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RESEARCH ARTICLE

Naringenin inhibited migration and invasion of glioblastoma cells through multiple mechanisms

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Abstract

Glioblastoma (GBM) is the most mortality brain cancer in the world. Due to high invasion and drug resistance cause the poor prognosis of GBM. Naringenin, an ingredient of citrus, exhibits many cellular functions such as antioxidant, anti-inflammation, and anticancer. Naringenin inhibits the migration of bladder and lung cancer via modulation of MMP-2 and/or MMP-9 activities, Naringenin inhibits migration and trigger apoptosis in gastric cancer cells through downregulation of AKT pathway. However, the effects of naringenin in GBM still remain to be elucidated. In this study, we reveal the molecular mechanisms of naringenin in the inhibition of migration and invasion in GBM. No overt alternation of cell proliferation was found in of GBM 8901 cells treated with different concentration of naringenin. Slight decreased cell viability was found in GBM 8401 cell treated with 200 and 300 μM naringenin. Significant reduction of migration and invasion as assayed by Boyden chamber analysis was found in of GBM cells treated with 100, 200, and 300 μM naringenin. Zymography analysis also revealed that the activities of MMP-2 and MMP-9 of GBM cells were significantly inhibited in response to 100, 200, or 300 μM naringenin treatment. Proteins of MMP-2 and MMP-9 were downregulated in naringenin treated GBM cells. In addition, naringenin also attenuated the activities of ERK and p38. Naringenin decreased mesenchymal markers (snail and slug) expression as revealed by Western blot analysis. Taken together, our findings indicated that naringenin eliminated the migration and invasion of GBM cells through multiple mechanisms including inhibition of MMPs, ERK, and p38 activities and modulation of EMT markers. Our results also suggested that naringenin may be a potential agent to prevent metastasis of GBM.

KEYWORDS

glioblastoma, invasion, migration, naringenin

Yen-Yu Chen and Yuh-Ming Chang, contributed equally to this work.

1 | INTRODUCTION

Glioblastoma (GBM) is the most common cancer in the brain and central nerve system.[1] The overall 5 year survival rate is around 5%, which makes the mortality of GBM is very high. In general, the treatment of GBM is dependent on resection, chemotherapy, and radiotherapy.[1] However, because of rapid growth, high invasion ability, and drug resistance, the prognosis of GBM is very poor. Searching a new GBM therapeutic agent with less toxicity and more effective is an urgent issue.

Now, using nature products from vegetables and traditional medicines in prevention of migration of GBM have received intensive focus. Flavonoids are nature bioactive compounds found in most vegetable, fruits, and traditional medicine and exert multiple biological functions such as anti-inflammation,[2] antioxidant,[2] and antitumors.[3] Naringin decreased the migration of GBM cells by suppression of matrix metalloproteinase 2 (MMP-2) and 9 (MMP-9) activities and inhibition of p38 activities.[4,5] Furthermore, naringin also diminished the focal adhesion kinase activities to induce apoptosis and inhibit migration of GBM cells.[6] Treated with nobiletin repressed the migration of U87 and U251 GBM cells via downregulation of AKT/GSK3β/β-catenin signal.[7] Administration of nobiletin also inhibited cancer metastasis of mice bearing with U87 GBM cells.[7] Sublethal dose of quercetin inhibited migration and invasion of U251 GBM cell by decreasing of vascular endothelial growth factor A (VEGFA), MMP-2, and MMP-9 expression.[8]

Naringenin, one of the flavonoids, is abundant in citrus, grape, and tomato. Emerging documents have been shown that naringenin suppress cancer cell proliferation and/or migration. Downregulation of MMP-2 and MMP-9 and subsequently inhibited migration in gastric cancer SGC-7901 cells in response to naringenin treatment was found.[9] Incubation with naringenin also triggered apoptosis in gastric cancer SGC-7901 via inhibition of AKT activities.[9] Combination of naringenin and gemcitabine enhanced the tumor growth factor-1 (TGF-1) β-induced drug resistant to undergo apoptosis.[10] In addition, naringenin blocked TGF-1/SMAD3 downstream signals, reduced mesenchymal makers' expression, and attenuated MMP-2 and MMP-9 activities, and consequently suppressed migration and invasion in pancreatic cancer cells.[10] Recent reports also indicated that naringenin decreased AKT and MMP-2 activities and inhibited migration in bladder cancer[11] and lung cancer cells.[12]

Till now, the effects of naringenin on GBM still remained unclear. In the present study, we demonstrated that naringenin diminished migration and invasion of GBM cells by decreased MMP-2 and MMP-9 activities, reversed mesenchymal markers' expression, downregulated ERK, and p38 activities. Our results highlighted the molecular mechanisms of naringenin on the migration and invasion of GBM cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human 8901 and 8401 GBM cancer cells were purchased from American Type Culture Collection (Manassas, Virginia) and maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. The cell line was grown at 37°C under a humidified atmosphere with 5% CO_2 to 80%-90% confluence.

