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Naringenin Attenuated Prostate Cancer Invasion *via* Reversal of Epithelial–to–Mesenchymal Transition and Inhibited uPA Activity

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Abstract. *Background: Prostate cancer is highly prevalent with a high mortality among males worldwide. Naringenin has been demonstrated to exhibit multiple cellular functions. In this study, we examined the effects of naringenin on prostate cancer. Materials and Methods: Transwell and zymography assays were used to detect cell migration and urokinase plasminogen activator (uPA) activity, respectively. Alternation of protein expression was measured by western blot analysis. Results: Transwell assay and zymography revealed that naringenin suppressed the migration and invasion of PC-3 cells and uPA activity in proportion to the concentration of naringenin. Western blot analysis indicated that naringenin up-regulated E-cadherin expression, but down-regulated the expression of vimentin, SNAIL family zinc finger 1 (SNAI1), SNAIL family zinc finger 2 (SNAI2), and TWIST family bHLH transcription factor 1 (TWIST1). Conclusion: Naringenin inhibited the migration and invasion of PC-3 cells by reversing expression of proteins involved in epithelial–to–mesenchymal transition and down-regulation of uPA activity. Thus, naringenin may be a promising anti-metastasis agent for prostate cancer.*

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Prostate cancer is the second leading cause of cancer-related death among men in Western countries (1). The 5-year survival rate dramatically decreases when cancer becomes invasive and metastasizes. Therefore, the development of non-toxic agents to retard prostate tumor migration and invasion is urgently needed.

Epithelial–to–mesenchymal transition (EMT) plays a critical role in embryotic development, wound healing, cancer metastasis, and drug resistance (2, 3). During EMT, cancer cells lose their epithelial characteristics, such as cell–cell adhesion and polarity, and gain mesenchymal phenotypes to facilitate their migration and invasion (2, 3). Several transcriptional factors, such as SNAIL family zinc finger 1 (SNAI1), SNAI2, and TWIST family bHLH transcription factor 1 (TWIST1) are involved in the EMT (2). These transcriptional factors can bind to the promoter region and suppress the expression of E-cadherin, an epithelial marker for cell–cell adhesion (4). However, these transcriptional factors up-regulate the expression of mesenchymal markers, such as vimentin and N-cadherin (4). E-cadherin expression was lost in prostate cancer with high grade and poor progression (5). By contrast, SNAI1 expression was higher in prostate cancer samples with advanced grade (6). The overexpression of TWIST1 is an independent prognostic factor for the recurrence-free survival of patients with prostate cancer (7).

Naringenin, a bioactive compound in grapefruit and orange, exhibits many cellular effects, such as anticancer, anti-inflammation, and anti-atherogenic effects (8). The treatment of bladder cancer and lung cancer cells with naringenin has been shown to inhibit migration by reducing matrix metalloproteinase 2 (MMP2) and MMP9 activities and protein kinase B (PKB/AKT) (9, 10). Very recently, Lim *et al*. (11) demonstrated that naringenin induced apoptosis through a mitochondrial-dependent pathway and modulated extracellular signal-regulated kinase (ERK) and AKT activities in PC3 and LNCaP prostate cancer cells. In addition, naringenin inhibited prostate cancer migration by regulating of voltage-gated sodium channels (12). To the best of our knowledge, although the effects of naringenin have been demonstrated in several human cancer types, its effects on prostate cancer migration and the mechanisms underlying these effects remain to be elucidated.

Materials and Methods

Cells and reagents. The PC-3 human prostate cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The PC-3 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37˚C and incubated under 5% CO2. Antibodies against E-cadherin, vimentin, SNAI1, SNAI2, and TWIST1 were obtained from Santa Cruz (Santa Cruz, CA, USA). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell viability assay. PC-3 cells were seeded in 24-well plates at a density of 2×10^4/ml and treated with 0, 25, 50, 100, 200, and 300 μM naringenin for 24, 48, and 72 h. The cells were subsequently treated with 5.0 g/I 3-(4,5dimethylthiazol-2-xyl)-2,5 diphenyl-tetrazolium bromide (MTT) and incubated at 37˚C for an additional 2.5 h. The purplish-blue sediments were dissolved in 1 ml of isopropanol, and the absorbance was determined at 563 nm. Based on the absorbance, cell viability was expressed as a percentage of control from three independent experiments.

