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Lupeol suppresses migration and invasion *via* p38/MAPK and PI3K/Akt signaling pathways in human osteosarcoma U-2 OS cells

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ABSTRACT

Lupeol, one of the common components from the fruits and natural foods, has been reported to exert antitumor activities in many human cancer cell lines; however, its effects on osteosarcoma cell metastasis were not elucidated. In the present study, lupeol at 10–25 μM induced cell morphological changes and decreased total viable cell number in U-2 OS cells. Lupeol (5–15 μM) suppressed cell mobility, migration, and invasion by wound healing and transwell chamber assays, respectively. Lupeol inhibited the activities of MMP-2 and −9 in U-2 OS cells by gelatin zymography assay. Lupeol significantly decreased PI3K, pAKT, β-catenin, and increased GSK3β. Furthermore, lupeol decreased the expressions of Ras, p-Raf-1, p-p38, and β-catenin. Lupeol also decreased uPA, MMP-2, MMP-9, and N-cadherin but increased VE-cadherin in U-2 OS cells. Based on these observations, we suggest that lupeol can be used in anti-metastasis of human osteosarcoma cells in the future.

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Osteosarcoma (OS), one of the most frequent malignant primary bone tumors in children [1], is observed to have a second incidence peak in over 60 years [2]. OS exhibits high destructive and metastatic potential [3,4] to damage surrounding tissues and to metastasize especially to the lung [5,6]. Currently, the exact etiology of OS in human population remains unclear, that was one of the obstacles for the OS treatment. Moreover, for the administration of chemotherapy, about 80% of OS patients with metastatic disease at diagnosis suffer relapse [7]. Currently, both surgical techniques and adjuvant chemotherapy have been improved; however, OS is still a primary cause of mortality for patients [8]. Therefore, new drugs or combination therapies are needed for patients with OS.

Metastasis involves multistep process initiated by local adhesion, migration, and invasion that also accompany a variety of proteases such as matrix metalloproteinases (MMPs) and urokinase, that degraded the extracellular matrix (ECM) and basement membrane [9]. ECM acts as a mechanical barrier which needs to be degraded through matrix metalloproteinase (MMP) system [10] for cell movement. That is a vital step in the metastatic process [11]. Other factors also play an important role in the process of metastasis, such as epithelial-mesenchymal transition (EMT) which is associated with cell polarity and cell-cell junction [12]. MMPs upregulation also promotes cancer metastasis [13–15]. In osteosarcoma cell lines, MMP-2 and MMP-9 are overexpressed and associated with enhanced metastatic ability [16]. Therefore, to suppress the factors or pathway associated with EMT and MMPs may be part of potential strategies for inhibiting tumor metastasis.

Numerous reports have shown that natural products from fruits and vegetables can prevent and/or treat certain cancers [17–19]. Lupeol (Lup-20(29)-en -3b-ol), a dietary triterpene, present in common fruits and vegetables [20,21] has anti-inflammatory and anti-cancer activities [22]. Lupeol has anticancer activity against many human cancer cells, and the major biofunctions of lupeol included the induction of cell cycle arrest and apoptosis [23–28]. Lupeol (2 mg/animal) significantly decreased tumor volume and suppressed local metastasis of head and neck squamous cell carcinoma cells in an orthotopic nude mouse model [29]. Lupeol has shown to induce apoptotic cell death and inhibit the migration and invasion of gallbladder carcinoma (GBC-SD) cells *via* the suppression of EGFR/MMP-9 signaling [30].

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However, very limited studies have been performed about the effect of lupeol on cell migration and invasion and its molecular mechanism on human osteosarcoma cells. Therefore, the aim of the present study is to focus on the possible effects and molecular mechanisms of lupeol on migration and invasion of osteosarcoma U-2 OS cells *in vitro*. These findings may provide the anti-metastasis mechanisms of lupeol for the clinical treatment of metastasis of human osteosarcoma in the future.

Methods

Test chemicals, reagents, and culture medium

Lupeol, dimethyl sulfoxide (DMSO), Tris-HCl, trypan blue, trypsin, propidium iodide (PI), Ethylenediaminetetraacetic acid (EDTA), gelatin, and Coomassie blue R-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). McCoy's 5A medium, penicillin-streptomycin and fetal bovine serum (FBS) were obtained from GIBCO/Invitrogen Life Technologies (Carlsbad, CA, USA). Antibodies against MMP-2 and MMP-9 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and against PI3K, p-AKT, p-PTEN, GSK3α/β, β-catenin, Ras, p-Raf-1, p-p38, TIMP-1, uPA, VE-cadherin, N-cadherin, β-actin, and peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell line and culture

Human osteosarcoma cell line (U-2 OS) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). U-2 OS cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/mL streptomycin and 100 units/mL penicillin in a humidified atmosphere containing a 5% CO_2 at 37°C [31].

Cell morphological changes and viability assays

U-2 OS cells (1×10^5 cells/well) were seeded in 12-well plates with McCoy's 5A medium and left to attach for 24 h and were incubated with lupeol at final concentrations (0, 5, 10, 15, 20 and 25 μM) in triplicate for 12 and 24 h. After incubation, cells were directly examined under contrast-phase microcopy at 200× and photographed. Cells were harvested, washed, counted, and stained with PI (5 μg/mL) for measuring total viability by using flow cytometry (Becton-Dickinson, San Jose, CA, USA) as described previously [31].

