Abstract. Kaempferol is a natural flavonoid that possesses anti-proliferative and apoptosis-inducing activities in several cancer cell lines. In the present study, we investigated the anti-metastatic activity of kaempferol and its molecular mechanism(s) of action in human osteosarcoma cells. Kaempferol displayed inhibitory effects on the invasion and adhesion of U-2 osteosarcoma (OS) cells in a concentration-dependent manner by Matrigel Transwell assay and cell adhesion assay. Kaempferol also inhibited the migration of U-2 OS cells in a concentration-dependent manner at different treatment time points by wound-healing assay. Additional experiments showed that kaempferol treatment reduced the enzymatic activities and protein levels of matrix metalloproteinase (MMP)-2, MMP-9 and urokinase plasminogen activator (uPA) by gelatin and casein-plasminogen zymography assays and western blot analyses. Kaempferol also downregulated the mRNA levels of MMP-2 and MMP-9 by quantitative PCR analyses. Furthermore, kaempferol was able to reduce the protein phosphorylation of ERK, p38 and JNK by western blotting. By electrophoretic mobility-shift assay (EMSA), we demonstrated that kaempferol decreased the DNA binding activity of AP-1, an action likely to result in the reduced expression of MMP-2, MMP-9 and uPA. Collectively, our data showed that kaempferol attenuated the MAPK signaling pathways including ERK, JNK and p38 and resulted in the decreased DNA binding ability of AP-1, and hence, the downregulation in the expression and enzymatic activities of MMP-2, MMP-9 and uPA, contributing to the inhibition of metastasis of U-2 OS cells. Our results suggest a potential role of kaempferol in the therapy of tumor metastasis of OS.

Introduction

Osteosarcoma (OS) is the most common primary solid malignancy of bone which originates from the malignant transformation of mesenchymal cells that are destined to develop into osteoid and bone (1). This disease primarily afflicts children and adolescents and is responsible for approximately 20% of all types of bone cancer (2,3). In addition, OS is the second highest cause of cancer-related mortality in these age groups, mainly due to the fatal metastasis to the lung (4). Clinical studies have shown that approximately 80% of OS tumors may finally gain metastatic potential (5), while the remaining 20% of OS tumors stay regional and never metastasize, indicating an inherent metastatic potential exists in OS tumors. Thus, OS is a highly metastatic tumor, and pulmonary metastases are the most common cause of mortality. Through combined treatment of surgery with chemotherapy, and sometimes radiotherapy, the average 5-year disease-free survival rate for OS patients without metastatic disease is 60-70%, while the 5-year survival rate of patients with metastatic disease is as low as 10-20% (6). Furthermore, OS patients with metastases have a poor prognosis and their long-term survival rates are approximately 10-30% (7). Therefore, there is an urgent need to develop novel drugs to treat OS patients with metastasis.

Flavonoids are plant secondary metabolites that play a role in the defense against microorganisms or pests (8) and are part of oxidative stress protection pathways (9). Flavonoids can be divided into several subgroups including flavones, flavonols, flavanones, flavanols, isoflavones, and anthocyanidins (10). Studies have shown that flavonoids exhibit numerous biological and pharmacological properties (11), such as anti-viral (12), anticancer (13), anti-oxidative (14), anti-inflammatory (15), anti-allergic (16), anti-microbial (17), lipolytic (18), and hepatoprotective activities (19), as well as the prevention of cardiovascular diseases (20). Kaempferol, 3,4',5,
7-tetrahydroxyflavone, a natural flavonoid, has been isolated from various plant sources including caper, kale cress, broccoli, tomatoes, hop, red grapes, grapefruit, strawberries, apples and *Ginkgo biloba* (21). Kaempferol can also be found as an ingredient of honey, green and black tea (21-26). Kaempferol and its derivatives are known for their anti-oxidant, anti-inflammatory, anti-microbial, anti-diabetic, neuroprotective, and cardioprotective activities (27). Furthermore, it has been reported that kaempferol has anti-proliferation activity and can induce apoptosis in several human cancer cell lines, such as non-small cell lung cancer (28), esophageal cancer (29), leukemia (30), oral cavity cancer (31), prostate cancer (32), colon cancer (33) and OS (34).