2.2 | MTT assay

The 8901 and 8401 cells were seeded in 24-well plates at a density of 2×10^4/mL. These cells were incubated with 0, 25, 50, 100, 200 and 300 μM naringenin for 24 hours. Following the treatment, the cells were incubated with fresh medium containing 5.0 g/L MTT, and incubated at 37°C for an additional 2 hours. After washing with PBS, the sediments were dissolved in 1 mL isopropanol and the absorbance at 563 nm was determined. The relative viability rate was determined based on the absorbance of 563 nm of each sediment compared with vehicle-treated groups (Figure 1).

2.3 | Western blot analysis

Naringenin-treated 8901 cells were lysed in 150 μL RIPA lysis buffer (Thermo Fisher Scientific, Inc., Waltham, Massachusetts). The lysates were centrifugated at 14 000*g* for 10 minutes and the supernatants

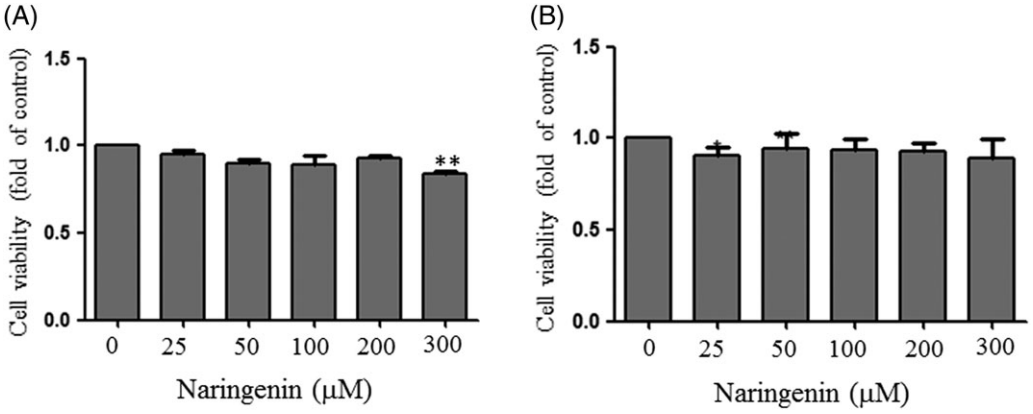

FIGURE 1 The effects of naringenin on proliferation of GBM cells. A, GBM 8401 and B, GBM 8901 cells were treated with indicated concentration of naringenin for 24 hours. The cell viability was measured by MTT assay. Data represented means ± SD from three independent experiments. **: $P < .01$

were collected. The protein concentration was measured using Bio-Rad Protein Assay Dye Reagent Concentrate in accordance with the manufacturer's recommendations (Bio-Rad, Hercules, California). A total of 50 μg protein was separated by a 10% polyacrylamide gel and transferred onto a nitrocellulose membranes (Merk Millipore, Darmstadt, Germany). The membrane was blocked with PBS with 0.5% non-fat milk for 1 hour at room temperature. After washing with PBST (PBS containing 0.1% Tween-20), the membrane was incubated with primary antibodies at 4°C overnight. The membrane was then washed with PBST and reacted with horse radish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California). After extensively washing with PBST the positive signal was determined by enhanced chemiluminescence (GE Healthcare Life Sciences, Chalfont, UK) and β-actin expression was used as an internal control.

