Morin, a flavonoid from Moraceae, suppresses growth and invasion of the highly metastatic breast cancer cell line MDA-MB-231 partly through suppression of the Akt pathway

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Abstract. Morin, a flavonoid found in figs and other Moraceae, displays a variety of biological actions, such as anti-oxidant, anti-inflammatory and anti-carcinogenic. However, the anticancer effects of morin and in particular its anti-metastatic effects are not well known. Therefore, in the present study, we investigated the anticancer effects of morin on highly metastatic human breast cancer cells. Our results showed that morin significantly inhibited the colony forming ability of highly metastatic MDA-MB-231 breast cancer cells from low doses (50 µM) without cytotoxicity. In addition, morin changed MDA-MB-231 cell morphology from mesenchymal shape to epithelial shape and inhibited the invasion of MDA-MB-231 cells in a dose-dependent manner. Morin decreased matrix metalloproteinase-9 (MMP-9) secretion and expression of the mesenchymal marker N-cadherin of MDA-MB-231 cells, suggesting that morin might suppress the EMT process. Furthermore, morin significantly decreased the phosphorylation of Akt, and inhibition of the Akt pathway significantly reduced MDA-MB-231 invasion. In an in vivo xenograft mouse model, morin suppressed MDA-MB-231 cancer cell progression. Taken together, our findings suggest that morin exhibits an inhibitory effect on the cancer progression and EMT process of highly metastatic breast cancer cells at least in part through inhibiting Akt activation. This study provides evidence that morin may have anticancer effects against metastatic breast cancer.

Introduction

Cancer is one of the leading causes of death. Breast cancer is the most common cancer diagnosed in North American and Western European women (1,2), and Asian populations generally have the lowest risk, but rates in this population have been steadily increasing. Particularly, in Korea, the incidence of breast cancer has increased 4-fold from 1996 to 2010, showing the highest growth rate of breast cancer among OECD countries (3). Majority of primary breast cancers (70-80%) are estrogen receptor (ER)-positive (+), and ER\(^-\) breast cancers generally have a better prognosis and are responsive to anti-estrogen therapy. In contrast, ER-independent (ER\(^-\)) breast cancers including refractory cancer to antiestrogen therapy are more aggressive, possess high metastatic potential (4,5). Most of these patients eventually die of metastatic disease.
Increasing evidence shows that breast cancer cells undergo an epithelial-to-mesenchymal transition (EMT) to metastasize, and this process is frequently observed in the most aggressive subtype, estrogen receptor-negative (ER)/progesterone receptor (PR)-negative (PR)/human epithelial growth factor receptor 2-negative (HER2) triple-negative breast cancer (TNBC) (6).

Numerous materials isolated from plants are being investigated for their therapeutic application against cancer metastasis. Among compounds of known structure, flavonoids deserve special attention because they are present in practically all dietary plants, fruits and root. The flavonoids, including morin (7,8), are non-toxic (9,10) and display a variety of biological actions including anti-carcinogenic (11-13). Mulberry trees are widely cultivated in East Asia and the white mulberry, *Morus alba* L. is a rich source of many bioactive phytochemicals. Five phenolic constituents, including maclurin, rutin, isoquercitrin, resveratrol and morin, have been identified in ethanolic extract of mulberry twigs to account for its potential oxidation capability; among them, maclurin and morin have been shown to be superior to the others (14).

Morin (3,5,7,2',4'-pentahydroxyflavone) is a kind of flavonoid found in figs and other Moraceae which are used as herbal medicines. It has certain biological activities, including anti-oxidant properties (15,16) and anti-inflammatoriy effects (17,18). Morin also acts as an anti-mutagen (19,20) and has an anti-promotion activity in a liver carcinogenesis model (21). Most of all, the favorable safety profile of this natural compound (8) makes it a potential candidate worthy of further investigations. Such beneficial effects of morin could be expected to work in *in vitro* and *in vivo* cancer model. However, the effect of morin on cancer growth and metastasis is not well known. Therefore, in the present study, we aimed to investigate the effect of morin on the cancer growth and invasion in highly metastatic human breast cancer cells MDA-MB-231. As mentioned above, most lethal and aggressive subtype of breast cancer is ER/PR/HER2 TNBC and MDA-MB-231 is a well-known TNBC. Thus, MDA-MB-231 was used in this study.

