Abstract. Breast cancer is the most commonly diagnosed cancer worldwide. Despite the use of chemotherapeutic drugs, drug resistance has been observed in numerous patients with breast cancer. Epithelial-mesenchymal transition (EMT) is an important initiation step in the process of metastasis, whereby cancer cells move away from the original tumor site. Therefore, the discovery of new substances that suppress EMT is a promising avenue for cancer treatment. The present study investigated the effect of hispidulin, a polyphenolic flavonoid, on EMT in human breast cancer cells in vitro (MCF-7 and HCC38). The EMT-associated mRNA and protein expression levels were measured using reverse transcription-quantitative PCR or western blot analysis. Hispidulin treatment increased the expression levels of EMT-associated epithelial markers and decreased the expression levels of mesenchymal markers in both cells. Transforming growth factor-β1 (TGF-β1) treatment increased breast cancer cell viability (assessed via MTS assay) and EMT induction. However, hispidulin and TGF-β1 co-treatment increased the expression levels of E-cadherin and occludin, while downregulating vimentin expression. Additionally, hispidulin treatment inhibited TGF-β1-induced Smad2/3 signaling and cell migration in both breast cancer cell lines. Overall, the current findings suggested that hispidulin may inhibit EMT and cell migration by suppressing the Smad2/3 signaling pathway in breast cancer cells.

Introduction

Breast cancer is the leading cause of cancer-related deaths in women worldwide (1). Breast cancer risk is increased by prolonged estrogen exposure, such as early menarche, late menopause, having no childbirth experience, or increased estrogen levels in adipocytes due to obesity (2,3). In addition, mutated BRCA1 and BRCA2 genes are associated with breast cancer risk (4). Management strategies for breast cancer include surgery, radiotherapy, chemotherapy, endocrine treatment, and targeted treatment (5). Among these treatments, anticancer drugs have been widely used for breast cancer treatment. For example, tamoxifen acts on the estrogen pathway and is applied in estrogen receptor-positive breast cancer patients (6). Patients who have high levels of metastatic human epidermal growth factor receptor 2 (HER2)/neu are prescribed trastuzumab, which blocks the HER2 signal and improves patient outcomes (7). However, these chemotherapy drugs have side effects including early menopause, depression, rash, and renal toxicity.

Epithelial-mesenchymal transition (EMT) is a process associated with the disruption of cell junctions and the loss of cell polarity, which increases cell mobility and allows them to acquire stem cell-like properties (8). As its name suggests, EMT causes the loss of epithelial markers (E-cadherin and occludin) and acquisition of mesenchymal markers (fibronectin, vimentin and N-cadherin) (9). EMT is induced by numerous growth factors and related signaling pathways, including transforming growth factor-β1 (TGF-β1) (10). TGF-β1 plays a prominent role in breast cancer progression and bone metastasis (11).

Hispidulin (4',5,7-trihydroxyl-6-methoxyflavone) is a phenolic flavonoid compound widely used in traditional medicine (12). Several studies have shown that hispidulin has anti-obesity, antioxidant, anti-mutagenic, anti-inflammatory, and anti-tumor effects (13-17). Hispidulin also has anticonvulsant, neuroprotective, and anti-osteoporosis effects (18-21). However, the role of hispidulin in EMT has not yet been studied in breast cancer. In this study, we used two breast cancer cell lines, luminal type cells (MCF-7) with strong cell-to-cell adhesion and an aggregated structure, and claudin-low type cells (HCC38) with an invasive form and high metastatic properties due to weak cohesion between cells. We aimed to investigate the effect of hispidulin on EMT and cell migration induced by TGF-β1 treatment in breast cancer cells.

Materials and methods

Reagents and antibodies. Hispidulin was purchased from Sigma-Aldrich; Merck KGaA and was dissolved in dimethyl sul-
oxide (DMSO) and mixed with fresh medium to achieve the desired final concentrations. TGF-β1 was purchased from R&D Systems. The following primary antibodies were used in the experiment: anti-β-actin and anti-total-Smad2/3 (Santa Cruz Biotechnology, Inc.), anti-phospho-Smad2/3 (Cell Signaling Technology, Inc.), anti-vimentin (Sigma-Aldrich; Merck KGaA), anti-E-cadherin, and anti-occludin (BD Biosciences). The secondary antibodies used were horseradish peroxidase (HRP-conjugated anti-mouse; Santa Cruz Biotechnology, Inc.) and HRP-conjugated anti-rabbit (Cell Signaling Technology, Inc.).

Cell culture. The two breast cancer cell lines used in this study (MCF-7 and HCC38) were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 1% penicillin, streptomycin, and 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO₂ atmosphere.