It is well known that natural phenolic compounds from plants are able to prevent cancer metastasis (35) and, among them, flavonoids are effective natural agents with anti-invasive and/or anti-metastatic activities against various types of cancer such as brain, breast, lung, colon, cervical, prostate cancer and melanoma (10). As a member of flavonoids, kaempferol displayed anti-invasive activity by reducing MMP-3 activity in MDA-MB-231 human invasive breast carcinoma cells (36). Kaempferol can also suppress the HGF-induced phosphorylation of Met and Akt, leading to the inhibition of cell migration of medulloblastoma DAOY cells (37). In addition, kaempferol is able to repress the TPA-induced invasion and migration by prohibiting the PKCα/ERK/NF-κB signaling pathway in GBM8401 glioblastoma cells (38). However, the anti-metastatic effects of kaempferol on OS remain unclear. In this study, we presented evidence to show that kaempferol was able to inhibit invasion, migration and adhesion of U-2 OS cells. Further evidence indicated that kaempferol suppressed p38, JNK as well as the AP-1 signaling pathways and thus reduced the expression and enzymatic activities of matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA), contributing to the inhibition of OS metastasis.

Materials and methods

**Chemicals and reagents.** Kaempferol was purchased from Sigma-Aldrich (St. Louis, MO, USA) and solubilized in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Antibodies against MMP-2, MMP-9, β-actin, uPA and GAPDH were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against phospho-JNK (Thr183/Tyr185), phospho-p38 (Thr183/Tyr185), and phospho-ERK (Thr202/Tyr204) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). HRP-coupled secondary antibodies such as rabbit anti-mouse IgG, goat anti-rabbit IgG, and donkey anti-goat IgG were obtained from Santa Cruz Biotechnology Inc. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich. RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco-BRL (Carlsbad, CA, USA). All other chemicals were obtained from Sigma-Aldrich and Merck KGaA (Darmstadt, Germany) unless otherwise indicated.

**Cell culture.** Human OS cell line, U-2 OS, was purchased from the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan) and cultured in McCoy's 5A medium, supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine (all from Gibco-BRL), and incubated at 37°C in a humidified chamber with 5% CO₂ (39).

**Cell invasion assay.** The membrane of each Transwell insert was washed with 1X PBS and coated with Matrigel (2 mg/ml; BD Matrigel™ Invasion chamber). Cells (2.5x10⁵) were seeded into the chamber of the insert and incubated with 0.5 ml of complete McCoy's 5A medium in each Transwell. Cells were treated with various concentrations of kaempferol (0, 25, 50 and 100 μM) for 48 h and cells inside the chamber were removed. Invaded cells were fixed with 4% formaldehyde in PBS and stained with 0.1% of hematoxylin, photographed and the number of invaded cells was counted and the relative cell invasion was calculated (40,41).

**Cell adhesion assay.** We used cell-matrix adhesion assay to determine cell adhesion. U-2 OS cells (2.5x10⁵/ml) were treated with various concentrations of kaempferol (0, 25, 50 and 100 μM) for 48 h, and then seeded for 2 h onto 24-well plates that were pre-coated with 150 µl type I collagen (10 μg/ml) (EMD Millipore). Subsequently, non-adherent cells were removed and adherent cells were washed with PBS and fixed in 70% ethanol for 15 min. Fixed cells were stained with 0.2% crystal violet for 10 min, and then lysed in 0.2% Triton X-100 for 30 min. The absorbance of the lysed solution was measured at 550 nm by a microplate reader and used to calculate the relative cell viability. Each treatment was in duplicate, and 3 independent experiments were performed (40,41).