2.4 | Gelatin zymography assay

Cells were incubated in serum-free medium and treated with the indicated naringenin concentration for 24 hours. We obtained 500 μL of conditional medium and incubated it with 5 μL of PBS-washed Gelatin Sepharose 4B beads (GE Healthcare Life Sciences, Pittsburg, Pennsylvania) at 4°C overnight. The beads were washed with PBS, and then 15 μL of non-reducing sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, and 0.003% bromophenol blue) was added. The complexes were separated using 8% sodium dodecyl sulfate-polyacrylamide gel containing 0.1% gelatin. The gel was washed with washing buffer (2.5% Triton X-100) twice with gentle agitation at room temperature for 30 minutes and incubated with reaction buffer (40 mM Tris-HCl [pH 8.0], 10 mM $CaCl_2$, 0.01% NaN_3) for 12 hours at 37°C. The gel was then stained with Coomassie brilliant blue R250. The degraded zones represented the MMP-2 and -9 activities.

2.5 | Migration assay

Cells were treated with different concentrations of naringenin for 24 hours and seeded at a density of 5 × 105/mL in the upper chamber of the 48-well Boyden chamber (Merk Millipore, Darmstadt, Germany). The lower chamber contained 20% FBS. Next, the chamber was incubated at 37°C for 24 hours and the cells that migrated to the lower surface of the membrane were fixed in methanol for 10 minutes and stained with 10% Giemsa for 1 hour. The positive signals were measured by Image J software.

2.6 | Statistical analysis

The data are represented as the mean ± SD of three independent experiments and were evaluated by one-way ANOVA with Tukey's honest difference post hoc test using SPSS 14.0 software (SPSS, Inc., Chicago, Illinois). $P < .05$ was used to indicate a statistically significant difference.

3 | RESULTS

3.1 | No overt alternation of cell proliferation of 8901 and 801 cells in response to naringenin treatment

To address whether naringenin affects the proliferation of GBM 8901 and 8401 cells, MTT assay was performed. No significant difference

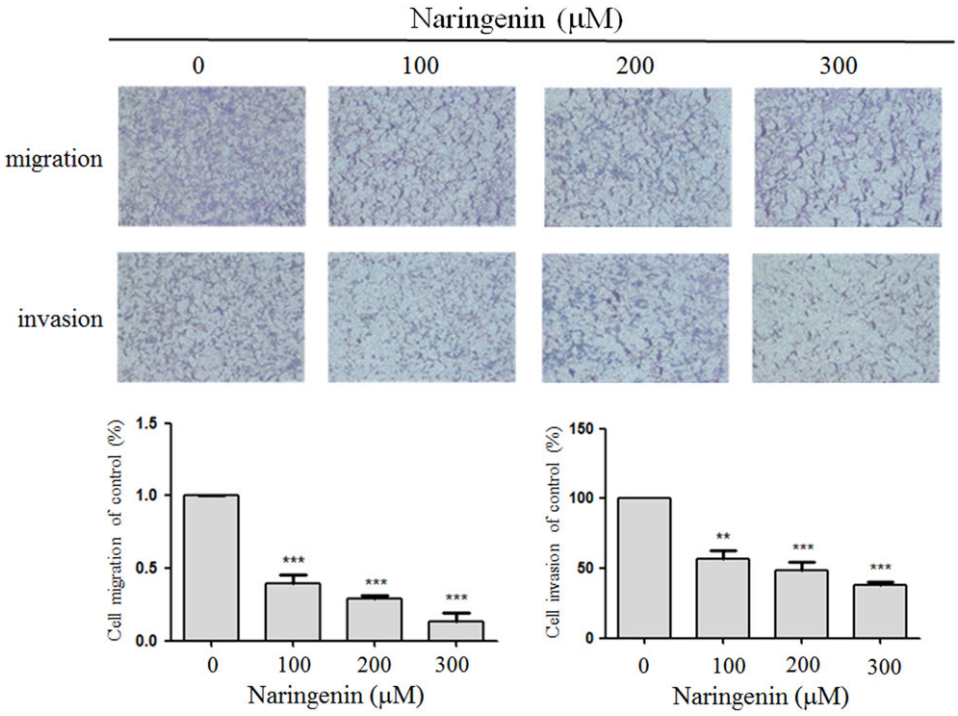

FIGURE 2 Naringenin repressed the migration and invasion of GBM cells. GBM 8901 cells were treated with indicated concentration of naringenin for 24 hours. A total of 5 × 10^3 cells were conducted migration (24 hours) and invasion (48 hours) analysis using transwell assay. Data represented means ± SD from three independent experiments. **: $P < .01$; ***: $P < .001$ [Color figure can be viewed at wileyonlinelibrary.com]

