Evaluation of the antimetastatic and anticancer activities of morin in HER2-overexpressing breast cancer SK-BR-3 cells

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Abstract. Morin (2',3,4',5,7-pentahydroxyflavone), a flavonoid isolated from members of the Moraceae family and the leaves of *Cudranaia tricuspidata* Buread, is a well-known natural substance with anti-inflammatory, antioxidative, antimetastasis, and anticancer effects. However, its anticancer activity has not been comprehensively investigated in human epidermal growth factor receptor 2 (HER2)-overexpressing breast cancer cells. Here, we evaluated the effects of morin on metastasis and cell viability in HER-2-overexpressing human breast cancer SK-BR-3 cells. Our results revealed that morin (150-200 μM) prevented endothelial growth factor (EGF)-induced metastatic potential and suppressed cell migration and MMP-9 activity by inhibiting the EGFR signaling pathway in SK-BR-3 cells by gelatin zymography, wound healing assay and western blotting. Interestingly, morin-induced reductions in cell viability were found to be associated with inhibition of the HER2/EGFR signaling pathway by sulforhodamine B assay and western blotting. Morin also induced the phosphorylation of H2A.X and downregulated the expression levels of RAD51 and survivin, which implied morin-induced DNA damage and that this damage accumulated in HER-2-overexpressing SK-BR-3 cells. Western blot analysis and fluorescent immunocytochemisty showed that morin also activated autophagy after 24 h of treatment and this was maintained at 48 h when activation of apoptosis via PARP cleavage resulted in the activation of caspase-3 and -7, which was associated with the release of cytochrome *c* to the cytosol from mitochondria. In addition, the phosphorylation of p38 and JNK was enhanced in the HER-2-overexpressing SK-BR-3 cells by morin after 24 and 48 h of treatment, which suggested p38 and JNK participate in morin-induced cell death. Taken together, the present investigation indicates that morin is a powerful therapeutic candidate for the treatment of HER2-overexpressing breast cancer because it suppresses the EGFR signaling pathway, induces cell death by inhibiting the HER2/EGFR signaling pathway, and suppresses metastatic potential.

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Introduction

Breast cancer is the second leading cause of cancer-related mortality in women (1). The disease may be characterized as estrogen receptor (ER) and progesterone receptor (PR)-positive, human epidermal growth factor receptor 2 (HER2)-positive, or triple-negative (ER-, PR- and HER2-negative). HER2-positive breast cancer is diagnosed in 15-20% of breast cancer patients and is more aggressive than hormone receptor (HR)-positive breast cancer (2). Although the prognosis of HER2-positive breast cancer patients has dramatically improved by the development of HER2-targeted therapy (trastuzumab), there are still major issues that remain to be resolved such as adverse side effects, which include cardiotoxicity and drug resistance (3,4). Drug resistance remains a key obstacle to the treatment of relapsed breast cancer (5,6). Furthermore, many HER2-positive breast cancer patients fail to respond to trastuzumab (7), and thus, a novel therapeutic strategy is needed to address adverse side effects and the development of drug resistance in HER2-positive breast cancer.

Metastasis is another important determining factor of prognosis and recurrence in cancer. Although the 5-year survival rate in breast cancer patients has been increased by therapeutic advancements, the 5-year survival rate of patients diagnosed with distant metastasis is appreciably lower than that of non-metastatic breast cancer patients (8).

Combinatorial treatments with trastuzumab and other drugs, such as doxorubicin and paclitaxel, have been used to reduce recurrence risk and improve prognosis in HER2-positive breast cancer (9,10). However, adverse side effects and metastasis rates remain unacceptably high. Furthermore, several investigators have reported that tumor relapse and metastasis should be promoted by chemotherapy (11). Therefore, efforts are needed to identify novel drug candidates with anticancer and antimetastatic activities.

Morin (2',3,4',5,7-pentahydroxyflavone) is a flavonoid isolated from almonds, bilberries, members of the Moraceae family, and the leaves of *Cudranaia tricuspidata*

Buread. Several investigators have reported that it has hepatoprotective, antihypertensive, anti-inflammatory, and antiangiogenic activities (12-14). Furthermore, we showed previously that morin exhibits antimetastatic activity in 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated MCF-7 breast cancer cells (15), and Jin *et al* reported that morin inhibited the invasion and growth of metastatic human breast cancer MDA-MB-231 cells (16). However, the therapeutic effect of morin in HER2-positive breast cancer treatment has not been evaluated. Therefore, we assessed the effects of morin on endothelial growth factor (EGF)-induced metastatic potential and cell death in HER2-overexpressing human breast cancer SK-BR-3 cells to confirm whether it should be used in the treatment of all types of breast cancer.