Scratch wound healing assay for cell mobility

Cell mobility characteristic was assayed by scratch wound healing protocol as described previously [31]. U-2 OS cells were seeded in a 12-well plate at a density of 3×10^5 cells/well until reaching a 100% confluency monolayer which were replaced with serum-free McCoy's 5A culture medium. Cell monolayers were wounded by scratching with a 200 μL pipette tip and using PBS to wash for removing cell debris and then treated with 5, 10, and 15 μM lupeol for 24 h. Control group (0 μM) was treated with same dilutions of DMSO alone. In the denuded zone, cell images were photographed using a phase-contrast microscope at different time points (0 and 24 h) as described previously [31].

Gelatin zymography assay for gelatinolytic (MMP-2 and −9) activity

U-2 OS cells (1×10^5cells/well) were kept in a 12-well plate at 37°C in a CO_2 incubator for 24 h and then replaced with serum-free McCoy's 5A culture medium containing lupeol (0, 5, 10 and 15 μM) for 12 and 24 h. Each conditioned medium was collected and subjected to 10% SDS-PAGE gel containing 0.2% gelatin and run in the SDS running buffer. Gels were washed twice in renaturing solution containing 2.5% Triton X-100 in dH_2O at 25°C for 30 min, then incubated with zymogen developing buffer (550 mM Tris (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.02% Brij 35) for 24 h at 37°C. Gel bands corresponding to MMP-2 and −9 activities were stained using 0.2% Coomassie Brilliant Blue for 30 min at RT, de-stained with de-staining solution (50% v/v methanol and 10% v/v acetic acid), and then photographed. The band of gelatinolytic activity was measured using NIH Image J software, version 1.47 (National Institutes of Health, Bethesda, MA, USA) [31,32].

Transwell assay for the measurement of cell migration and invasion

The cell migratory and invasion ability were assayed by a commercial Transwell chamber insert (8 μm pore size, Millipore, Temecula, CA, USA). The chambers were coated with collagens (Sigma Chemical Co.; St. Louis, MO, USA) for the determination of migratory ability and with Matrigel for the invasion test. U-2 OS cells (5×10^4 cells/well) suspended in serum-free McCoy's 5A medium and different concentrations of lupeol (0, 5, 10, and 15 μM) were placed in the upper chamber which were coated with 50 μL collagen. An 800 μL of McCoy's medium with 10% FBS was placed in the lower chamber for 24 h and cells adhering to the upper surface of the membrane were removed by cotton swab. The

migrated cells (on the underside of the filter) were first fixed with 100% methanol, stained by 0.1% crystal violet solution, and finally photographed under light microscopy. Finally, the numbers of stained cells were counted under a microscope. For cell invasion, all subsequent steps were performed in the same manner as described for cell migration assay except filters were coated with Matrigel [31,32].

Western blotting analysis for cell metastasis-associated protein expressions

Approximately U-2 OS cells (1×10^6cells/dish) were seeded in 10 cm culture dishes for 24 h, and cells were incubated with lupeol (0 and 15 μM) for 0, 2, 4, 6, 8, 12 and 24 h. After incubation, cells were washed with cold PBS and lysed with lysis buffer (50 mM Tris-HCl pH 7.5, 400 mM NaCl, 2 mM EGTA, 1 mM EDTA, 1 mM DTT, and protease inhibitor cocktail) (Roche) on ice. The cell lysates were centrifuged and quantitated total protein concentration according to the Bio-Rad protein assay reagent and kit as described previously [31,32]. The protein was quantified, and equal amount (30 μg) of proteins were electrophoresed onto 8–12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to PVDF membranes. Membranes were probed using specific primary antibodies against PI3K, p-AKT, p-PTEN, GSK3α/β, Ras, p-Raf-1, p-p38, TIMP-1, uPA, MMP-2, MMP-9, VE-cadherin, N-cadherin, β-catenin and β-actin at 4°C overnight. Membranes were followed by incubation with horseradish peroxidase-conjugated appropriate secondary antibodies (diluted 1:5000; Santa Cruz Biotechnology), and visualized by the ECL detection system. Image J software was used for densitometry of the blots [31,32].

Confocal laser microscopy assay for examining p-p38 expressions in U-2 OS cells

Western blotting had indicated that lupeol decreased the p-p38 expression in U-2 OS cells, herein, we further investigated the translocation and expression of p-p38. U-2 OS cells were treated with 15 μM of lupeol for 0, 2, 4, 6 and 8 h and were examined and photographed by confocal laser microscopy systems as described previously [33].

Statistical analysis

Data are presented as mean ± SD from three independent experiments. Differences between groups were analyzed by one-way analysis of variance and Dunnett test for multiple comparisons (SigmaPlot for Windows version 12.0; Systat Software, Inc., San Jose, CA). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ are determined as significant between lupeol-treated and control groups.

Results

Lupeol induces cell morphological changes and decreases total viability of U-2 OS cells

U-2 OS cells were treated with various concentrations of lupeol for 12 and 24 h, and cells were examined for morphological changes, photographed, and the percentage of viable cell number was counted and presented in Figure 1(a,b). Lupeol at 5–10 μM treatment at 12 and 24 h did not decrease total viable cells, however, 15–25 μM treatment of lupeol show significantly decreased total viable cell number and cell morphology changes in U-2 OS cells. Therefore, we selected 5–15 μM for further experiments. The reason for using 15 μM of lupeol is it acts like a positive control.

Lupeol inhibits cell mobility in U-2 OS cells

Wound healing assay was used for examining cell mobility and results are presented in Figure 2(a,b). Lupeol at the 5–15 μM significantly suppressed the closure rate of the scratch in U-2 OS cells when compared to control group and these effects are dose-dependent. Therefore, lupeol significantly inhibited the cell mobility of U-2 OS cells *in vitro*.