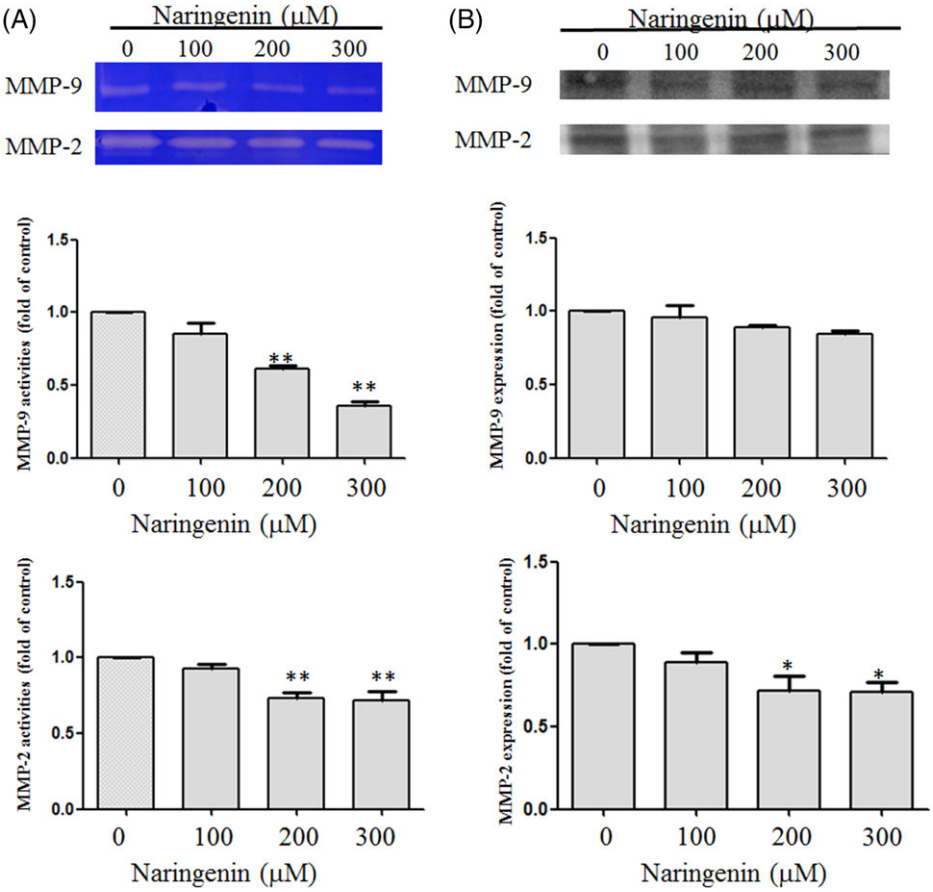

FIGURE 3 A, Naringenin inhibited the MMP-2 and MMP-9 activities and B, expression. GBM 8901 cells were treated with indicated concentration of naringenin for 24 hours. A, The conditional mediums were subjected into zymography analysis. B, Fifty μg cell lysates derived from naringenin treated cells were subjected to Western blot analysis using anti-MMP-2 or MMP-9 antibodies. Lower panel: Data represented means ± SD from three independent experiments. *: $P < .05$; **: $P < .01$. β-actin was loaded as internal control [Color figure can be viewed at wileyonlinelibrary.com]

of cell viability was found in 8901 cells treated with 25, 50, 100, 200, and 300 μM naringenin for 24 hours. Cell viability was slightly decreased in 8401 cell in response to 200 (92.7%), and 300 μM (83.8%) naringenin for 24 hours compared to vehicle treated group (Figure 1). To investigate the effect of naringenin on the migration and invasion of GBM cells, 8901 cells were chosen for further study.

3.2 | Naringenin inhibited migration and invasion of 8901 cells

Next, we conducted Boyden chamber assay to determine the effects of naringenin on migration and invasion properties of GBM 8901 cells. In the presence of 100, 200, and 300 μM naringenin, 8901 cell migration was significantly reduced to 39.5%, 28.7%, and 13.5% compared to vehicle group, respectively. Invasion abilities of 8901 cells also obviously suppressed by treated with 100, 200, and 300 μM naringenin (57.0%, 48.6%, and 37.6%, respectively) (Figure 2).