**Cell migration assay.** U-2 OS cells (2.5x10⁵) were seeded into 6-well plates and grown to 90% confluency. Cells were then scratched with a tip and treated with various concentrations of kaempferol (0, 25, 50 and 100 μM) in McCoy's 5A serum-free medium for 24 and 48 h. The cells were photographed and cells that migrated into the denuded zone were counted to calculate the relative cell migration. All treatments were in duplicate and three independent experiments were performed (40,41).

**Gelatin zymography analysis.** U-2 OS cells (1x10⁴) were seeded into 6-well plates for 4 h and treated with various concentrations of kaempferol (0, 25, 50, 75 and 100 μM) in serum-free McCoy's 5A medium for an additional 24 h. Culture medium was spun at 1,000 x g for 10 min at 4°C and supernatant was collected. Then, 5 μg of total protein was mixed with 2X sample buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 0.01% bromophenol blue) and resolved in an 8% SDS-polyacrylamide gel containing 1% gelatin. The gel was incubated with 2.5% Triton X-100 for 30 min, and incubated in zymogen developing buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM CaCl₂, 1 μM ZnCl₂, 0.02% Brij-35; Bio-Rad Laboratories; Hercules, CA, USA) at 37°C for 16-18 h. The gel was then rinsed with water and stained with 0.5% Coomassie Blue G-250 (0.5% Coomassie Blue G-250, 50% methanol, and 10% acetic acid) for 3 h, and de-stained in de-staining solution (50% methanol and 10% acetic acid) until clear zones were visualized. The gel was scanned by a scanning digitizing system and processed by using ImageJ software (NIH) (40,41).
**Casein-plasminogen zymography analysis.** U-2 OS cells (1x10^6) were seeded into 6-well plates and treated with various concentrations of kaempferol (0, 25, 50, 75 and 100 µM) in serum-free McCoy's 5A medium for 48 h. Protein in culture medium was collected as described above and used for assaying the uPA activity. Then, 30 µg of total proteins were electrophoresed in an 8% SDS-PAGE gel containing 2% casein and 20 µg/ml plasminogen and zymography was analyzed as described in the gelatin zymography analysis (40,41).

**Preparation of whole cell and nuclear protein lysate.** U-2 OS cells were treated with various concentrations of kaempferol for the indicated times, and cells were harvested for the preparation of whole cell lysate using extraction buffer containing ice-cold RIPA buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5) supplemented with the protease inhibitors including phenylmethylene sulfonyl fluoride (10 mg/ml), leupeptin (17 mg/ml) and sodium orthovanadate (10 mg/ml). Cells were completely re-suspended in extraction buffer and incubated in ice for 30 min with occasional mixing, and cell lysates were collected by a spin at 12,000 x g for 10 min at 4°C. Nuclear extracts were prepared by using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, Rockford, IL, USA). The obtained nuclear pellet was solubilized in nuclear extraction buffer (1.5 mM MgCl₂, 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 25% glycerol and 420 mM NaCl), and incubated in ice for 20 min, then centrifuged at 14,000 x g for 5 min. The supernatant, corresponding to the soluble nuclear fraction, was collected for the electrophoretic mobility shift assay (EMSA) of AP-1. The protein concentrations were determined by using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) (41).

**EMSA.** U-2 OS cells were seeded at a density of 5x10^6 the day prior to treatment. Cells were then treated with 100 µM of kaempferol for 12 h. Soluble nuclear fraction was prepared as described above. Biotin end-labeled oligonucleotide corresponding to the consensus AP-1 binding site (5'-CGCCTTGATGACTCAGCCGGAA-3') was prepared with the LightShift Chemiluminescent EMSA kit (Thermo Scientific) and used as the probe. Then, 5 µg of nuclear extract was incubated with biotin end-labeled duplex DNA, electrophoresed in a 6% polyacrylamide native gel, transferred to a positive nylon membrane, UV cross-linked, and incubated with streptavidin-HRP. Signals were developed by enhanced chemiluminescence using the ECL kit from Millipore (Billerica, MA, USA) (41).