Materials and methods

**Materials.** Morin (Fig. 1A) was obtained from Aging Tissue Bank (Pusan, Korea). Antibodies against N-cadherin, phospho- Akt, Akt, phospho-GSK3β and GSK3β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). BD Matrigel™ basement membrane matrix is supplied by BD Biosciences (San Diego, CA, USA). BD Matrigel™ is a rich source of numerous materials isolated from plants are being investigated for their therapeutic application against cancer metastasis. Among compounds of known structure, flavonoids deserve special attention because they are present in practically all dietary plants, fruits and root. The flavonoids, including morin (7,8), are non-toxic (9,10) and display a variety of biological actions including anti-carcinogenic (11-13). Mulberry trees are widely cultivated in East Asia and the white mulberry, *Morus alba* L. is a rich source of many bioactive phytochemicals. Five phenolic constituents, including maclurin, rutin, isoquercitrin, resveratrol and morin, have been identified in ethanolic extract of mulberry twigs to account for its potential oxidation capability; among them, maclurin and morin have been shown to be superior to the others (14).

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**Cell culture.** The human breast cancer cell MDA-MB-231 was grown in RPMI-1640 supplemented with 10% FBS, 100 IU/ml penicillin and 10 µg/ml streptomycin. The human umbilical endothelial cell line EA.hy 926 was grown in DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 10 µg/ml streptomycin. All cells were incubated in a humidified 5% CO₂ incubator.

**Cell proliferation assay.** Cells were seeded at 10⁴ cells per well in 24-well plates. After treatments, 50 µl of 5 mg/ml MTT solution was added to each well and incubated for 3 h. The supernatants were aspirated and the formazan crystals were dissolved with 200 µl of 4 N HCl-isopropanol in each well. The optical density of the colored product was measured at 570 nm, as suggested by the manufacturer, using an Infinite 200 microplate reader (Tecan Austria GmbH, Grödig, Austria).

**Colony formation assay.** Cells were seeded in 6-well plates at 1,000 cells/well. After serum starvation for 16 h, the cells were treated with morin at the indicated doses in a 37°C cell culture incubator. After 24 h, culture medium was discarded and changed with media every 2-3 days. After 1-2 weeks, cells were fixed and stained using crystal violet and photographed. The experiments were performed in triplicate.

**Western blot analysis.** Cells were washed in ice-cold PBS and lysed in PRO-PREP protein extraction solution (iNtRON Biotechnology, Seoul, Korea). The samples were centrifuged at 13,000 rpm, for 15 min at 4°C. An aliquot of the whole cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membrane. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 for 2 h at room temperature and incubated with primary antibodies at 1:1,000 in TBS containing 0.05% Tween-20 and 3% bovine serum albumin overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000) antibody for 1 h at room temperature. After washing, the membranes were developed using the ECL reagent (Bionote, Gyeonggi-do, Korea).

**Matrigel invasion assay.** The upper chamber of 24-well cell culture inserts (8-µm pore size, Falcon, Franklin Lakes, NJ, USA) were washed with a serum-free medium, coated with 100 µl of Matrigel (1 mg/ml) and dried for 30 min at 37°C. MDA-MB-231 cells treated with morin were collected; 2x10⁵ cells were loaded to the upper chambers filled with serum-free media, and 500 µl of RPMI media containing 10% FBS was added to the lower chambers. The invasion chambers were incubated for 24 h in a 37°C cell culture incubator. The non-invasive cells that remained on the upper surface of the insert membranes were removed by scrubbing. The cells on the lower insert membranes were stained with DAPI, and cells were counted under a fluorescence microscope. Each sample was measured in triplicate, and each experiment was repeated three times.

**Gelatin zymography.** Gelatin zymography was performed as described by Jin et al (22). Briefly, conditioned media were concentrated using a protein concentrator (Thermo Pierce, Rockford, IL, USA) and subjected to electrophoresis on 8% PAGE gels containing 1 mg/ml gelatin. Gels were washed twice with 2.5% Triton X-100, stained with 0.2% Coomassie Brilliant Blue and destained (50% methanol and 10% acetic acid).
acid). Gelatinolytic activity was detected as clear bands in the background of blue staining.

**Animal experiments.** Athymic nude mice were divided into 2 groups (5 mice/group) and received morin at the dose of 10 and 50 mg/kg (daily, i.p.), respectively for 7 days. Mice were injected subcutaneously with MDA-MB-231 (5x10⁶ cells/100 µl of serum-free RPMI). Tumors were allowed to grow until they reached 4 mm. At this point, mice were divided into 2 groups (7 mice/group): control and morin-treated mice. The mice were administered a daily i.p. injection of 10 mg/kg (non-toxic dose) morin for 45 days. The mice were sacrificed at day 45, and the tumors were extracted. Body weights and tumor volumes were measured every 3 days, starting at 7 days after injection. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Gyeongsang National University.

**Statistical evaluations.** Scanning densitometry was performed using Image Master® VDS (Pharmacia Biotech Inc., San Francisco, CA, USA). The treatment groups were compared using one-way analysis of variance and the post hoc test by Scheffe. P<0.05 was considered statistically significant. All data were expressed as the mean ± standard error (SEM).