Migration and invasion assay. PC-3 cells were treated with 0, 25, 50, 100, 200, and 300 μM concentration of naringenin for 48 h. The cells were detached by trypsin and plated into the upper section of a 48-well Boyden chamber at a density of 2.5×10^4/ml. After incubation at 37˚C for 16 h, the migrated cells were fixed with methanol and stained with Giemsa solution. For the invasion assay, the upper section of the Boyden chamber was coated with Matrigel (100 μg/cm^2), and the incubation time was 24 h. Three fields of each chamber were randomly selected, and the cell numbers were counted under a microscope.

Zymography assay. PC-3 cells were incubated with serum-free RPMI-1640 medium containing 0, 25, 50, 100, 200, and 300 μM naringenin for 24 h. The conditioned media were collected and subjected to zymography assay to detect uPA activity as previously reported (13).

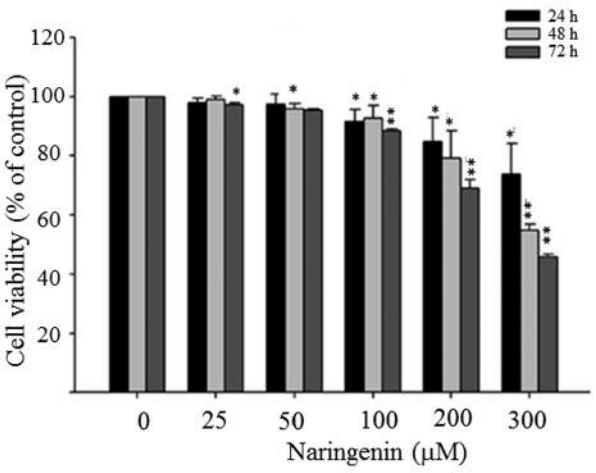

Figure 1. *Naringenin inhibited prostate cancer cell viability. PC-3 Human prostate cancer cells were treated with the indicated concentrations of Naringenin for 24, 48, and 72 h. Cell viability was measured by the MTT assay. Data represent means±S.D. from at least three independent experiments. Significantly different from the control (0 μM) at *p<0.05 and **p<0.001.*

Western blot analysis. PC-3 cells were treated with 0, 25, 50, 100, 200, and 300 μM concentrations of naringenin for 48 h. Cell lysates were collected, and an equal amount of proteins (50 μg) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted to determine EMT-related proteins, as described in a previous report (10).

Statistical analysis. The reported data are expressed as mean±standard deviation of three independent experiments and were evaluated by Student's *t*-test using SPSS software (Armonk, NY, USA). Differences with *p*-values less than 0.05 were considered to be statistically significant.

Results

MTT assay was performed to detect the effects of naringenin on the proliferation of PC-3. The cells were treated with different concentrations of naringenin for 24, 48, and 72 h. Compared with the vehicle-treated group, naringenin significantly reduced cell viability in a dose- and time-dependent manner (Figure 1). These findings suggest that naringenin reduced the proliferation of PC-3 cells.

