the relevant mechanisms of metastasis is required.

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