Materials and methods

Materials. Morin and sulforhodamine B (SRB) were obtained from Sigma-Aldrich; Merck KGaA, and trichloroacetic acid (TCA) was obtained from Samchun Pure Chemical Co., Ltd. Dulbecco's modified Eagle's medium (DMEM), antibiotic/antimycotic solution and trypsin were purchased from Welgene. Fetal bovine serum (FBS) was obtained from Cytiva (HyClone), and 30% polyacrylamide solution, protease inhibitor cocktail, and phosphatase inhibitor cocktail were from GenDEPOT. Epidermal growth factor (EGF) was purchased from R&D Systems. Primary antibodies for Akt (cat. no. 4691), phospho (p)-Akt (cat. no. 4060), EGFR (cat. no. 4267), p-EGFR (cat. no. 3777), extracellular signal-regulated kinase (ERK1/2; cat. no. 4695), p-ERK1/2 (cat. no. 4370), HER2 (cat. no. 2248), p-HER2 (cat. no. 2247), c-Jun N-terminal kinase (JNK; cat. no. 9258), p-JNK (cat. no. 4668), p38 mitogen-activated protein kinase (MAPK; cat. no. 8690), p-p38 MAPK (cat. no. 4511), microtubule-associated protein 1A/1B-light chain 3 (LC-3; cat. no. 4108), mammalian target of rapamycin (mTOR; cat. no. 2972), p-mTOR (cat. no. 2971), NF-κB (cat. no. 8242), p-NF-κB (cat. no. 3033), c-Jun (cat. no. 9165), p-H2A.X (cat. no. 9718), 5'adenosine monophosphate-activated protein kinase (AMPK; cat. no. 2532), p-AMPK (cat. no. 2535), ATG7 (cat. no. 8558), beclin-1 (cat. no. 3495), ribosomal protein S6 (cat. no. 2217), p-S6 (cat. no. 4858), survivin (cat. no. 2808), signal transducer and activator of transcription 3 (STAT3; cat. no. 4904), p-STAT3 (cat. no. 9145), p62 (cat. no. 7695), B-cell lymphoma-extra large (Bcl-xL; cat. no. 2764), Bcl-2 associated X protein (Bax; cat. no. 2772), caspase-3 (cat. no. 9662), caspase-7 (cat. no. 9492), poly(ADP-ribose) polymerase (PARP; cat. no. 9542), cytochrome *c* (cat. no. 11940), cytochrome *c* oxidase (COX) IV (cat. no. 4850), and GAPDH (cat. no. 5174) were purchased from Cell Signaling Technology. DNA repair protein RAD51 homolog 1 (RAD51; cat. no. sc-8349) and goat anti-mouse immunoglobulin G-horseradish peroxidase-conjugated antibody (cat. no. sc-2005) were from Santa Cruz Biotechnology, Inc. and goat anti-rabbit immunoglobulin G-horseradish peroxidase-conjugated antibody (cat. no. 31460) was from Thermo Fisher Scientific, Inc.

Cell culture. Human breast cancer SK-BR-3 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were routinely grown in DMEM supplemented with 10% FBS and 1% antimycotic/antibiotic solution at 37˚C in a 5% CO_2 atmosphere.

Analysis of the cytotoxicity of morin. The cytotoxicity of morin was assessed using an SRB assay. Briefly, $5x10^3$ HER2-overexpressing SK-BR-3 cells suspended in culture medium (DMEM supplemented with 10% FBS) were seeded into the wells of a 96-well plate and cultured for 24 h to facilitate attachment. Then, the culture medium was replaced with 5% FBS supplemented-DMEM containing morin (50 to 200 μM) and the cells were further cultured for 24, 48, or 72 h. Cells were then fixed with 20% TCA for 1 h, washed with tap water, and dried for 2 h. Then, cells were stained by 0.4% SRB solution at room temperature for 30 min. The stained cells were washed by 1% acetic acid and dried completely in room temperature. SRB was dissolved by 100 μl of 10 mM Tris-HCl (pH 5.0) buffer, and absorption at 510 nm was measured by SpectraMax M2e (Molecular Devices).

Cell migration assay. The cells ($1x10^6$/well) were plated into collagen-coated 6-well plates and grown for 24 h in 10% FBS-supplemented DMEM. Cell monolayers were scratched using a 1-ml micropipette tip, washed twice with phosphate-buffered saline, and further incubated in serum-free DMEM for 24 h. The serum-free DMEM was then removed and cell monolayers were incubated in 1% FBS-supplemented DMEM containing different concentrations (50-200 μM) of morin for 1 h and then treated with 20 ng/ml of EGF. Plates were then further kept for 48 h. Scratches were photographed at 0 and 48 h after EGF treatment using a Nikon light microscope (magnification, x40; Nikon Corp.).