A previous report showed that naringenin abolished the migration of MAT-LyLu prostate cells (12). Transwell assay was performed to determine whether naringenin reduced the migration and invasion of PC-3. The PC-3 cells were pretreated with 25, 50, 100, 200, and 300 μM of naringenin for 48 h. Equal amounts of alive cells were plated in the upper chamber, and migration and invasion were detected after 16 h. Treatment with naringenin significantly reduced cell

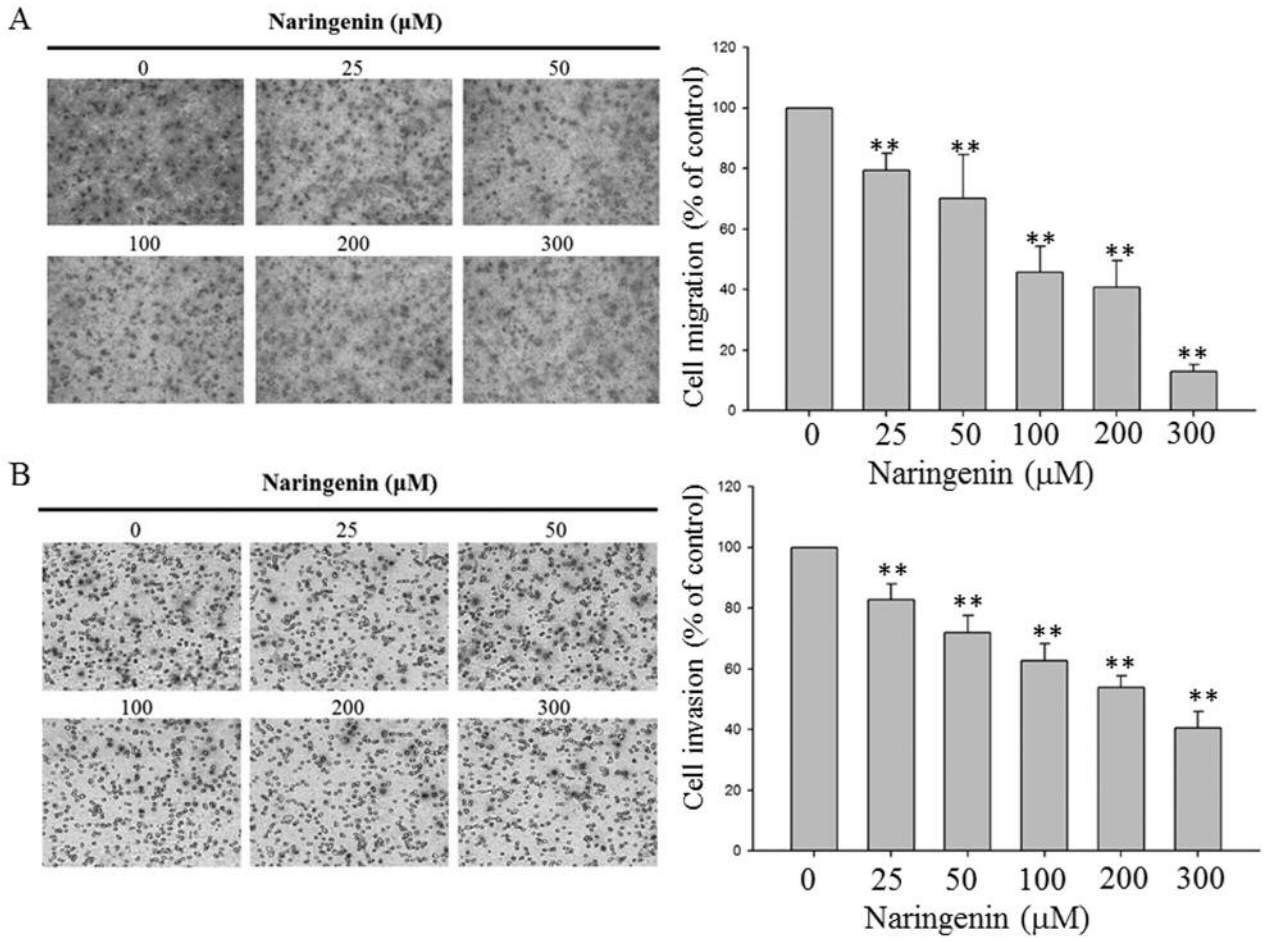

Figure 2. *Naringenin inhibited migration (A) and invasion (B) of PC-3 cells. Migration and invasion were assayed by Boyden chamber as mentioned in the Materials and Methods section. Data represent means±S.D. from at least three independent experiments. Significantly different from the control (0 μM) at *p<0.05.*

migration compared with the vehicle-treated group (Figure 2). The invasive ability was also significantly reduced response to naringenin (Figure 2). Naringenin dose-dependently inhibited the migration and invasion of PC-3 cells.

