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ORIGINAL ARTICLE

Naringenin Reduced Migration in Osteosarcoma Cells through Downregulation of Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 and Snail

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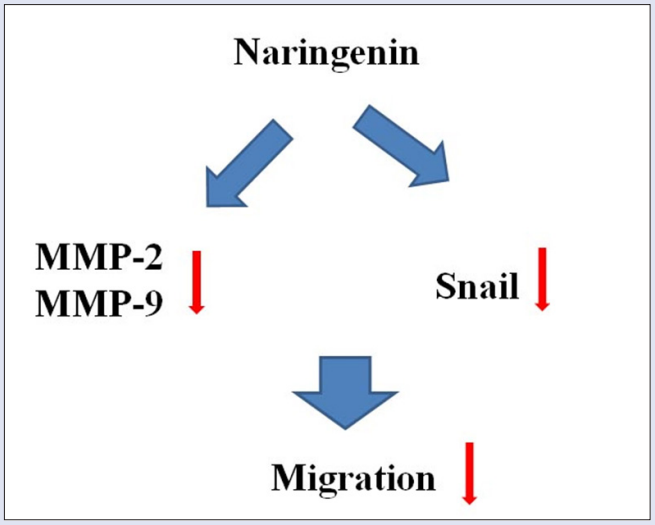
ABSTRACT

Background: Osteosarcoma is one of the most malignant cancers in children. Naringenin exhibits several cellular functions. **Objectives:** In this study, we investigate the effects of naringenin on osteosarcoma. **Materials and Methods:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide (MTT) assay was performed to detect the cell viability. Zymography assay and transwell assay were used to measure the matrix metalloproteinase (MMP) activity and migration ability. Western blot analysis was used to determine the expression of migration-related proteins. **Results:** No overt alternation of cytotoxicity in response to different concentrations of naringenin for 24 h was found by MTT assay. MMP-2 and MMP-9 activities and expression were significantly repressed by naringenin in a dose-dependent manner, as evidenced by zymography and Western blot analysis. Naringenin dose dependently reduced the expression of mesenchymal marker Snail. **Conclusion:** These results indicate that naringenin exhibited antimigration property through inhibition of MMP-2 and MMP-9 and downregulation of Snail expression. Thus, naringenin may be a potential inhibitor of metastasis of osteosarcoma.

Key words: Matrix metalloproteinase, migration, naringenin, osteosarcoma, Snail

SUMMARY

- Herein, we investigate the mechanisms of naringenin on the migration of osteosarcoma cells. No overt alternation of 143B cell proliferation of osteosarcoma was found in response to naringenin exposure. Zymography analysis revealed that matrix metalloproteinase (MMP)-2 and MMP-9 activities were repressed in corporation to naringenin concentration. Naringenin dose dependently inhibited the migration of osteosarcoma 143B cells. Naringenin also reduced the expression of MMP-2 and MMP-9 and Snail. Collectedly, our findings suggested that naringenin could be a potential agent to prevent metastasis of osteosarcoma.



Abbreviations used: ATCC: American Type Culture Collection; DMEM: Dulbecco's Modified Eagle Medium; EMT: Epithelial-to-mesenchymal transition; GADPH: Glyceraldehyde-3-phosphate dehydrogenase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MMP: Matrix metalloproteinase; PBS: Phosphate-buffered saline; TGF: Tumor growth factor; uPA: Urokinase plasminogen activator.

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INTRODUCTION

Osteosarcoma is one of the most malignant bone tumors and predominant in children and adolescents.[1] In general, treatment of osteosarcoma is based on surgical resection, chemotherapy, and radiotherapy.[1] These treatments may lead to severe side effects. Although the mortality rate has improved in early stage of osteosarcoma, the prognosis of advanced stages remains poor due to metastasis, chemoresistance, and recurrence. Developing a more effective drug with less side effects for inhibition of metastasis of osteosarcoma is an important issue.