Gelatin zymography. Matrix metalloproteinase-9 (MMP-9) activity was evaluated by gelatin zymography. The cells ($5x10^5$/well) were seeded into 6-well plates and allowed to attach for 24 h. Cells were serum-starved for 24 h and then treated with various concentrations (50-200 μM) of morin dissolved in serum-free DMEM for 24 h. The serum-free DMEM was then transferred to new 15-ml conical tubes, centrifuged to remove floating debris, and subjected to 8% non-reducing SDS-polyacrylamide gel (containing 0.1% (v/v) gelatin) electrophoresis (PAGE). The SDS in polyacrylamide gel was then removed by immersing in 0.25% Triton X-100 solution for 40 min and then kept in reaction buffer [5 mM $CaCl_2$, 0.04% NaN_3 and 50 mM Tris-HCl] overnight at 37˚C. The gel was then stained with Coomassie brilliant blue R solution for 1 h to visualize the bands, which were photographed using an LAS-4000 image analyzer (Fujifilm Corp.). Band densities were determined using Scion Image software (Alpha 4.0.3.2; Scion Corp.).

Western blotting. HER-2-overexpressing SK-BR-3 cells were seeded into 6-well plates at $5x10^5$ cells/well, allowed to attach for 24 h, and then serum-starved for 6 h. The cells were treated with various concentrations (50-200 μM) of morin dissolved in serum-free DMEM for 24 h, and then treated with 20 ng/ml of EGF for 30 min to investigate the effect of morin on the metastasis-regulating signaling pathway triggered by EGF. To study the effect of morin on cell survival, HER-2-overexpressing SK-BR-3 cells were treated with 50 to 200 μM of morin in

DMEM supplemented with 2% FBS for 24, 48, or 72 h. Cells were then lysed with RIPA lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 2 mM ethylenediaminetetraacetic acid] (Biosesang) containing protease inhibitor cocktail and phosphatase inhibitor cocktail, and lysates were centrifuged at 13,000 rpm for 10 min at 4˚C. Supernatants (whole cell lysates) were removed and stored at -80˚C until required. Total protein concentrations in whole cell lysates were calculated using the bicinchoninic acid (BCA) method using Pierce™ BCA Protein Assay Kits (Thermo Fischer Scientific, Inc.). Same amounts of total protein were subjected to 6, 8, 10 or 15% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were incubated in Tris-buffered saline-Tween (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; TBS-T) containing 1% BSA. Target proteins were probed overnight by the antibodies diluted at 1:3,000 in 5% non-fat dry milk or 1% BSA in TBS-T at 4˚C. Then, secondary antibody solutions diluted at 1:5,000 in TBS were covered and incubated onto the membrane for 1 h after washing three times for 5 min by TBST. Handmade chemiluminescent substrate [100 mM Tris (pH 8.5; BioShop, Canada, Inc.), 1.25 mM luminol, 198 μM coumaric acid and 0.01% hydrogen peroxide (all from Sigma-Aldrich; Merck KGaA)] was used for detecting the target protein bands and each band was photographed using Luminescent Image Analyzer LAS-4000 (Fujifilm Corp.). Band densities were determined using Scion Image software (Alpha 4.0.3.2).

Mitochondrial fractionation. HER-2-overexpressing SK-BR-3 cells ($1x10^6$) were plated into 100-mm dishes and allowed to attach for 24 h. Culture medium was then removed; cells were treated with morin at 50-200 μM for 48 h at 37˚C. After detaching cells with a scraper, they were collected by centrifugation at 800 x g for 5 min. Mitochondrial fractionation was performed using a Mitochondria/Cytosol Fractionation Kit (BioVision Inc.).

Fluorescent immunocytochemistry. The cellular expression levels of LC-3I/II and SQSTM1/p62 in SK-BR-3 cells were assessed by fluorescent immunocytochemistry to confirm whether morin had triggered autophagy. Initially, coverslips were sterilized with 70% ethanol in PBS and UVB radiated for 10 min, coated with collagen, and placed in 6-well plates. Cells were seeded onto coverslips, allowed to attach for 24 h, treated with morin at 0 or 200 μM for 24 or 48 h, serially fixed with ice-cold methanol and acetone for 4 min and 2 min, respectively, blocked in PBS containing 10% FBS for 2 h, and washed three times with PBS for 5 min. Cells were then treated with 1:200 diluted primary antibodies for LC-3I/II and SQSTM1/p62 in PBS, incubated overnight at 4˚C, and probed for 2 h in a dark with 1:200 diluted Alexa 488 (Green)-conjugated goat anti-rabbit antibody in PBS. DAPI containing antifade mounting solution was dropped onto the coverslips, and expression levels of LC-3I/II and SQSTM1/p62 in cells were photographed under a fluorescence microscope (magnification, x200; Carl Zeiss).