Lupeol affects matrix metalloproteinase activity in U-2 OS cells

After U-2 OS cells were treated with 0, 5, 10 and 15 μM of lupeol for 12 and 24 h, conditioned medium was harvested for examining the gelatinase activities of MMP-2 and −9 by using gelatin zymography assay and the results are presented in Figure 3(a,b). Lupeol at 10 and 15 μM significantly inhibited MMP-2 activity at 12 h treatment but only at 15 μM significantly reduced MMP-2 activity at 24 h treatment (Figure 3(a)). However, lupeol only at 15 μM significantly reduced MMP-9 activity at 12 and 24 h treatment in U-2 OS cells.

Lupeol affects cell migration and invasion in U-2 OS cells

The transwell chambers were used for assaying cell migration and invasion of U-2 OS cells after exposure to various concentrations of lupeol for 24 h. Figure 4(a) indicates that lupeol at 5–15 μM significantly suppressed the migration of U-2 OS cells around 18–82% compared to untreated (control) cells. Figure 4(b) indicates that lupeol at 5–15 μM significantly inhibited the invasion of U-2 OS cells about 20–70% compared to control groups. Both results indicated that lupeol reduced cell migration and invasion in a dose-dependent manner (Figure. 4(a,b)).

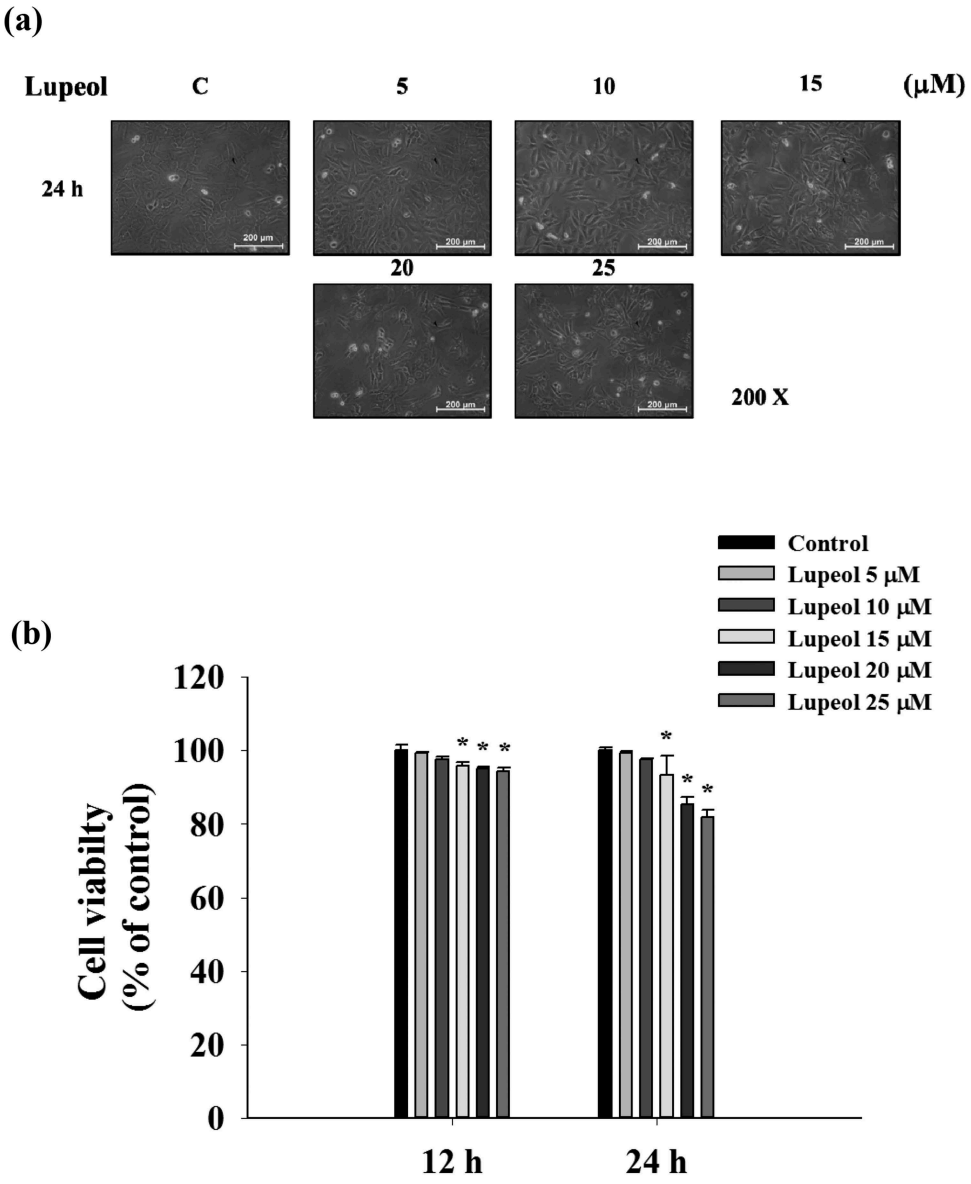

Figure 1. Lupeol induced cell morphological changes and decreased cell viability of U-2 OS cells.
Cells (1×10^5 cells/well) were incubated with lupeol (0, 5, 10, 15, 20, and 25 μM) for 24 h. Cells were examined for morphological changes (a) and were harvested for total cell viability (b) as described in materials and methods. *$p < 0.05$, significant difference between lupeol-treated groups and the control as analyzed by as analyzed by one-way ANOVA.