Cancer cells can secrete high levels of uPA to degrade the extracellular matrix for their migration and invasion (14). A zymography assay was conducted to test whether naringenin also affected uPA activity. In the presence of 25, 50, 100, 200, and 300 μM naringenin, the uPA activities were reduced to around 75% compared with that in the vehicle-treated group (Figure 3).

EMT has a critical role in cell migration (2). Thus, western blot analysis was performed to test whether naringenin affected the expression of EMT-related proteins. Exposure to 300 μM naringenin significantly up-regulated E-cadherin expression, whereas low concentrations of naringenin had no effects on E-cadherin expression (Figure 4). By contrast, expression of mesenchymal markers, namely, SNAI1, SNAI2, TWIST1, and vimentin, was reduced in proportion to the concentration of naringenin. Therefore, naringenin appears to reverse EMT.

Discussion

Given its high metastatic ability, it is surprising that prostate cancer is one of the leading causes of cancer-related deaths among men worldwide. The anti-metastatic effects of extracts derived from dietary foods have recently received great attention. In this study, we demonstrated that naringenin diminished the migration of PC-3 cells by inhibiting uPA activity, down-regulating expression of mesenchymal-like markers, and up-regulating E-cadherin.

In this study, we demonstrated that naringenin significantly attenuated migration and invasion of PC-3 cells. Our

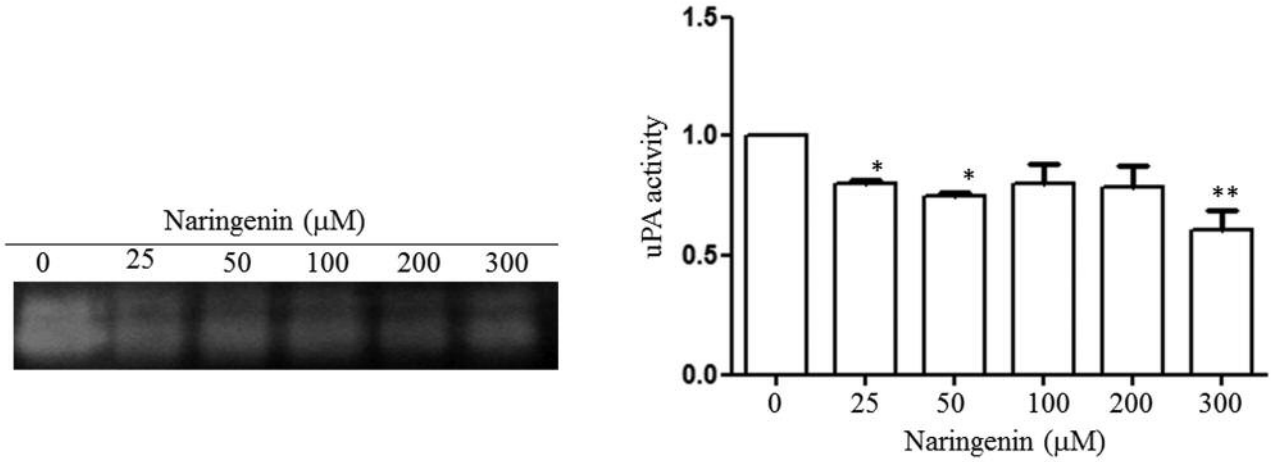

Figure 3. *Naringenin reduced urokinase plasminogen activator (uPA) activity. PC-3 cells were treated with indicated concentration of naringenin in serum-free medium for 24 h. Conditional serum was collected and subjected into casein zymography analysis. Casein data represent means±S.D. from at least three independent experiments. Significantly different from the control (0 μM) at *p<0.05 and **p<0.001.*