**Western blotting.** Whole cell lysate was prepared from treated cells as described above, electrophoresed in sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore). The membranes were then incubated in blocking buffer (5% non-fat milk and 0.1% Tween-20 in Tris-buffered saline) for 1 h and incubated with primary antibody in 1% non-fat milk (with 0.1% Tween-20 in Tris-buffered saline) at 4°C overnight. Membranes were washed with 0.1% Tween-20 in Tris-buffered saline 3 times for 10 min before incubating with HRP-conjugated secondary antibody at room temperature for 1 h. Protein signals were detected by enhanced chemiluminescence (ECL) (41).

**Quantitative real-time PCR analyses of MMP-2 and MMP-9 genes.** U-2 OS cells were treated with 0 and 100 µM of kaempferol for 24 h and cells were collected for the isolation of total RNAs using the Qiagen RNeasy mini kit. cDNAs were obtained using the High Capacity cDNA reverse transcription kit according to the standard protocol provided by the supplier (Applied Biosystems, Foster City, CA, USA). Then, 1 µl of reverse-transcribed cDNA was mixed with 2X SYBR-Green PCR master mix (Applied Biosystems) and 200 nM of forward and reverse primers for the quantitative PCR according to the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C, 1 min at 60°C. PCR reaction was performed on an Applied Biosystems 7300 Real-Time PCR system in triplicate and fold changes of the expression were derived using the comparative C<sub>T</sub> method (42,43). Primers used were: human MMP-2-forward, CCCCAGACAGGTTGAC and reverse, GCTTGCGAGGGAAGAAGTTG; human MMP-9-forward, CGCTGGGCCTTAGATCTTCCC and reverse, AGGTGGATACTACGTGATT; human GAPDH-forward, ACACCCCCTCCTACACCTTT and reverse, TAGCCAAATTCGTTGTGCTACC (40).

**Statistical analysis.** The student's t-test was used to analyze differences between treated and control groups. P<0.05 was considered to indicate a statistically significant difference (39, 40).

**Results**

**Kaempferol inhibits invasion, migration and adhesion of U-2 OS cells.** We performed the Matrigel-coated Transwell assay to determine the effects of kaempferol on cell invasion. As shown in Fig. 1A, treatment of U-2 OS cells with increasing concentrations of kaempferol decreased the cell invasion in a concentration-dependent manner. It is possible that kaempferol inhibits migration of U-2 OS cells, leading to the inhibition of cell invasion. To address this possibility, cells were treated with different concentrations of kaempferol for 24 and 48 h and the effects of kaempferol on cell migration were analyzed by wound-healing assay. As shown in Fig. 1B, cells that migrated into the denuded zones were decreased by kaempferol treatment in a concentration-dependent manner after 24 h of treatment. We observed the same phenomenon after 48 h of drug treatment. To examine whether kaempferol also affects cell adhesion, U-2 OS cells were treated with different concentrations of kaempferol and the ability of cells to adhere to extracellular matrix (ECM) was examined by cell-matrix adhesion assay. The result showed that kaempferol treatment inhibited adhesion of cells onto collagen matrix in a concentration-dependent manner, with >60% inhibition following treatment with 100 µM of kaempferol (Fig. 1C).

**Kaempferol reduces the enzymatic activities of MMP-2, MMP-9 and uPA of U-2 OS cells.** During cancer metastasis, MMPs are produced to degrade ECM, a critical step for cancer invasion. Kaempferol inhibited the enzymatic activities of both MMP-2 and MMP-9 by gelatin zymography.
analysis (Fig. 2A and B). More than 50% of enzymatic activity of MMP-2 was inhibited after 50 µM of kaempferol treatment. MMP-9 activity was more sensitive to kaempferol than MMP-2, with >60% inhibition at 50 µM and >80% inhibition at 75 and 100 µM. After binding to its cognate receptor on the cell membrane, uPA is activated to convert plasminogen into plasmin, which in turn degrades ECM and cleaves pro-MMPs, finally leading to cancer metastasis (44). As revealed by casein-plasminogen zymography, kaempferol also reduced the enzymatic activity of uPA, with >50% inhibition at drug concentrations >75 µM (Fig. 2C).