Lupeol affects key metastasis-related proteins in U-2 OS cells

For further investigating the inhibition of cell migration and invasion of lupeol of U-2 OS cells and whether it was *via* the effects of associated protein expression, western blotting was performed and results are present in Figure 5. Results indicated that lupeol (15 μM) significantly decreased PI3K at 2 h, pAKT at 2 and 4 h, p-PTEN at 8 h, and increased GSK3β at 4–8 h treatment (Figure 5(a)). In addition, lupeol decreased the expressions of Ras, p-Raf-1, and p-p-38 (Figure 5(b)). Lupeol decreased uPA at 12 and 24 h, decreased MMP-2 at 12 h and decreased MMP-9 at 12 and 24 h treatment (Figure 5(c)). Lupeol decreased N-cadherin and increased VE-cadherin at 12 and 24 h (Figure 5(d)). Furthermore, lupeol (15 μM) decreased β-catenin level in the nucleus and decreased p-p38 level in the cytoplasm and nucleus in U-2 OS cells (Figure 6(a)). Based on these findings, lupeol inhibited cell metastasis of U-2 OS cells may be through multiple signaling pathways.

Lupeol decreases p-p38 proteins in U-2 OS cells

In order to confirm whether or not p-p38 involved the reduced cell migration and invasion, U-2 OS cells were incubated with 15 μM of lupeol for 2, 4, 6 and 8 h and then cells were harvested for examining by confocal laser microscopy and results are presented in Figure 6(b). The level (fluorescence intensity) of p-p38 in cytoplasm and nucleus was decreased in a time-dependent manner.

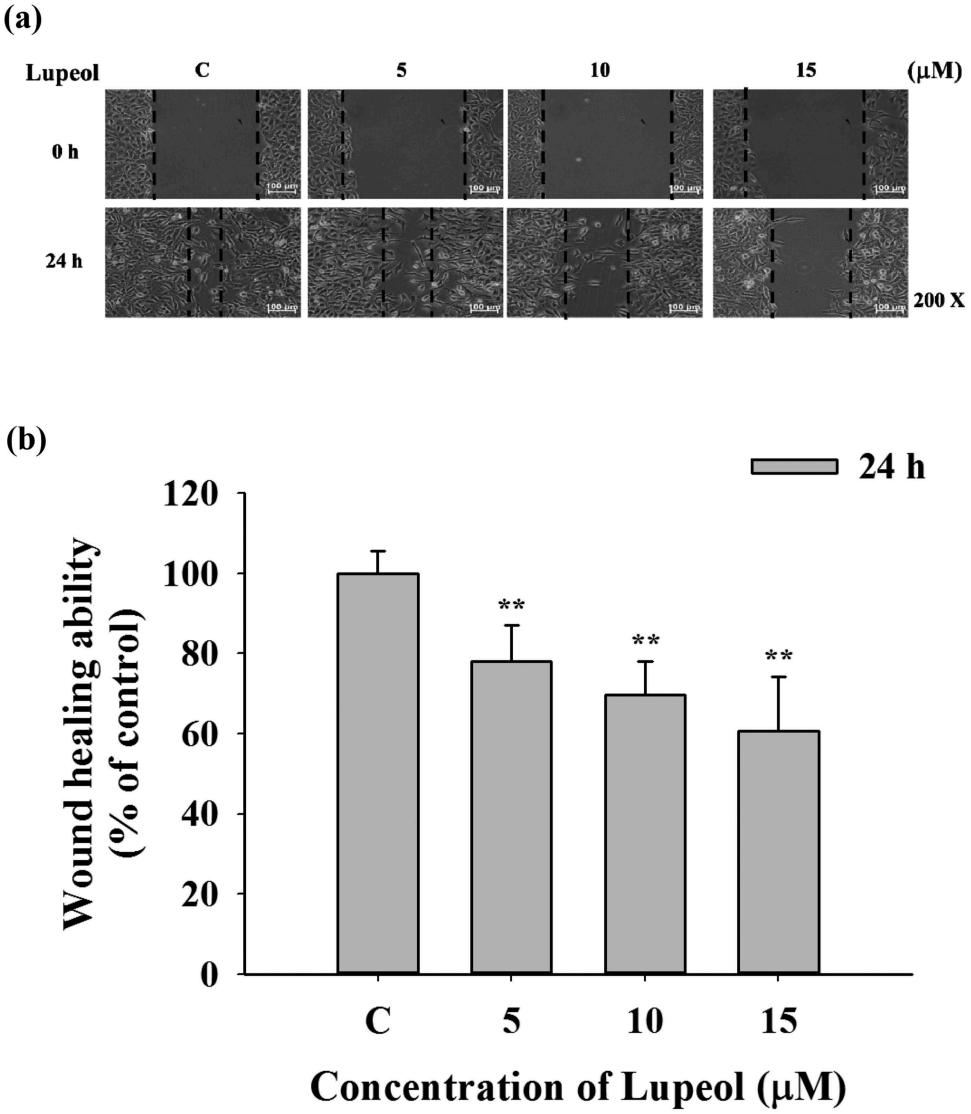

Figure 2. Lupeol inhibited *in vitro* wound closure of U-2 OS cells. Cells (3×10^5cells/well) were kept in 12-well plate for 24 h, scratched (wounded), and were incubated with lupeol (0, 5, 10, and 15 μM) for 24 h.
The relative wound closures were photographed using phase contrast microscopy (a) and percentage of healing area were calculated (b) as described in materials and methods. **p < 0.01, significant difference between lupeol-treated groups and the control as analyzed by one-way ANOVA.