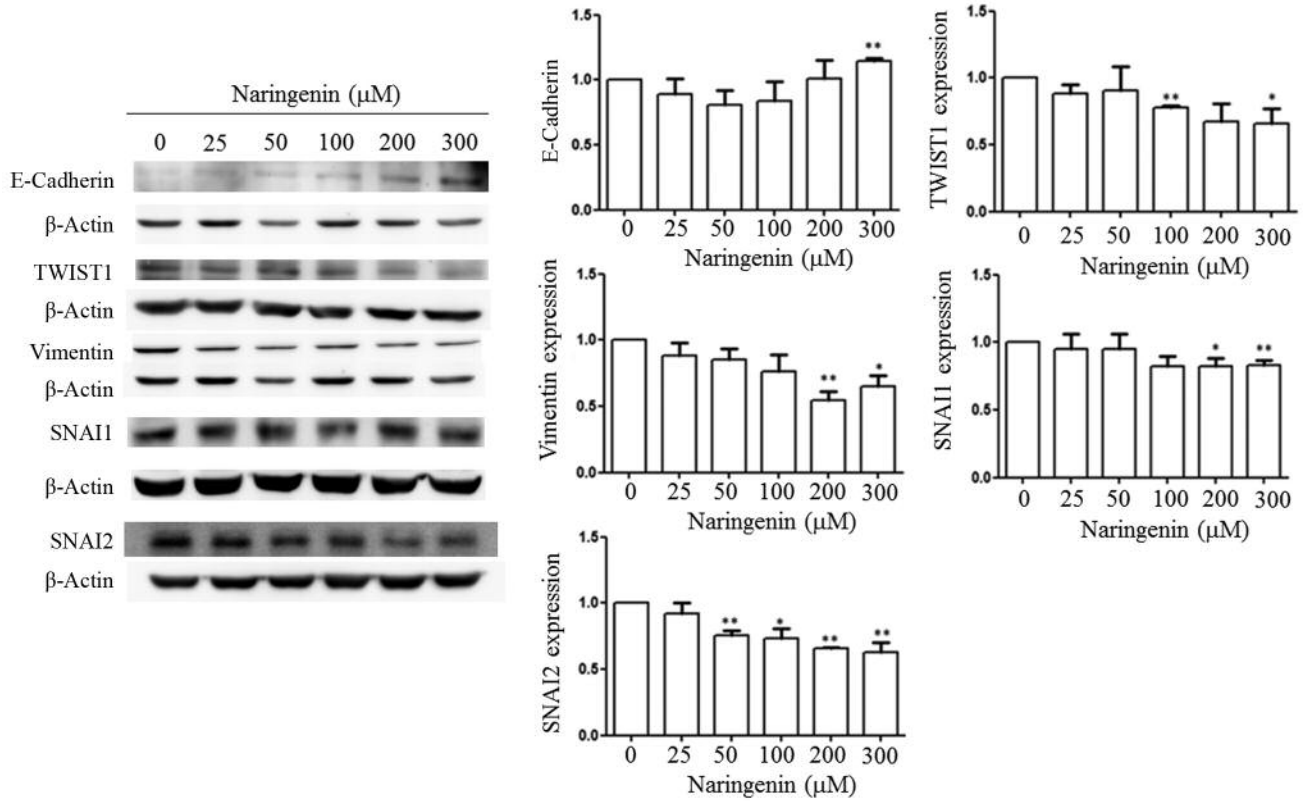

Figure 4. *Naringenin reversed the expression of epithelial–to–mesenchymal transition markers. PC-3 cells were exposed to the indicated concentration of naringenin for 48 h. Cell lysates were extracted and western blot analysis was performed and using antibodies against E-cadherin (epithelial marker) and mesenchymal markers SNAIL family zinc finger 1 (SNAI1), SNAI2, TWIST family bHLH transcription factor 1 (TWIST1), vimentin, and TWIST1. Data represent means±S.D. from at least three independent experiments. Significantly different from the control (0 μM) at *p<0.05 and **p<0.001.*