Naringenin, a major bioactive flavonoid from *Citrus*, has been shown to exert several cellular functions including antioxidant, anti-inflammation, and antitumor. Naringenin triggered apoptosis in epidermoid carcinoma,[2] pancreatic cancer,[3] and placental choriocarcinoma.[4] A recent study reported that naringenin combined with hesperetin inhibited tumor proliferation and migration through downregulation of focal adhesion kinase (FAK) and p38 but without any side effects on normal cells.[5] Lou *et al.*[6] indicated that naringenin diminished the migration of pancreatic cancer cells through repression of epithelial-to-mesenchymal transition (EMT) induced by tumor growth factor-β. Exposure to naringenin repressed the activity of AKT, downregulated the matrix metalloproteinase (MMP)-2 and MMP-9 expression, and decreased the proliferation and migration of gastric cancer cells.[7] Through inhibition of MMP-2 and MMP-9, naringenin suppressed the migration of cancer cells in lung cancer[8] and glioblastoma.[9] In addition, Han *et al.* showed that naringenin reduced the invasion of prostate PC-3 cells by eliminating the activity of urokinase plasminogen activator (uPA).[10] Finally, a recent report found that naringenin inhibited wound healing in fish intestinal epithelial cell sheets.[11] This suggests that naringenin might broadly target animal cell migration.

A previous report demonstrated that naringenin reduced the proliferation of MG-63 osteosarcoma cells.[12] Daily administration of 20 mg of naringenin decreased the proliferation and recurrence of cancer through downregulation of reactive oxygen species level in patients with osteosarcoma who underwent surgery.[12] However, the effects and molecular mechanisms of naringenin on the migration of osteosarcoma remain to be elucidated. In the current study, we investigated the antimigration effect of naringenin on osteosarcoma 143B cells. Our results revealed that naringenin eliminated the migrated osteosarcoma cells by decreased MMP-2 and MMP-9 activities and reduced the mesenchymal marker such as Snail expression.

MATERIALS AND METHODS

Materials

Naringenin was purchased from Sigma-Aldrich (Merck, KGaA). Primary antibodies against β-actin (Santa Cruz Biotechnology, Inc.), glyceraldehyde-3-phosphate dehydrogenase (GADPH, Proteintech), Snail, and MMP-9 were obtained from GeneTex (GeneTex International Corporation (Hsinchu City, Taiwan)). MMP-2 was purchased from Boster (Boster Biological Technology).

Cell culture

The human osteosarcoma 143B (also called CVCL_2270) cells original derived from osteosarcoma of a 13-year-old female were obtained from American Type Culture Collection[13] and cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 mg/ml streptomycin (Thermo Fisher Scientific, Inc.). The 143B cells were maintained under an atmosphere containing 5% CO_2 at 37°C.

3-(4,5-dimethy lthiazol-2-yl)-2,5-dipheny ltetrazolium bromide assay

Human 143B cells were seeded in a density of 2 × 104/well in a 24-well plate. After attachment for overnight, the cells were exposed to 0, 25, 50, 100, 200, and 300 μM naringenin for 24 h and then replaced with fresh medium containing 5.0 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, Merck, KGaA) for additional 2 h. The formazans were dissolved in 1 ml isopropanol and detected absorbance at 563 nm. Cell viability was expressed as a percentage of control based on the value of absorbance in each well.

Gelatin zymography assay

The 143B cells were exposed to serum-free medium containing indicated concentration of naringenin for 24 h. The conditional medium was collected and then separated by 8% sodium dodecyl sulfate–polyacrylamide gel containing 0.1% gelatin. After washing with washing buffer (2.5% Triton-X-100), the gel was merged in buffer containing 40 mM Tris-HCl (pH 8.0), 10 mM $CaCl_2$, and 0.01% NaN_3 for 12 h at 37°C. The gel was stained with Coomassie Brilliant Blue R250. The clear bands indicated the activities of MMP-2 and MMP-9.