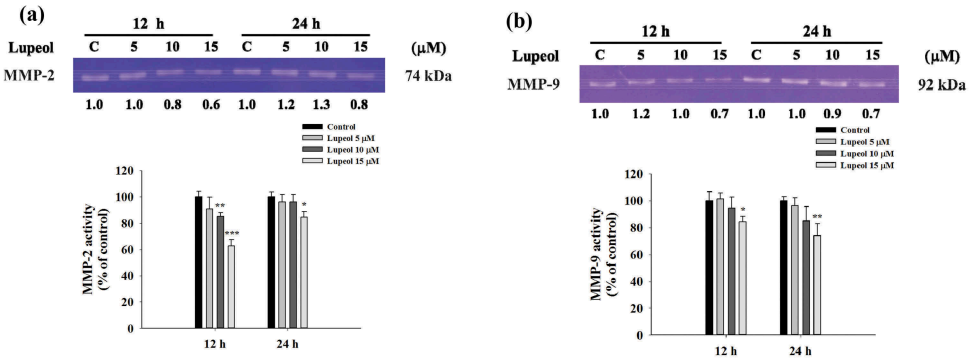

Figure 3. Lupeol inhibited MMP-2 and −9 activities of U-2 OS cells.
Cells (1×10^5 cells/well) were placed at 12-well plate and treated with lupeol (0, 5, 10, and 15 μM) for 24 h and then conditioned medium was harvested for gelatin zymography assay as described in materials and methods. Representative gelatin gel pictures: MMP-2 (a) and MMP-9 (b). *p < 0.05, **p < 0.01, ***p < 0.001, significant difference between lupeol-treated groups and the control as analyzed by one-way ANOVA.

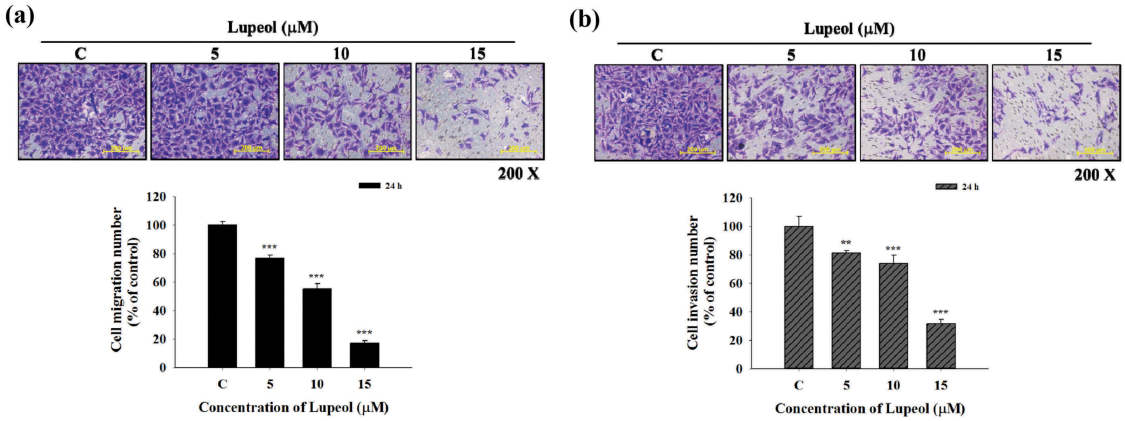

Figure 4. Lupeol suppressed cell migration and invasion of U-2 OS cells.
Cells (5×10^4 cells/well) were placed on transwell insert coated with collagen for migration or Matrigel for invasion and were treated with lupeol (0, 5, 10, and 15 μM) for 24 h. U-2 OS cells penetrated to the lower surface of the transwell membrane for migration (a) and invasion (b) were stained with crystal violet and were photographed under a light microscope at 200 × and penetrated cells were counted as described in materials and methods. **$p < 0.01$, ***$p < 0.001$, significant difference between lupeol-treated groups and the control as analyzed by one-way ANOVA.

Discussion

Cell metastasis is the spread of cancer cells from primary site to other sites to form tumors at distant sites. Patients with invasive and metastatic tumor could lead to being relatively resistant for chemotherapy. Overall, cancer metastasis accounts for more than 90% of cancer mortality [34]. Therefore, one of the strategies for chemotherapy is to block cancer cell migration and invasion, furthermore, another potential strategy is to inhibit the molecular pathways involved in cancer metastasis [35,36].

Currently, the treatment of patients with osteosarcoma include surgery, chemotherapy, and adjuvant postoperative chemotherapy, however, those treatments remain still unsatisfactory due to cancer cell metastasis to lung and cancer cell forming multi-drug resistance [37]. Furthermore, the understanding of molecular biomarker and signaling pathway of cancer cell in osteosarcoma patients in the early stages are still unclear [38]. The other drawback for therapy for patients with osteosarcoma is the side effects; therefore, more studies have focused on finding new compound from natural products for treatment of osteosarcoma. Lupeol is obtained in natural plants, and it has been shown to induce cancer cell apoptosis and inhibit cell migration and invasion in many human cancer cell lines. However, there is no evidence to show lupeol suppress cell migration and invasion in human osteosarcoma cells. Therefore, we investigated the effects of lupeol on cell migration and invasion in U-2 OS cells *in vitro*.

We treated U-2 OS cells with various concentrations of lupeol for 12 and 24 h and cell morphological changes and total viable cell number were examined and results indicated that lupeol induced cell morphological changes and decreased total viable cell number (Figure 1(a,b)) and these findings are in agreement with other reports [23–28]. Therefore, we selected the concentration (5–15 μM) of lupeol for further experiments.

Cancer cell mobility is involved in tumor metastasis. In this study, wound healing cell mobility assay was used for examining cell mobility [30,31]. Results indicated that lupeol inhibited cell mobility of U-2 OS cells at 5–15 μM at 24 h treatment (Figure 2(a,b)) in a dose-dependent manner (Figure 2(b)). This is a first finding regarding lupeol suppressing the cell mobility of U-2 OS cells *in vitro*.