3.3 | Naringenin inhibited both protein concentration and activities of MMP-2 and MMP-9

Documents have been shown that MM-2 and MMP-9 play an important role in cancer migration and invasion.[13] To investigate whether naringenin affected the protein level and activities of MMP-2 and MMP-9, we performed Western blot and zymography analysis using GBM 8901 cells. In the presence of 100, 200, and 300 μM naringenin, the activities of the MMP-9 was obviously reduced to 80.3%, 61.3%, and 35.0% exposure to 100, 200, and 300 μM naringenin, respectively. Similarly, MMP-2 was significant reduced to 92.8%, 73.0%, and 71.9%, respectively. As shown in Figure 3B, Western blot analysis revealed that naringenin reduced the protein level of MMP-9 (95.7% in 100 μM, 89.0% in 200 μM, and 84.3% in 300 μM compared to vehicle group) and MMP-2 (89.0% in 100 μM, 71.7% in 200 μM, and 71.3% in 300 μM compared to vehicle group).

3.4 | Naringenin altered the expression of mesenchymal markers

Epithelial to mesenchymal transition (EMT) was critical for migration and invasion of cancer cells.[14] To determine the effects of naringenin on EMT markers' expression, Western blot analysis was performed. The protein of mesenchymal marker slug was significantly decreased in proportion to naringenin concentration whereas snail was significantly reduced in 100 μM naringenin treated group (Figure 4). However, no effect of naringenin on epithelial marker such as E-cadherin expression was found (data not shown).

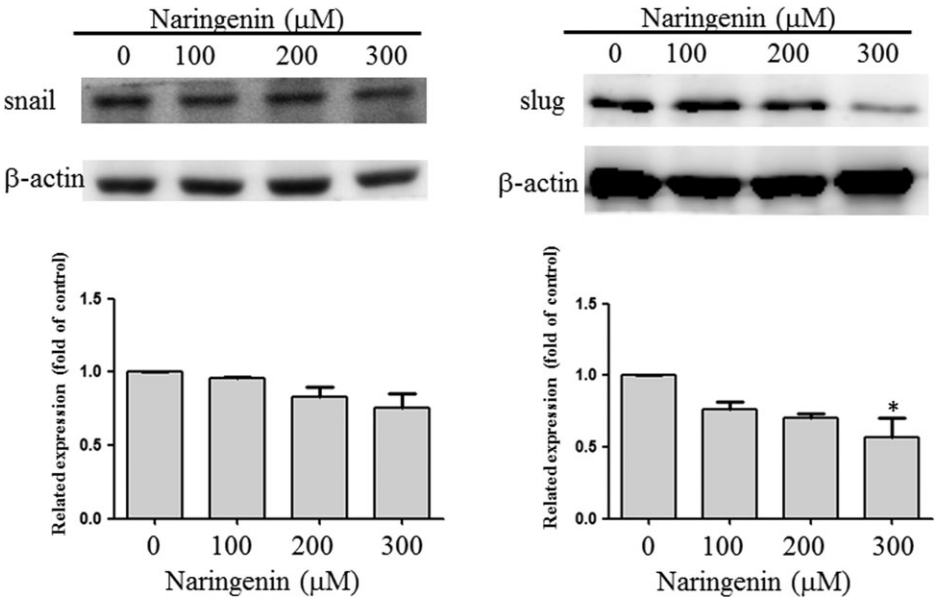

FIGURE 4 Naringenin repressed the mesenchymal markers expression. Cells treated with indicated concentration of naringenin for 24 hours. Fifty μg cell lysates derived from naringenin treated cells were subjected to Western blot analysis using antibodies as indicated. Lower panel: Data represented means ± SD from three independent experiments. *: $P < .05$. β-actin was loaded as internal control

3.5 | Naringenin inhibited MAPKs activities

It is well known that activation of mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinases (ERK1/2) and p38 involved in cancer metastasis.[15] The phosphorylated of ERK and p38 are active hence increases in their phosphorylation status implicated their activation. To test whether naringenin modulate the activities MAPKs, Western blot analysis was conducted. ERK1/2, and p38 activities was repressed in response to 100, 200, and 300 μM naringenin treatment.

4 | DISCUSSION

GBM is one of the most malignant brain tumors with high mortality. Naringenin, the abundant bioactive compound in citrus, has been shown to exhibit anticancer activities. In present study, we demonstrated the molecular mechanisms of naringenin on the migration and invasion of GBM cells. Naringenin significantly suppressed the migration and invasion of GBM cells as evidenced by Boyden chamber assay. Treatment with naringenin also reduced the activities of MMP-2 and MMP-9. Western blot analysis revealed that naringenin inhibited the expression of MMP-2, MMP-9, snail, and slug expression. Naringenin also reduced the activities of MAPKs and p38.