Cell viability assay. Breast cancer cell viability was assessed using the 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay, as previously described (22). Briefly, cells were plated (2x10⁵ cells/well) in 96-well plates and allowed to grow overnight. Cells were treated with various concentrations of hispidulin and/or TGF-β1 for 24 h. Next, MTS reagent was added to each well according to the manufacturer's protocol.

Western blot. After treatment with hispidulin and/or TGF-β1 for 24 h, cell lysates were collected and prepared. Protein concentration was quantified using the Bradford assay (Bio-Rad Laboratories, Inc.). After SDS-PAGE, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore). After blocking with 5% non-fat dry milk, the membranes were probed with different primary antibodies. Membranes were incubated with an HRP-conjugated...
secondary antibody for 1 h, and protein detection was performed using enhanced chemiluminescence (ECL) solution (PerkinElmer).

**Confocal microscopy.** Fluorescent images were examined as previously described (22). Briefly, the treated breast cancer cells were fixed in 4% paraformaldehyde for 1 h. Then, the cells were permeabilized with 0.5% Triton X-100, blocked in 1% bovine serum albumin (BSA), and stained with the primary antibody overnight at 4˚C. Cells were treated with secondary antibody for 1 h and then mounted using 4',6-diamidino-2-phenylindole (DAPI). Cells were observed using confocal microscopy (LSM-700; Carl Zeiss).

**Wound healing assay.** Each breast cancer cell line (4x10^5 cells) was seeded in a 12-well plate and allowed to grow until approximately 90% confluence. Then, the media was removed and the monolayer was scratched with a pipette tip. 10 ng/ml TGF-β1 and hispidulin (1.25 and 2.5 µM)-treated cells were maintained in 1% FBS-containing media for 24 h. The media was then removed and the cells were washed with phosphate-buffered saline (PBS). After fixing with methanol, the cells were stained with Giemsa stain solution (Sigma-Aldrich; Merck KGaA) for 1 h. Cells were then observed and photographed using an optical microscope (CKX41; Olympus Corporation) and analyzed using Image J software (NIH).

**Transwell migration assay.** Breast cancer cells were plated in the upper chamber of transwell plates containing TGF-β1 (10 ng/ml) and hispidulin (1.25 and 2.5 µM) (Costar). The lower chamber was supplemented with serum-free media containing 5% FBS. After 24 h of incubation, the cells were fixed with methanol. Cells were stained with hematoxylin (Sigma-Aldrich; Merck KGaA) and eosin (Sigma-Aldrich; Merck KGaA). Cells were then photographed using an optical microscope (CKX41; Olympus Corporation).

**Statistical analysis.** Graph Pad Prism 6 Software (GraphPad Software, Inc.) was used for statistical analysis. All experiments were repeated at least three times and the data are expressed as the mean ± SD. Student’s t-test was used to determine the differences between the control and experimental groups. *P<0.05 vs. DMSO-control group. EMT, epithelial-mesenchymal transition.
were performed three times, independently. One-way ANOVA with Bonferroni post-hoc test was used for statistical analysis of the data. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Hispidulin inhibits breast cancer cell viability.** The chemical structure of hispidulin is shown in Fig. 1A. We first determined the inhibitory effect of hispidulin on the viability of breast cancer cells. MCF-7 and HCC38 cancer cells were treated with various concentrations of hispidulin for 24 h. We observed that the 50% inhibitory concentration (IC\textsubscript{50}) values of hispidulin in MCF-7 and HCC38 cells were 25.44±0.23 and 65.42±0.31 µM, respectively. The inhibition rate of hispidulin in MCF-7 and HCC38 breast cell lines increased with increasing concentration. Next, we determined the TGF-β1-induced inhibitory effect of hispidulin on cell viability. As shown in Fig. 1B, MCF-7 and HCC38 cells showed a significant increase in growth after treatment with only TGF-β1 compared to treatment with DMSO control for 24 h. However, treatment with both TGF-β1 and hispidulin caused a significant growth reduction compared to the TGF-β1-treated group in both cell lines.