MMPs play an important role in cancer cell metastasis, herein, we have found that lupeol suppressed the cell mobility, and the activities and the expression of MMP-2 and −9, which are key elements for cell metastasis. Therefore, we try to examine whether or not lupeol inhibited cell mobility are involved in the inhibitions of MMP-2 and −9. MMP-2 and MMP-9 gelatinase activity can be measured by using gelatin zymography. Results indicated that lupeol at 10 and 15 μM significantly reduced MMP-2 activity at 12 h but only at 15 μM reduced MMP-2 activity at 24 h treatment; lupeol only at 15 μM significantly reduced MMP-9 activity at 12 and 24 h treatment (Figure 3(a,b)). According to the results of gelatin zymography, there may be other factor, such as urokinase plasminogen activator (uPA), involved in the inhibition of cell mobility in lupeol-treated U-2 OS cells.

Chamber filter system was used for examining cell migration and invasion. Results from Figure 4(a,b) indicated that lupeol at 5–15 μM after 24 h treatment significantly inhibited cell migration and invasion in U-2 OS cells *in vitro* and these effects are in a dose-dependent manner. Many signaling pathways were involved in the metastasis of tumors such as PI3K/AKT/mTOR, ERK/MAPK, and Slit-Robo pathways [39,40]. Therefore, we used western blotting to examine the protein expression

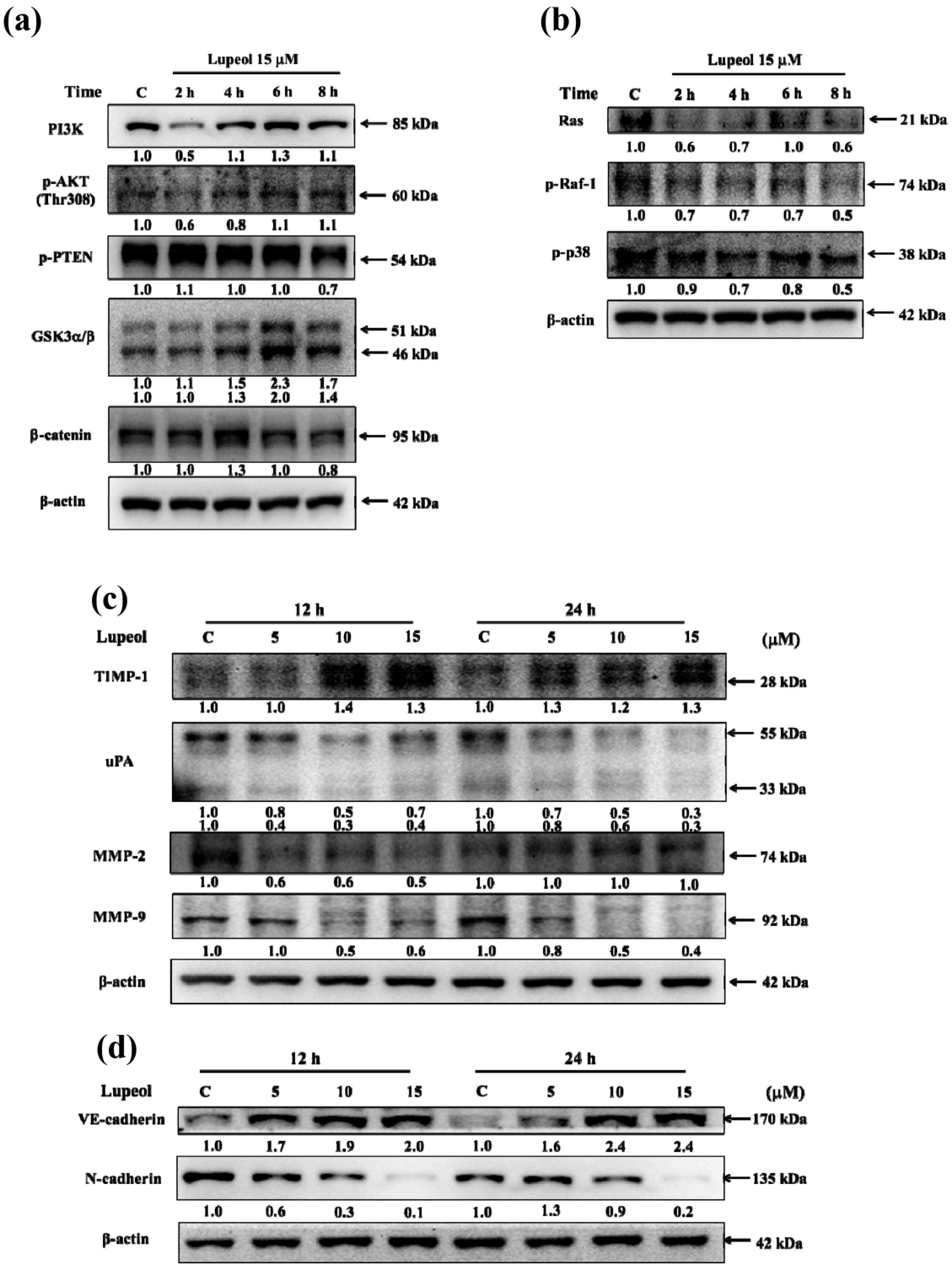

Figure 5. Lupeol affected the levels of associated proteins in migration and invasion of U-2 OS cells.
Cells (1×10^6 cells/dish) were treated with lupeol (15 μM) for 2, 4, 6 and 8 h. Cells were harvested for quantitated total protein and were performed by SDS-PAGE as described in the Materials and Methods. The levels of PI3K, p-AKT^{Thr308}, p-PTEN and GSK3α/β (a); Ras, p-Raf-1 and p-p-38 (b) were analyzed by western blotting. Cells (1×10^6 cells/dish) were treated with different concentrations of lupeol (0, 5, 10, and 15 μM) for 12 and 24 h. Cells were harvested and the levels of TIMP-1, uPA, MMP-2 and MMP-9 (c); VE-cadherin and N-cadherin (d) were estimated by western blotting as described in Materials and Methods.