Kaempferol reduces the expression of MMP-2, MMP-9 and uPA of U-2 OS cells. Since kaempferol reduced the enzymatic
activities of MMP-2, MMP-9 and uPA, we hypothesized that kaempferol inhibits the expression of MMPs and uPA. Cells were treated with different concentrations of kaempferol (0, 25, 50, 75 and 100 µM) for 48 h. Cells were collected for the preparation of cell lysates and then subjected to western blot analyses for MMP-2, MMP-9 and uPA. GAPDH served as the loading control. (B) Kaempferol decreased the gene expression of MMP-2 and MMP-9. Cells were treated with 0 and 100 µM of kaempferol for 24 h and gene expression of MMP-2 and MMP-9 was assessed by quantitative RT-PCR.

Kaempferol inhibits protein phosphorylation of ERK, p38 and JNK in U-2 OS cells. It has been reported that the MAPK signaling pathway, which includes ERK1/2, C-Jun N-terminal kinase, and p38 kinase pathways, can activate the downstream signaling cascade to increase the expression of MMP family proteins such as MMP-2, MMP-7 and MMP-9, contributing to cancer invasion and metastasis (45,46). We therefore determined the effects of kaempferol on the protein phosphorylation of ERK, JNK and p38. U-2 OS cells were treated with 100 µM of kaempferol for 12 h. Nuclear extracts were obtained to analyze the AP-1 DNA binding activity using biotin end-labeled AP-1 oligonucleotides in electrophoretic mobility-shift assay (EMSA). Lane 1, 0 µM of kaempferol (control). Lane 2, 100 µM of kaempferol. The strong signals on the bottom were excessive free probes. Results from 3 independent experiments were comparable.

Kaempferol inhibits the DNA binding activity of AP-1 in U-2 OS cells. It is known that the promoters of MMP-2, MMP-9 and uPA harbor several consensus binding sites for AP-1 transcription factor (47-49). One possibility is that kaempferol can suppress the DNA binding activity of AP-1, leading to the reduced expression of MMP-2 and MMP-9. Cells were treated with 0 and 100 µM of kaempferol and nuclear extracts were prepared for the EMSA of AP-1 using consensus AP-1 specific oligonucleotides as a
Osteosarcoma (OS) is the most frequent primary bone tumor that afflicts children and young adults. It is a highly aggressive tumor that metastasizes primarily to the lungs. Current therapy for the disease involves various combinations of surgery and chemotherapy. The dosage of therapeutic drugs is high, often leading to severe side-effects. Radiotherapy may also be included, but most of the tumors are not sensitive to irradiation. Therefore, there is a pressing need for novel agents for the therapy of the disease, particularly for OS patients with metastatic disease. In the present study, we found that the natural flavonoid, kaempferol, was able to inhibit the invasion, migration and adhesion of U-2 OS cells and could have a potential to treat OS metastasis.