Our results indicated that naringenin reduced migration of GBM through multiple mechanisms.

Naringenin triggered the C6 glioma cells cerebrally implanted in rat to undergo apoptosis by upregulating connexin 43, reducing the Bcl-2/Bax ratio, and increasing the activities of caspase-3 and -9.[16] Administering naringenin also attenuated the expression of protein kinase C, cyclin D1, and cyclin-dependent protein kinase 4, which in turn suppressed the proliferation of cerebrally implanted C6 glioma cells in rats.[17] Stompor et al.[18] reported that treatment with naringenin at 150, 250, and 500 μM for 24 hours reduced the cell viability to 60%, 50%, and below 20%, respectively, in GBM U-118 MG cells. However, no effects of naringenin on cell viability of 8901 were found. One possible reason for this difference is the disparate genetic backgrounds of U-118 MG and 8901 cells. The U-118 MG cells were derived from a 50-year-old male with grade IV GBM. By contrast, the 8901 cells were derived from a 31-year-old female with GBM and bone marrow metastasis. In addition, naringenin caused minimal but remarkably decreased viability of 8401 cells, which were derived from the same patient as that of the 8901 cells but without metastasis. Our findings suggested that the 8901 cells were resistant to naringenin treatment because of their high malignancy and metastatic ability.

Degradation of extracellular matrix (ECM) plays an important role for cancer to invasion and metastasis.[13] MMPs especially for MMP-2 and MMP-9 are the major enzymes for degradation of ECM.[13] It has been shown that active MMP-9 was detected in 50% of GBM patients whereas no active MMP-9 was found in normal control.[19] Musumeci et al.[20] demonstrated that increased MMP-2 and MMP-9 was found in 52% and 76% stage IV GBM patients compared to control. Expression of MMP-2 was shown to be higher in GBM compared to normal brain tissues and was correlated with poor prognosis.[21] Recently, using extracts derived from traditional medicine or dietary food to repress GBM migration via downregulation of MMPs activities was received great attention. Desmethylanhydroicaritin from *Sophora flavescens* exhibited antimigration of U87MG cells by inhibition of MMP-2 and MMP-9 activities and protein expression.[22] Naringenin also has been shown to suppress migration via downregulation of MMPs expression in bladder and lung cancer.[11,12] In line with these observations, treatment with naringenin significantly decreased the activities and expression of MMP-2 and-9 in a dose-dependent manner. Interestingly, our results showed that naringenin inhibited the activity but not protein expression of MMP-9. Hong et al.[23] showed by molecular docking and surface plasmon resonance measure analysis that wogonin, a bioactive flavonoid with a similar structure to that of naringenin, interacts with