of metastasis-associated protein within 24-h lupeol-treated U-2OS cells. The expressions of metastasis-associated pathways were analyzed in short-time treatment and those of matrix metallopeptidases at 24 h. Results indicated that lupeol decreased PI3K expression at 2 h treatment and then increased PI3K levels at 4–8 h treatment (Figure 5(a)). Lupeol decreased p-AKT at 2–4 h treatment and then increased it at 6–8 h treatment (Figure 5(a)). These results were consistent with the reports that the activated PI3K/AKT signaling was associated with cancer cell invasion and oncogenesis [41–43]. Furthermore, lupeol increased GSK3α/β at 2–8

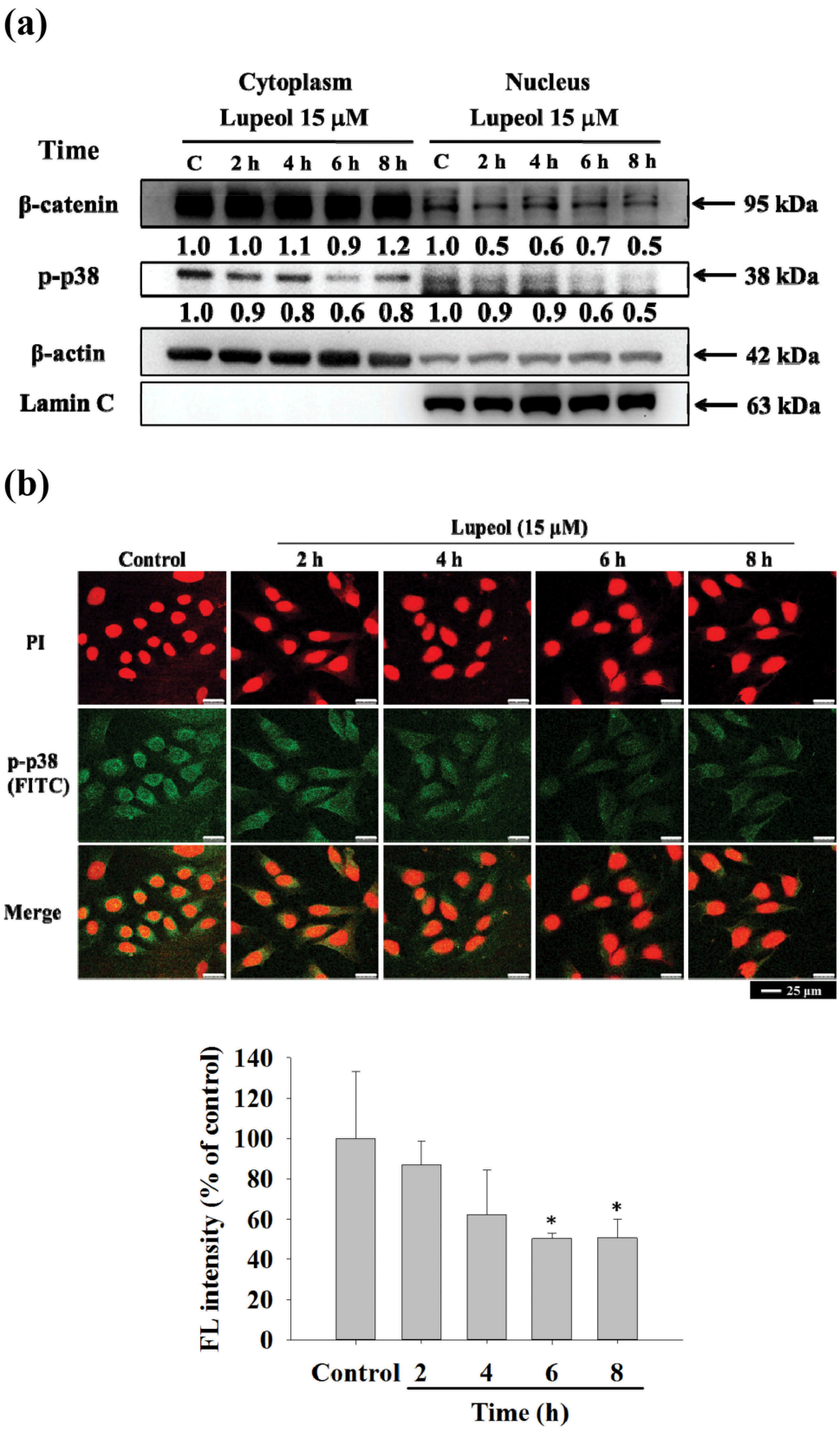

Figure 6. Lupeol affected the levels of associated proteins in cell migration and invasion of U-2 OS cells.
Cells (1×10^6 cells/dish) were treated with lupeol (15 μM) for 2, 4, 6, and 8 h. Cells were harvested, cytoplasmic and nuclear proteins were individually extracted, and quantitated total protein for SDS-PAGE as described in the materials and methods. The levels of β-catenin and p-p38 (a) expressions were estimated by western blotting. Or cells were cultured and treated with lupeol (15 μM) for 2, 4, 6, and 8 h and examined the p-p38 levels and fluorescence intensity (b) by confocal laser microscopy as described in the materials and methods. β-actin and lamin C were the internal control of cytoplasmic and nuclear protein, respectively.