Statistical analysis. The data were statistically analyzed with one-way ANOVA test, followed by Turkey post-hoc test, using SPSS V20.0 software (IBM Corp.). The values are presented as the means ± SD. P-value of less than 0.05 was considered to indicate a significant difference.

Results

Effects of morin on cell viability and metastatic potential. Cytotoxicity of morin in HER-2-overexpressing SK-BR-3 cells was evaluated using the SRB assay. Results showed that SK-BR-3 cell viability was significantly and concentration-dependently reduced by morin after treatment for 48 and 72 h (Fig. 1A), whereas only a slight decrease in cell viability was observed at 24 h (Fig. 1A). In the wound healing assay, EGF-induced cell migration was effectively and concentration-dependently suppressed by morin (Fig. 1B). Furthermore, MMP-9 activity, which is an important factor in breast cancer metastasis, was significantly reduced by 150 and 200 μM of morin. We also evaluated the effect of morin on MMP-2 activity but the activity was not detectable (data not shown). These results suggest that morin suppressed EGF-induced metastatic potential in SK-BR-3 cells by inhibiting cell migration and MMP-9 activity.

Morin inhibits EGF-induced metastatic potential mediated by the EGFR signaling pathway. EGF-triggered metastatic potential is primarily governed by the EGFR signaling pathway. Therefore, the effect of morin on the EGF-triggered EGFR signaling pathway was investigated. As shown in Fig. 2, the EGF-induced phosphorylation of EGFR (p-EGFR) was significantly suppressed by morin at concentrations >150 μM. Furthermore, EGF-induced levels of p-STAT3 and p-JNK1/2 were concentration-dependently suppressed by morin, which also reduced c-Jun protein levels (Fig. 2). However, the significant phosphorylation of Akt was not observed. These results suggest that the antimetastatic effects of morin in EGF-treated SK-BR-3 cells were mediated by inhibition of the EGFR signaling pathway.

Morin reduces the protein expression levels of HER2 and EGFR. HER2 protein is targeted to treat HER2-overexpressing breast cancer and plays important roles in cell growth and proliferation (7), and EGFR is also used as a therapeutic target in several other cancer types (17). Therefore, we evaluated the effect of morin on the expression and phosphorylation of HER2 and EGFR. The result showed that morin (150-200 μM) decreased the protein expression and the phosphorylation of HER2 after treatment for 24 and 48 h and reduced the phosphorylation of EGFR at 24 and 48 h (Fig. 3). In addition, decreased EGFR expression by morin (100-200 μM) was also observed after treatment for 48 h. Furthermore, mTOR phosphorylation at 24 and 48 h and protein expression at 48 h were significantly reduced by morin (Fig. 3). This result shows that the observed morin-induced reduction in SK-BR-3 cell viability was associated with inhibition of the HER2/EGFR signaling pathway.

Morin-induced cell death is associated with the induction of DNA damage. Previous investigations have shown that the death of HER2-overexpressing breast cancer cells is induced by the downregulation of HER2, the induction of DNA damage, and the downregulation of DNA repair enzyme (18,19). Therefore, we investigated whether morin induces DNA damage in