**Hispidulin treatment increases the expression of epithelial markers in breast cancer cells.** We studied the effect of hispidulin on EMT markers in MCF-7 and HCC38 cells. Interestingly, 2.5 µM of hispidulin significantly increased the mRNA expression of epithelial markers such as E-cadherin and occludin in MCF-7 and HCC38 cells (Fig. 2A). Moreover, hispidulin decreased the expression of vimentin mRNA in HCC38 cells. As shown in Fig. 2B, hispidulin increased the protein expression of E-cadherin and occludin in MCF-7 cells, and similar results were obtained in HCC38 cells. Vimentin protein expression was significantly decreased after hispidulin treatment in HCC38 cells.
Hispidulin suppresses EMT through TGF-β1 in breast cancer cells. TGF-β1 plays a key role in maintaining the balance between cell differentiation and regeneration in normal epithelial cells (24). However, TGF-β1 promotes EMT, invasion, and immunosuppression in advanced cancers (25). We investigated the effect of hispidulin treatment on TGF-β1-induced EMT in breast cancer cells. As shown in Fig. 3, both breast cancer cells treated with both TGF-β1 and hispidulin had increased protein expression of E-cadherin compared to the cells treated with TGF-β1 alone. After TGF-β1 and hispidulin co-treatment, the expression of occludin increased compared to TGF-β1 treatment alone in HCC38 cells. Occludin protein expression was assessed in MCF-7 cells, but no significant changes were found among the treatment groups (Fig. 3). TGF-β1 and hispidulin co-treatment decreased vimentin protein expression in HCC38 cells. To confirm these results, immunofluorescence was performed. As shown in Fig. 4, TGF-β1 and hispidulin co-treatment increased the expression of E-cadherin compared to TGF-β1 treatment alone in both MCF-7 and HCC38 cells. These data suggest that hispidulin inhibits TGF-β1-induced EMT.

Hispidulin downregulates TGF-β1-induced Smad signaling. TGF-β1 induces EMT via Smad signaling pathways (26,27). TGF-β signaling is initiated upon the interaction of TGF-β1 with transmembrane kinase receptors, causing the activation of downstream signaling pathways involving Smad2 and/or 3 (Smad2/3) and Smad4. Our data showed that phospho-Smad2/3 protein expression was significantly increased after TGF-β1 treatment compared to the DMSO (control) treatment, but the protein expression of total Smad2/3 did not change in either cancer cell line (Fig. 5). However, TGF-β1 and hispidulin co-treatment significantly inhibited the phosphorylation of Smad2/3 protein in breast cancer cells.
Hispidulin inhibits TGF-β1-induced cell migration in breast cancer cells. To assess whether hispidulin influences cell migration, we performed migration assays in breast cancer cells. The wound area in cells treated with TGF-β1 was significantly reduced compared to that in cells treated with DMSO (control) (Fig. 6A). After TGF-β1 and hispidulin co-treatment, the wound area was significantly increased compared to that in the TGF-β1 treatment group. In addition, the percentage of cell migration in cells treated with TGF-β1 and hispidulin was significantly suppressed compared to the TGF-β1 treatment according to the Transwell assay in both breast cancer cell lines (Fig. 6B).

Discussion

Recent studies have shown that bioactive compounds in natural products have anticancer activities, and these substances can be effective in preventing and treating cancer (28). The natural compound hispidulin is a phenolic flavonoid found in Lamiaceae plants and has shown various effects against cancers (29-33). However, studies on the effect of hispidulin on EMT in breast cancer are limited. In this study, we studied the effect of hispidulin on EMT in breast cancer cells. In this study, we studied the effect of hispidulin on two breast cancer cell lines: MCF-7 of the luminal A type [estrogen receptor (ER)+, progesterone receptor (PR)+, human epidermal growth factor receptor 2 (HER2)+], and HCC38 of the claudin-low type (ER-, PR-, HER2-) (34). Our results, for the first time, demonstrated that hispidulin inhibits EMT and cell migration through E-cadherin-mediated reorganization after TGF-β1 treatment in breast cancer cells.

EMT is an essential process in the early stages of embryonic development, but uncontrolled EMT is associated with tumorigenesis (35). During EMT, cancer cells lose the properties of epithelial cells and acquire the properties of mesenchymal cells, allowing them to migrate to other organs through blood vessels or lymph nodes, causing metastasis and recurrence (36). Most cancer-related deaths are due to metastasis, which includes cell discharge from the primary tumor to the circulatory system, survival in the circulatory system, adaptation in new organs, initiation and maintenance of growth, and angiogenesis of metastasized tumors (7,9,37). Low expression of E-cadherin plays an important role in cancer metastasis and is associated with a reduced survival rate in breast cancer patients (38,39). Occludin is an integral membrane protein located in tight junctions that physically creates a barrier between cells (40). Loss of occludin affects bone metastasis in cancer patients (41). It has been shown that vimentin, a type III intermediate fibrous protein, is extensively expressed in triple-negative breast cancer subtypes and is correlated with tumor invasiveness and resistance to chemotherapy (42,43). In our study, hispidulin treatment increased the expression of E-cadherin and occludin and downregulated vimentin, with or without TGF-β1 treatment (Fig. 5). These results showed that hispidulin modulated EMT in breast cancers.