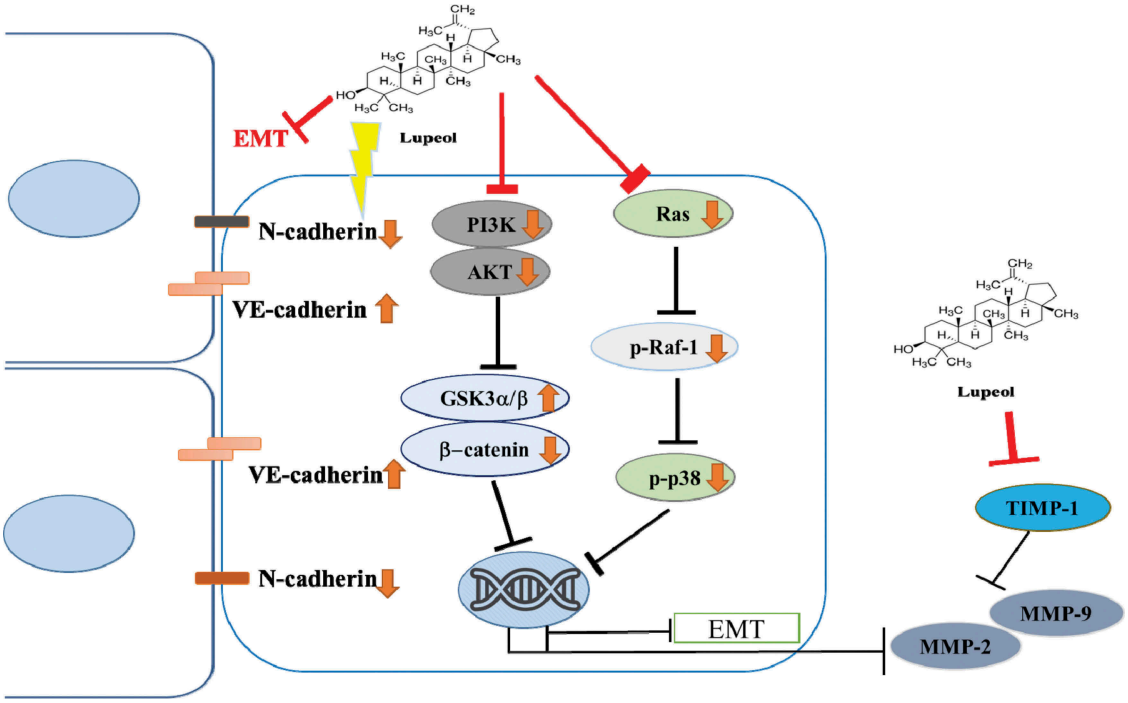

Figure 7. The possible signaling pathways for lupeol suppressed cell migration and invasion in U-2 OS cells *in vitro*.

h treatment (Figure 5(a)). It has been recognized that PI3K/Akt/GSK-3β signaling pathways are overactive in cancer cells, and it leads to reduce apoptosis and allows cell to proliferate and to promote cancer cell invasion and metastasis [44–47]. Thus, if agents can induce the inhibition of the PI3K/Akt/GSK-3β signaling pathway, that may present beneficial effects on treating cancer patients with advanced metastasis.

Results also showed that lupeol decreased Ras, p-Raf-1 and p-p38 expression in U-2 OS cells (Figure 5(b)). We also used confocal laser microscopy assay to confirm that lupeol significantly reduced the level of p-p38 in U-2 OS cells *in vitro* (Figure 6(b)). Ras/Raf/MEK/ERK may play an important role in osteosarcoma lung metastasis [48]. Blocking the Ras/MAPK kinase cascade have been reported to be a feasible and promising approach for osteosarcoma treatment [49]. The p38-MAPK signaling pathways are involved in a variety of cellular responses including cell apoptosis [50] and cell metastasis, and the p38 MAPK are phosphorylated and activated by dual kinases MKK3 and MKK6 at threonine and tyrosine regions [51].

Lupeol significantly inhibited the expression of β-catenin in U-2 OS cells (Figure 5(c)). Thus, lupeol inhibited U-2 OS cell migration and invasion may also through the inhibition of β-catenin. The activation of Wnt/β-catenin induced alterations to the actin cytoskeleton may lead to the acquisition of a migratory phenotype [52]. Furthermore, mesenchymal stem cells promote metastatic growth and chemoresistance of cholangiocarcinoma cells *via* activation of Wnt/β-catenin signaling [53]. Based on these results, lupeol indicated that activation of Wnt/β-catenin signaling may lead to cancer cell metastasis.

To summarize, lupeol significantly inhibited cell mobility, migration and invasion of U-2 OS cells *in vitro* that involved in inhibiting β-catenin and p-p-38 by PI3K/Akt/GSK-3β and p38/MAPK signaling pathway, respectively. Furthermore, the reduced levels of p-p38 and β-catenin led to the inhibition of MMP-2 and MMP-9 *in vitro*. Overall, the possible signaling pathways of lupeol reduced the cell mobility, migration and invasion of U-2 OS cells are outlined in Figure 7.

Author contributions

Study conception and design: M.J.H., W.W.H., and J.G.C.; Acquisition of data: M.J.H., S.F.P. and F.S.C.; Analysis and interpretation of data: F.J.T., C.Y.H., C.H.T., J.S.Y., and Y. M.H.; Drafting of manuscript: S.F.P., W.W.H., and J.G.C.; Critical revision: W.W.H. and J.G.C. All authors discussed the results and commented on the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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