Migration assay

Human 143B cells were pretreated with 0, 25, 50, 100, 200, and 300 μM naringenin for 24 h. A total of 3 × 10^4 cells was seeded into the upper wells of Boyden chamber (Merck Millipore) and then incubated for 24 h at 37°C. Migrated cells in the lower chamber were fixed, stained with Giemsa solution, and pictured with microscopy. The migrated cells from three independent experiments were counted.

Western blot analysis

Human 143B cells were incubated with 0, 25, 50, 100, 200, and 300 μM naringenin for 24 h. The cell lysates were extracted by RIPA buffer. Fifty milligrams of protein was separated by electrophoresis and transferred into polyvinylidene fluoride membrane and then probed with first antibodies at 4°C for overnight. After washing with phosphate-buffered saline, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody. The positive bands were detected by Western Lightning Plus-enhanced chemiluminescence kit (cat. no. NEL 105001EA, PerkinElmer, Inc.). The intensity of each band was measured by Multi Gauge version 3.0 software (FUJIFILM, Tokyo, Japan).

Statistical analysis

Data represented as means ± standard division obtained from three independent experiences were calculated by one-way ANOVA by Prism software (GraphPad Software, San Diego, CA, USA). A significant difference was set as $P < 0.05$.

RESULTS

Naringenin exerted no cytotoxicity in osteosarcoma cells

MTT assay was conducted to determine the cytotoxicity effects of naringenin on osteosarcoma cells. As shown in Figure 1, the cell viability was 0.96 ± 0.03, 0.95 ± 0.03, 0.90 ± 0.03, 0.91 ± 0.02, and 0.93 ± 0.06 in the presence of 25, 50, 100, 200, and 300 μM naringenin for 24 h, respectively. Our findings indicated the lack of overt alternation of cell viability in osteosarcoma 143B cells in response to naringenin.

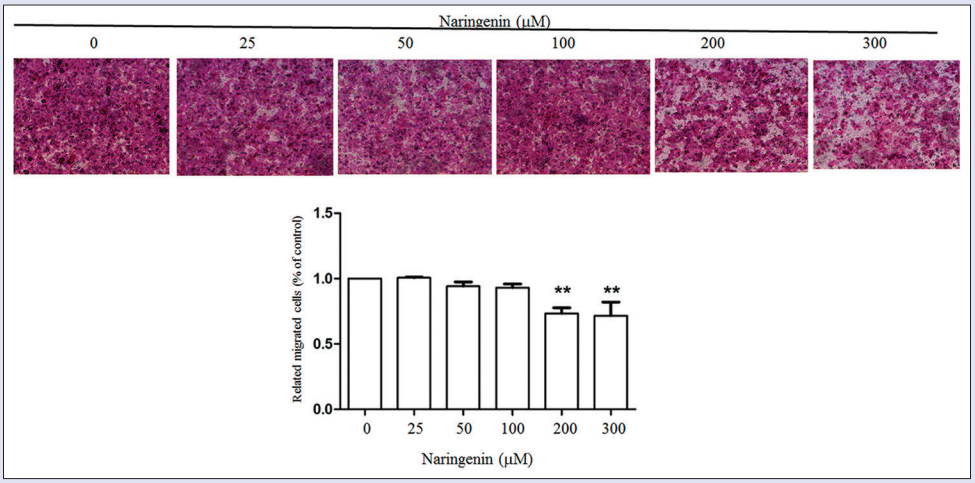

Figure 1: The cytotoxicity effect of naringenin in osteosarcoma cells. Human osteosarcoma 143B cells were treated with indicated concentration of naringenin for 24 h. The cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data represented as means standard division obtained from three independent experiments. Significance was set as $P < 0.05$. *denoted as $P < 0.05$