observations are in line with that of Gumushan Aktas and Akgun whereby naringenin (5 and 10 μM) diminished migration of MAT-LyLu prostate cancer cells, accompanied with inhibition of voltage-gated sodium channel SCN9A expression (12). Lim *et al*. demonstrated that naringenin at 50 μM reduced the migration of PC-3, but not LNCaP cells (11). Controversially, naringenin at 5, 10, 20, 50, and 100 μM reduced cell viability of serum-starved PC-3 cells and LNCaP cells (11). In contrast, our results indicated that naringenin slightly reduced cell viabilities only at 100, 200, and 300 μM concentrations. This difference in observations between the present study and those of Lim *et al*. (11) might result from an increase inPC-3 cell sensitivity to naringenin treatment after serum starvation. Collectedly, our findings indicated that naringenin inhibited migration and invasion of PC-3 cells.

The degradation of the extracellular matrix by proteinases, such as MMPs and uPA, has a critical role in the migration and invasion of cancer cells (15). Through immunohistochemical staining, Kumano *et al*. demonstrated that uPA expression was correlated with advanced stages and lymph node metastasis in prostate cancer (16). Several extracts from dietary foods were recently shown to have anti-metastatic effects on prostate cancer. Epigallocatechin gallate, a polyphenol in green tea, cooperated with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to reduce the migration and invasion of prostate cancer cells by down-regulating uPA (17). Quercetin similarly repressed the expression of uPA and subsequently inhibited the migration of PC-3 cells (18). Similarly, our finding indicated that naringenin treatment reduced the activity of uPA as assayed by zymography analysis and subsequently attenuated the migration and invasion of PC-3 cells.

During EMT, cancer cells acquire mesenchymal-like phenotypes to promote their migration and invasion, eventually causing metastasis in secondary organs (19). Transcriptional factors, such as SNAI1, SNAI2, and TWIST1, up-regulate expression of mesenchymal markers and down-regulate that of epithelial markers (19, 20). In prostate cancer, SNAI1 expression is associated with Gleason grade (21), and up-regulation of *SNAI1* expression has been correlated with the progression of prostate cancer (22). Kwok *et al*. also demonstrated that TWIST1 expression increased in prostate cancer with bone metastasis (23). Accumulating reports have shown that polyphenols from dietary foods can attenuate the migration of prostate cancer cells by reversing the EMT. Genistein dose-dependently suppressed the invasion of prostate cancer cells by reversing EMT (24). Wu *et al*. also demonstrated that silibinin enhanced cytokeratin-18 expression and down-regulated vimentin, *MMP2*, and *SNAI2* (25). These processes reduce the metastatic properties of PC-3 cells. Similarly, naringenin treatment up-regulated expression of epithelial markers, such as E-cadherin, and down-regulated the four mesenchymal markers. Moreover, naringenin reversed the EMT and reduced the migration of PC-3 cells.

In conclusion, naringenin attenuated the migration and invasion of PC-3 cells by inhibiting uPA activity, down-regulating mesenchymal regulators, namely, *SNAI1, SNAI2*, and *TWIST1*, and elevating that of epithelial markers, such as E-cadherin. To the best of our knowledge, this is the first report that highlights the molecular mechanisms of naringenin in the migration of PC-3. These results also provide evidence for naringenin as a potential therapeutic agent for the treatment of prostate cancer metastasis.

Conflicts of Interest

The Authors declared no conflict of interest in regard to this study.

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