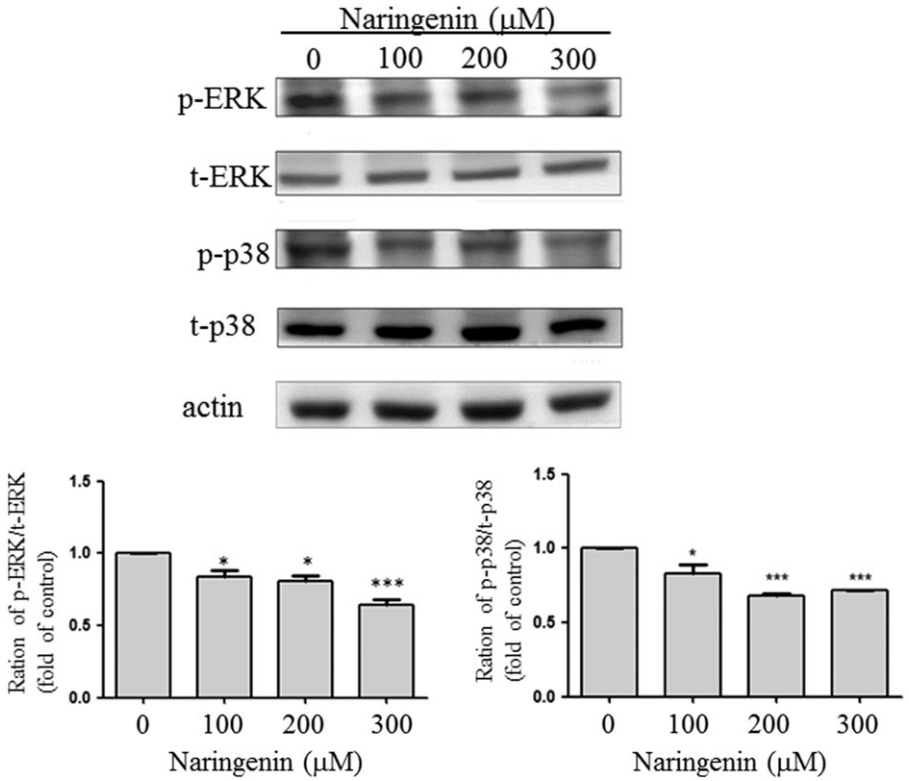

FIGURE 5 Naringenin inhibited the activities of ERK and P38. Cells treated with indicated concentration of naringenin for 24 hours. Fifty μg cell lysates derived from naringenin treated cells were subjected to Western blot analysis using antibodies as indicated. Lower panel: Data represented means ± SD from three independent experiments. *: $P < .05$; **: $P < .01$; ***: $P < .001$. β-actin was loaded as internal control

MMP-9. Upon binding with wogonin, MMP-9 was reduced in activity but without overt alteration of protein expression. By contrast, wogonin did not interact with MMP-2.[23] One possible reason is that naringenin maybe bind to and inhibit the activities of MMP-9 without affect MMP-9 expression like wogonin.

EMT plays a critical role in several cellular functions such as embryo development, tissue regeneration, and cancer formation.[24] During EMT progression, cells lost the epithelial markers such as E-cadherin, claudin, and cytokeratin and gain of mesenchymal markers such as snail, slug and vimentin, and finally facilitate cells to gain of migration and invasion abilities.[14] Increased slug mRNA expression was found to correlate with histologic grade and more invasive phenotype in GBM samples. Overexpression of slug promoted cell proliferation and migration in GBM cells.[25] Downregulation of snail by small interference RNA inhibited the proliferation and migration of GBM cells.[26] Treatment with honokiol suppressed the expression of snail, β-catenin, and N-cadherin and increased the expression of E-cadherin in U87MG GBM cells.[27] Moreover, honokiol attenuated the invasion of U87MG cells.[27] Kukoamine A significantly reduced the expression of mesenchymal markers such as snail, vimentin, and N-cadherin and increased the expression of epithelial markers such as E-cadherin and subsequently inhibited the migration of human GBM U251 and WJ1 cells.[28] Jorda et al.[29,30] indicated that snail bound to the promoter region and enhanced the expression of MMP-9 in Madin Darby canine kidney epithelial cells and Hep G2 cells. Snail also upregulated the expression of MMP-2 in hepatocellular carcinoma cells.[31] In this study, treated with naringenin reduced the expression of snail and slug then decrease the expression of MMP-2 and MMP-9 and eventually repressed migration of GBM cells.

MAPKs regulate a wide range of cellular functions such as proliferation, migration, survival, and apoptosis.[32] Documents have been demonstrated that inhibition of MAPKs signals by flavonoids can reduce GBM migration. Nobiletin attenuated migration and invasion of GBM cells via inhibition of MAPKs including ERK and p38 activities.[33] Quercetin suppressed ERK activities and then inhibited migration of U87MG cells.[34] In line with these observations, in this study, we showed that naringenin dose-dependently repressed the activities of ERK and p38. Moreover, ERK and p38 were also involved in MMP-2 and MMP-9 expression. Lin et al.[35] demonstrated that 12-O-tetradecanoylphorbol-13-acetate (TPA) elevated MMP-9 expression through ERK-pathway in GBM 8401 cells. Blocked ERK activities diminished TPA effects.[35] Collectedly, our results indicated that naringenin also inhibited ERK and p38 activities and finally reduced migration of GBM cells.

In summary, our findings provided the first evidences that naringenin repressed the migration and invasion of GBM cells through multiple mechanisms such as inhibited ERK1/2 and p38 activities, decreased snail and slug expression, diminished MMP-2 and -9 activities and expression. Our findings also indicated that naringenin could be a potential agent to repress metastasis of GBM and maybe useful as an anti-GBM drug.

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CONFLICT OF INTEREST

These authors declaimed no conflict of interests.

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