**Results**

Morin inhibits the colony forming ability of the human breast cancer MDA-MB-231 cells without cytotoxicity to human endothelial cells. First, we examined the cell viability of...
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MDA-MB-231 cells and normal endothelial cells in response to morin. When MDA-MB-231 cells and endothelial cells were treated with indicated doses of morin (10, 50, 100 and 200 µM) for 24 h, the results revealed that morin did not affect cell viability at the doses <100 µM both in MDA-MB-231 cells and endothelial cells. High concentrations (100 and 200 µM) of morin reduced cell viability of MDA-MB-231 a little but rather increased cell viability in endothelial cells (Fig. 1B). Thus, we investigated the effect of morin on the ability of MDA-MB-231 cells to form colonies. Interestingly, morin effectively inhibited colony formation of MDA-MB-231 cells in a dose-dependent manner (Fig. 1C).

Morin affects cell morphology and inhibits the invasion of MDA-MB-231 breast cancer cells. Next, we observed changes of MDA-MB-231 cells in morphology after morin treatment. As shown in Fig. 2A, morin induced morphologic changes of MDA-MB-231 cells from mesenchymal form to epithelial form in a dose-dependent manner. We then determined the effects of morin on MDA-MB-231 cell invasion because cancer cell invasion is the first step for cancer metastasis. Matrigel invasion assays revealed that morin significantly inhibited cell invasion in a dose-dependent manner (Fig. 2B). Moreover, morin also effectively inhibited TNF-α-induced MDA-MB-231 cell invasion in a similar manner (Fig. 2C).
Morin decreases matrix metalloproteinase-9 (MMP-9) secretion and the mesenchymal marker N-cadherin expression involved in cancer metastasis. Proteolytic digestion of the extracellular matrix (ECM) by secreted MMPs is one of major steps for cancer invasion (23,24). MMP-2, -3 and -9 are also biomarkers for EMT (25). In particular, MMP-9 expression is associated with pathological processes, including inflammation, atherosclerosis and tumor-cell invasion and metastasis (26-28). Thus, we examined the effect of morin on the activity of the secreted MMP-9 from MDA-MB-231 cells in the presence of TNF-α or not by gelatin zymographic analysis. Morin dose-dependently suppressed the gelatinolytic activities of secreted MMP-9 in MDA-MB-231 cells. MMP-9 secretion augmented by TNF-α was also significantly inhibited by a low dose of morin (10 µM) (Fig. 3A). In addition, we also assessed the changes in EMT biomarkers to confirm that morin has inhibitory effects on EMT. Fig. 3B showed that morin inhibited mesenchymal markers N-cadherin, but did not influence the expressions of either vimentin or E-cadherin of MDA-MB-231 cells (data not shown). These results suggest that morin might suppress the invasion of highly metastatic MDA-MB-231 breast cancer cells through regulating the EMT process.

Morin inhibits the invasion of MDA-MB-231 breast cancer cells through inhibiting Akt pathway but not GSK3β pathway. Concerning the upstream signals that affect N-cadherin, β-catenin induces N-cadherin expression, and β-catenin is negatively regulated by GSK-3β. GSK-3β is regulated by intracellular signaling pathways including PI3K/Akt. In other
words, activation of PI3K/Akt results in the phosphorylation of GSK-3β (inactivation of GSK-3β), which in turn increases β-catenin protein levels. Thus, we investigated whether morin modulates Akt and GSK-3β pathways in MDA-MB-231. Morin reduced phosphorylated Akt level in MDA-MB-231 cells but failed to inhibit GSK-3β phosphorylation. In addition, TNF-α (10 ng/ml) enhanced Akt phosphorylation in MDA-MB-231, which was also significantly inhibited by morin. Morin did not reduce the TNF-α-mediated phosphorylation of GSK-3β (Fig. 4A and B). Then, we confirmed whether inhibition of Akt pathway could block the invasion of MDA-MB-231. As expected, inhibition of Akt pathway using LY294002 significantly decreased the invasion of MDA-MB-231 as well as TNF-α-enhanced invasion of MDA-MB-231 (Fig. 5A). In addition, LY294002 downregulated N-cadherin expression level (Fig. 5B). These results suggest that morin suppresses the invasion of MDA-MB-231 breast cancer cell through inhibiting Akt pathway and N-cadherin expression.

**Morin reduces breast cancer cell growth in xenograft mouse model in vivo.** To confirm the in vivo effect of morin in tumor progression, morin was injected into tumor-bearing mice for 45 days. Before that, to determine whether morin has a toxic effect in vivo, the mice received i.p. a daily injection of 10 or 50 mg/kg morin for 7 days (n=7/group). Both 10 and 50 mg/kg concentrations of morin caused no lethality in mice (Fig. 6A), however 50 mg/kg made the mice somewhat nervous. Thus, we used 10 mg/kg of morin for further experiments. Control animals developed significant tumor growth during the 45-day follow-up period, as shown by the tumor volume in Fig. 6B and C. In contrast, animals that received 10 mg/kg morin daily showed that tumor growth was significantly inhibited (Fig. 6B and C). Body weight does not show any significant differences between the groups.