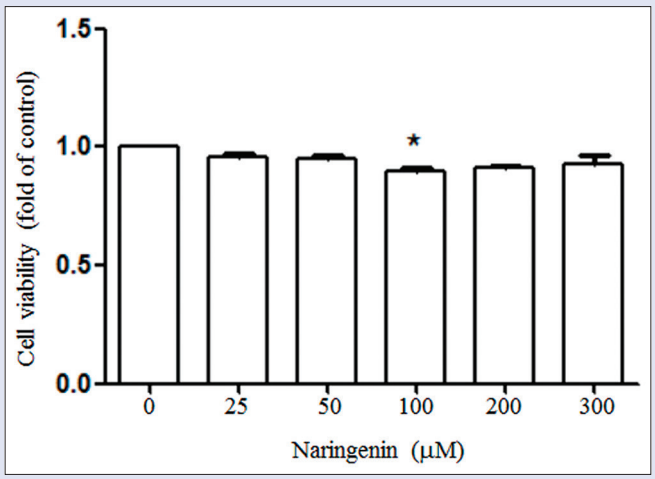

Figure 2: Naringenin diminished matrix metalloproteinase-2 and matrix metalloproteinase-9 activities. An equal amount of 143B cells were exposed to indicated concentration of naringenin in serum-free medium for 24 h. The matrix metalloproteinase-2 and matrix metalloproteinase-9 activities in conditional medium were analyzed by gelatin zymography assay. Data represented one of three independent experiments

Naringenin inhibited matrix metalloproteinase-2 and matrix metalloproteinase-9 activities of osteosarcoma cells

Previous works reported that naringenin repressed cell migration in several cancers by reducing MMP-2 and MMP-9 activities. To address whether naringenin also represses MMP-2 and MMP-9 activities, we conducted zymography analysis. In the presence of 25, 50, 100, 200, and 300 μM naringenin, the MMP-9 activities were reduced to 0.79, 0.64, 0.60, 0.30, and 0.36, respectively, compared with the control group. The MMP-2 activities were 0.88, 0.82, 0.81, 0.58, and 0.54 in response to 25, 50, 100, 200, and 300 μM naringenin treatment, respectively [Figure 2].

Naringenin attenuated migration of osteosarcoma cells

Previous reports indicated that naringenin repressed the migration of lung and bladder cancers.[8,14] Next, we have performed Boyden chamber assay to detect whether naringenin affects the migration of osteosarcoma cells. Naringenin obviously attenuated the migration of osteosarcoma cells in 200 and 300 μM concentration [Figure 3].

Naringenin inhibited migration-related protein expression

It has been shown that Snail bound to and activated the expressed of MMP-2 and MMP-9 and then facilitated migration of cancer cells.[15] Therefore, Western blot analysis was performed to further evaluate whether naringenin influences the expression of migration-related proteins such as MMP-2, MMP-9 and Snail. As shown in Figure 4, naringenin dramatically reduced the expression of MMP-2, MMP-9, and Snail in proportion to its concentration.

DISCUSSION

In this study, we revealed that naringenin suppressed the migration of osteosarcoma cells through downregulation of MMP-2 and MMP-9 activities. In addition, exposure to naringenin also reduced the expression of Snail1 (a mesenchymal marker).

Metastasis is the major risk factor for high mortality of osteosarcoma. MMPs degrade the basal membrane and extracellular matrix, which play an important role in cancer cell progression, migration, and metastasis.[16,17] The relationship of MMP expression and poor prognosis of osteosarcoma has been reported. Korpi *et al*. demonstrated the inverse relationship between MMP-2 expression and chemotherapy effects in patients with osteosarcoma.[18] The reverse transcription-quantitative polymerase chain reaction analysis demonstrated that the expression of MMP-2 was significantly correlated with lung metastasis and short survival rate.[19] A metaphase study reported the significant association of MMP-9 expression with survival rate in patients with osteosarcoma.[20] A recent work indicated that patients with osteosarcoma had increased