TGF-β1 acts as a tumor suppressor in normal epithelial cells, but promotes tumorigenesis, metastasis, cancer stem cell formation, and immune suppression in cancer cells (44). High levels of TGF-β1 are found in triple-negative breast carcinomas and are associated with metastasis and tumor progression (24,45,46). TGF-β1 also stimulates metastatic progression, leading to the development of chemoresistance in breast cancer stem cells (47). Moreover, TGF-β1 can induce EMT and increase cell motility (48). Our study revealed that TGF-β1 decreased the expression of E-cadherin and occludin proteins and increased the expression of vimentin in breast cancer cells (Fig. 5). However, hispidulin reversed the TGF-β1-induced EMT phenomenon. Therefore, hispidulin may be effective in inhibiting TGF-β1-induced EMT in breast cancer cells.
TGF-β1 induces EMT via the activation of Smad-dependent and Smad-independent signaling (46). TGF-β1 binds to TGF-β1 type II receptors (TβRII) and TGF-β1 type I receptors (TβRI) at serine and threonine residues. Smad2/3 is then phosphorylated and activates the expression of EMT-related transcription factors (49-52). Our data demonstrates that hispidulin inhibits the expression of phosphorylated-Smad2/3 induced by TGF-β1 in MCF-7 and HCC38 cell lines (Fig. 5). Some clinical studies have shown that TGF-β1-targeting anticancer compounds have therapeutic effects in breast cancer patients (53). Thus, we suggest that hispidulin may be a chemo-therapeutic via targeting TGF-β1 signaling in breast cancers. However, further experiments are needed to investigate non-Smad signaling in breast cancers.

Loosely connected mesenchymal cells developed through EMT can migrate and invade other tissues (54). The present study demonstrated that the motility of cells increases after TGF-β1 treatment; however, TGF-β1 and hispidulin co-treatment decreases cell migration in MCF-7 and HCC38 cells, as assessed through wound healing and Transwell migration assays (Fig. 5). EMT stimulates tumor cells to acquire stem

Figure 6. Hispidulin inhibited TGF-β1-induced breast cancer cell migration. (A) Wound healing assay in MCF-7 and HCC38 cells. Confluent monolayers of MCF-7 and HCC38 cells were treated with hispidulin and TGF-β1 for 0 and 24 h. Quantification of the scratched area was performed using ImageJ software. (B) Transwell assay in MCF-7 and HCC38 cells. Cells treated with hispidulin (1.25 or 2.5 µM) and TGF-β1 (10 ng/ml) were seeded into the Transwell upper chamber. The lower chamber was filled with 5% FBS-containing media. Following a 24 h incubation, migrated cells were stained with hematoxylin and eosin. Data are expressed as the mean ± SD of at least three independent experiments. Magnification, x10. *P<0.05 vs. DMSO-control group; #P<0.05 vs. TGF-β1-treated group. TGF-β1, transforming growth factor-β1.
cell-like properties and increases their resistance to standard chemotherapeutic drugs as well as conventional chemotherapy and radiotherapy (55,56). Our study demonstrated that the inhibitory effects of hispidulin on EMT can be modulated against both MCF-7 (moderately invasive) and HCC38 (highly invasive) breast cancers. Most studies using hispidulin have focused on anti-cancer effects, including apoptosis signaling including mitochondrial ROS, cell cycle mediated apoptosis using various cancer cells (57). However, the role of hispidulin in TGF-β1-induced EMT in human breast cells has not been elucidated. The concentration of hispidulin used in other cancer studies was over 10 μM (58). Our study showed that hispidulin was effective at concentrations below 5 μM for EMT inhibition. Therefore, this is the first study to show that the suppression of EMT using hispidulin is a strategy for preventing and treating breast cancers. Furthermore, several studies have shown that hispidulin, in various drug combinations, has synergistic effects against cancers (59). Hispidulin enhances the anticancer effect of chemotherapeutic drugs including gemcitabine, 5-fluorouracil, mitoxantrone, sunitinib, temozolomide, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (17,30,59-62).

However, there is a need for more research on the synergistic effects of hispidulin and breast cancer drugs. We found that hispidulin is rapidly absorbed in the stomach and intestines, with an absolute bioavailability of 4.02% after oral administration (63). Therefore, hispidulin requires additional strategies to enhance its efficacy for practical clinical use in chemoprevention and chemotherapy.

In conclusion, we show that hispidulin can block EMT and that this effect may be associated with a decrease in TGF-β1-induced signaling. Additionally, hispidulin inhibited breast cancer cell migration after TGF-β1 treatment. Thus, hispidulin may represent a novel anticancer agent for the treatment of early and late stage breast cancers.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL conceived and designed the study. HAK performed the experiments. JL and HAK analyzed the data and wrote the manuscript. JL and HAK confirm the authenticity of all the raw data. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the research.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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