Cancer metastasis is a complicated process that involves the loss of cell adhesion, the increase in cell migration, degradation of extracellular matrix (ECM), invasion of surrounding tissues, intravasation and circulation in the vascular and lymphatic systems, extravasation and finally the dwelling in distant organs (50). Interruption during the earlier steps of metastasis may be more promising for the treatment of cancer metastasis. Our data show that kaempferol can indeed attenuate the metastatic behavior of U-2 OS cells by inhibiting the attachment of cells to the ECM, suppressing cell migration, and blocking the invasion of cells (Fig. 1). The proteolysis of the ECM and basal membranes is a critical step in the invasive processes and the MMPs are responsible for ECM degradation. MMPs are zinc-dependent endopeptidases. According to their structure and substrate specificity, MMPs can be divided into several subgroups including gelatinases, collagenases, stromelysins, membrane-type MMPs and other MMPs (51). The MMP-2/MMP-9 double null mice have defects in tumor angiogenesis and invasion, outlining the importance the gelatinases MMP-2 (gelatinase-A) and MMP-9 (gelatinase -B) in tumor progression (52). Clinically, the expression levels of MMP-2 and MMP-9 in tumors are highly related to the metastatic potential (53-55). Increased expression levels of MMP-2 and MMP-9 were also identified as prognostic markers for a poor outcome of OS (56-58). MMP-9-positive OS patients have an overall 5-year survival rate of 28%, while that of MMP-9-negative OS patients is 79% (59). Therefore, MMP-2 and MMP-9 are suitable therapeutic targets. In the present study, we found that kaempferol can act on MMP-2 and MMP-9 by decreasing their enzymatic activities and expression at the transcriptional and translational levels (Figs. 2 and 3), leading to the inhibition of invasion of U-2 OS cells. These findings also outline the importance of MMP-2 and MMP-9 in OS metastasis.

Activation of MMPs is initiated by the binding of the uPA to the membrane-bound receptor, the urokinase plasminogen activator receptor (uPAR), and this binding leads to the activation of uPA to convert the plasminogen to plasmin, which in turn cleaves pro-MMPs into active MMPs. Thus, the association of uPA with its cognate receptor uPAR at the surface of cancer cells is considered to be significantly involved in tumor invasion and metastasis (44,60). It has been shown that the uPA-uPAR system plays a critical role in OS metastasis (61,62). In addition, the uPA levels are found to be in an inverse relationship with survival time in OS patients (62). Furthermore, reduction in primary tumor growth and pulmonary metastasis was observed in an in vivo OS model that was inoculated with uPAR antisense clones (63). Based on these observations, inhibition of uPA may be suitable for overcoming OS metastasis. Our results showed that kaempferol not only reduced the enzymatic activity of uPA (Fig. 2C), but also decreased the protein levels of uPA (Fig. 3A), possibly resulting in the inactivation of MMPs and hence the inhibition of invasion of U-2 OS cells.

MMPs (ERK, p38, JNK) are involved in MMPs or uPA promoter induction through AP-1 (47,48,64) and can regulate the activities of MMPs or uPA in various cell types (65-67). AP-1 is comprised of Fos and Jun proteins and functions as a transcription factor that controls cell proliferation, differentiation, and bone metabolism. Studies have shown that Fos and Jun are significantly upregulated in high-grade OS, as compared to benign osteoblastic lesions and low-grade OS (68,69) and tend to develop metastases (70). It was reported that inhibition of AP-1-mediated transcription resulted in reduced migration, invasion and metastasis in a murine model of OS (71,72). These data strongly suggest that MAPKs and AP-1 function as mediators of cancer metastasis and our current study indicated that kaempferol can inhibit the metastasis of OS cells by suppressing the activation of these two important mediators.

In summary, the inhibitory effects of kaempferol on the metastasis of OS are delineated in Fig. 5. The natural flavonoid kaempferol exhibits anti-metastasis activity by blocking the activation of MAPKs and AP-1 signaling pathways to reduce the expression and activities of MMPs and uPA and results in the inhibition of invasion, migration and adhesion of OS cells. Our previous studies have shown that kaempferol reduced the cell viability, induced cell apoptosis of U-2 OS cells and inhibited tumor growth in BALB/c nude mice inoculated with U-2 OS cells, but exhibited low cytotoxicity on human fetal osteoblast progenitor (hFOB) cells (34). In the present study, we further demonstrated that kaempferol inhibited the meta-

![Figure 5. The proposed signaling pathways of kaempferol-inhibited invasion, migration and adhesion in U-2 OS human osteosarcoma cells.](image-url)
static potential including invasion, migration and adhesion of U-2 OS cells. Due to its relative non-toxic, inexpensive nature and its ability to suppress cell growth and inhibit the metastatic potential of OS, kaempferol is a promising agent for the therapy of OS and metastasis of the disease.

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References