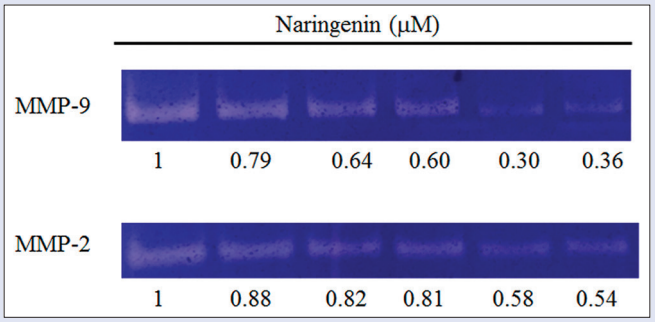

Figure 3: Naringenin attenuated migration of osteosarcoma cells. Human osteosarcoma cells were treated with indicated concentration of naringenin for 24 h. Equal amounts of cells were plated into the upper chamber and migrated to lower chamber in the presence of naringenin for 24 h. Migrated cells were pictured in at least three independent randomly selected fields. Data represented one of the three independent experiments. $^{**}P < 0.01$

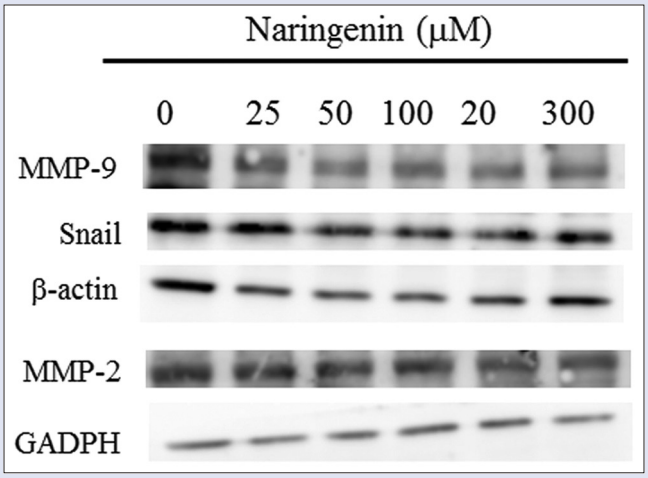

Figure 4: Naringenin alters the migration-related proteins' expression. 143B cells were treated with indicated concentration of naringenin for 24 h. The cell lysates were subjected to Western blot analysis using specific antibody. Data represented one of the three independent experiments

MMP-2/MMP-9 ratio, which was correlated with poor chemotherapy response.[21]

Flavonoids have received great attention because of their antimetastasis function in osteosarcoma cells. Deguelin, nobiletin, and tricetin diminished the migration of osteosarcoma cells by inhibiting MMP-2 and/or MMP-9.[22-24] In the present study, we showed that naringenin also reduced MMP-2 and MMP-9's activities. In line with our observation, naringenin repressed MMP activities and subsequently suppressed the migration of lung, bladder, and glioblastoma cells.[8,9,14] Our findings suggested that naringenin attenuated the migration of osteosarcoma by downregulating MMP-2 and MMP-9.

EMT plays an important role in several biological functions, such as embryo development and cancer metastasis.[25] During the EMT process, transcription factors, such as Snail, Slug, and Twist, enhanced the expression of mesenchymal markers and repressed the expression of epithelial markers.[25] Previous reports demonstrated that naringenin attenuated the expression of Snail and subsequently reduced the migration of tumors in glioblastoma and prostate cancers.[9,10] Moreover, naringenin attenuated the expression of Snail in a dose-dependent manner. In addition, Yokoyama *et al.*[26] demonstrated through luciferase reporter assay that Snail1 can bind to the promoter-262 to promoter-441 region and enhance MMP-2 expression. In hepatocellular carcinoma cells, Snail1 expression significantly increased the expression of MMP-1 and MMP-2.[27] Similarly, overexpression of Snail enhanced the MMP-9 level in Madin–Darby canine kidney cells.[28] Collectively, our results indicated that naringenin suppressed MMP-2 and MMP-9 levels through downregulation of Snail expression.

CONCLUSION

Our findings revealed that naringenin repressed the migration of osteosarcoma cells through downregulation of MMP-2, MMP-9, and Snail.

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Conflicts of interest

There are no conflicts of interest.

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