**Discussion**

Cancer metastasis is responsible for most cancer death rather than the primary tumors (29-31). Cancer metastasis is a complex process involving the coordinated responses among cancer cells, normal cells and ECM. Variable growth factors, MMPs and cytokines including TNF-α stimulate cancer metastasis (32-34). Considered that tumor metastasis is the main cause of mortality of cancer patients, it is more beneficial to develop drugs that are able to suppress the highly metastatic cancer cells progression. Indeed, human breast cancer cells MDA-MB-231 express putative aggressiveness markers and are well known as an ER-/PR-/HER2- TNBC. Therefore, in this study, we examined the anticancer effect of morin using the highly metastatic human breast cancer cells MDA-MB-231. Our results showed that morin significantly inhibited the ability of MDA-MB-231 cells to form colony and invade. In addition, morin suppressed the EMT process. Interestingly, morin has no severe cytotoxicity to cancer cells or normal endothelial cells; high concentrations (100 and 200 µM) of morin slightly reduced cell viability of MDA-MB-231 but rather increased cell viability in endothelial cells. These results suggest that morin could be used in cancer patients without serious cytotoxicity. Previous studies demonstrated that natural compounds could safely induce anticancer effects.

A family of extracellular matrix-degrading enzymes, the MMPs, has been implicated in inflammation and cancer (35). In particular, MMP-9 expression is associated with pathological processes, including inflammation, atherosclerosis and tumor cell invasion and metastasis (26-28). Increased levels of serum and tissue expression of MMP-9 are associated with a poor prognosis of breast cancer (36). Our results also showed that MMP-9 secretion was more prominent than MMP-2 in MDA-MB-231 (data not shown), which was effectively inhibited by morin.

For the evaluation of EMT process, it is important to assess the expression of E-cadherin an epithelial marker, but
E-cadherin expression level of MDA-MB-231 was too low to detect (22). Instead, we showed that morin downregulated N-cadherin expression in the MDA-MB-231. The expression of N-cadherin (37,38) is frequently upregulated in moderately-to-poorly invasive duct carcinomas (IDCs) (39,40) and HER2-amplified tumors. Accordingly, it is suggesting that N-cadherin might play a role in cancer invasion and EMT in breast cancer. In this respect, morin might downregulate the EMT process by suppressing N-cadherin expression. Activation of PI3K/Akt causes an inactivation of gSK-3β, which in turn...

Figure 6. Morin reduces breast cancer cell growth in a mouse model in vivo (A) Survival curves of mice treated with morin 10 or 50 mg/kg. Athymic nude mice were divided into 2 groups (5 mice/group): morin 10 mg/kg-treated mice and morin 50 mg/kg-treated ones. The mice received i.p. daily injection of morin for 7 days. (B) MDA-MB-231 cells (5x10⁶ cells in 100 µl of serum-free medium) were injected subcutaneously into athymic nude mice. Tumors were allowed to grow until they reached 4 mm. At this point, mice were divided into 2 groups (7 mice/group): control and morin-treated mice. The mice received i.p. a daily injection of 10 mg/kg morin for 45 days. The mice were sacrificed at day 45, and the tumors were extracted. (C) Tumor volumes and body weights were measured every 3 days during tumor development. Significance compared with control, *P<0.05, **P<0.01.
increases β-catenin protein levels, and finally results in the N-cadherin expression. Our results showed that morin significantly decreased phosphorylation of Akt in MDA-MB-231 cells as well as in TNF-α-stimulated MDA-MB-231 cells. Inhibition of Akt pathway using LY294002, a PI3K/Akt inhibitor, significantly reduced MDA-MB-231 invasion in the presence of TNF-α or not, suggesting that morin suppresses EMT at least in part through suppressing Akt pathway. However, morin did not affect GSK-3β phosphorylation. Without GSK-3β phosphorylation, MMP expression and activity can regulate N-cadherin function (41-43). In addition, gene expression of MMPs can be regulated via the Akt pathways (44,45). These findings support that morin may suppress the EMT process by inhibiting MMP-9 activity followed by N-cadherin expression through suppression of Akt pathway. Furthermore, MMP-9 is also involved in EMT. Finally, we confirmed the anticancer effect of morin using in vivo xenograft mouse model in which animals were injected with MDA-MB-231 cells. Taken together, our findings suggest that morin exhibits an inhibitory effect on the cancer progression by inhibiting EMT in the highly metastatic breast cancer MDA-MB-231 cells at least in part through inhibiting Akt activation. These findings suggest that morin may serve as an effective therapeutic strategy against metastatic breast cancer without side effects.

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