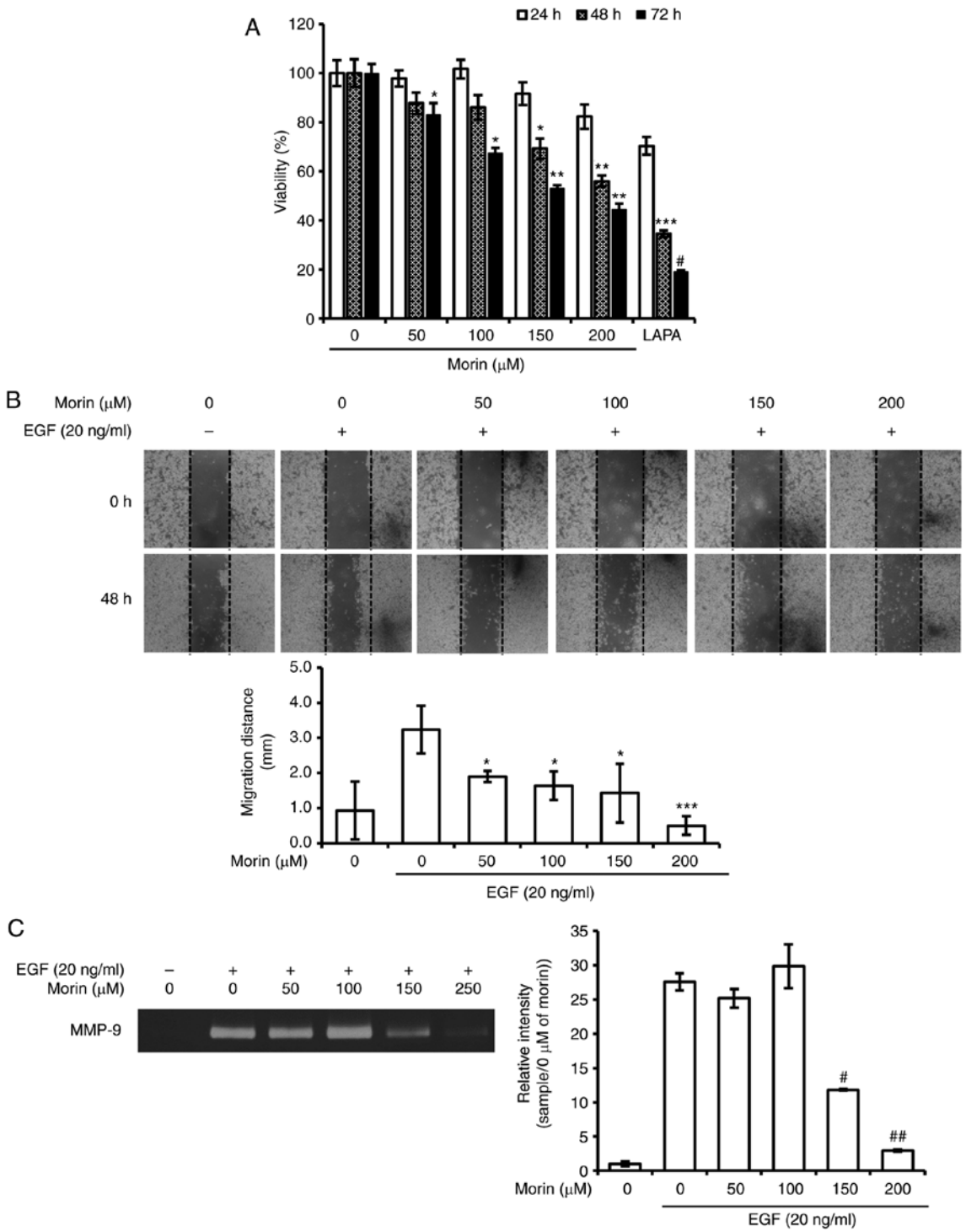

Figure 1. Effects of morin on the viability and metastatic potential of SK-BR-3 cells. (A) Cell viabilities were measured by using a sulforhodamine B based method. Cell viability assays were performed independently in triplicate. Data are presented as means ± SD; *P<0.05, **P<0.01, ***P<0.005 and $^{\#}$P<0.001 vs. 0 μM of morin. LAPA, lapatinib. (B) Scratch widths were observed after 0 and 48 h of morin treatment and photographed using a Nikon DS-U3 Digital Sight microscope camera. Magnification, x40. Migration distances were randomly measured at four different point in each sample. Data are presented as means ± SD; *P<0.05 and ***P<0.005 vs. 0 μM of morin with EGF, respectively. (C) Media were collected and subjected to 8% non-denaturing SDS-polyacrylamide gel (containing 0.1% gelatin) electrophoresis (PAGE). Gels were stained with Coomassie brilliant blue R and clear bands were observed and captured using an LAS-4000 image analyzer to determine MMP-9 activity. The experiments were performed independently in triplicate. Data are presented as means ± SD; $^{\#}$P<0.001 and $^{\#\#}$P<0.0001 vs. 0 μM of morin with EGF, respectively. EGF, epidermal growth factor; MMP-9, matrix metalloproteinase-9.

SK-BR-3 cells. As shown Fig. 4A, H2A.X phosphorylation, a marker for DNA damage, was concentration-dependently increased by morin (100-200 μM) following 24 or 48 h of treatment. Moreover, TUNEL-positive cells were observed in morin-treated SK-BR-3 cells (Fig. S1). In addition, levels of RAD51 and survivin proteins, which participate in DNA repair, were diminished by morin treatment (Fig. 4A). These results regarding the suppression of the protein expression levels of RAD51 and survivin imply that morin induced DNA damage accumulation.

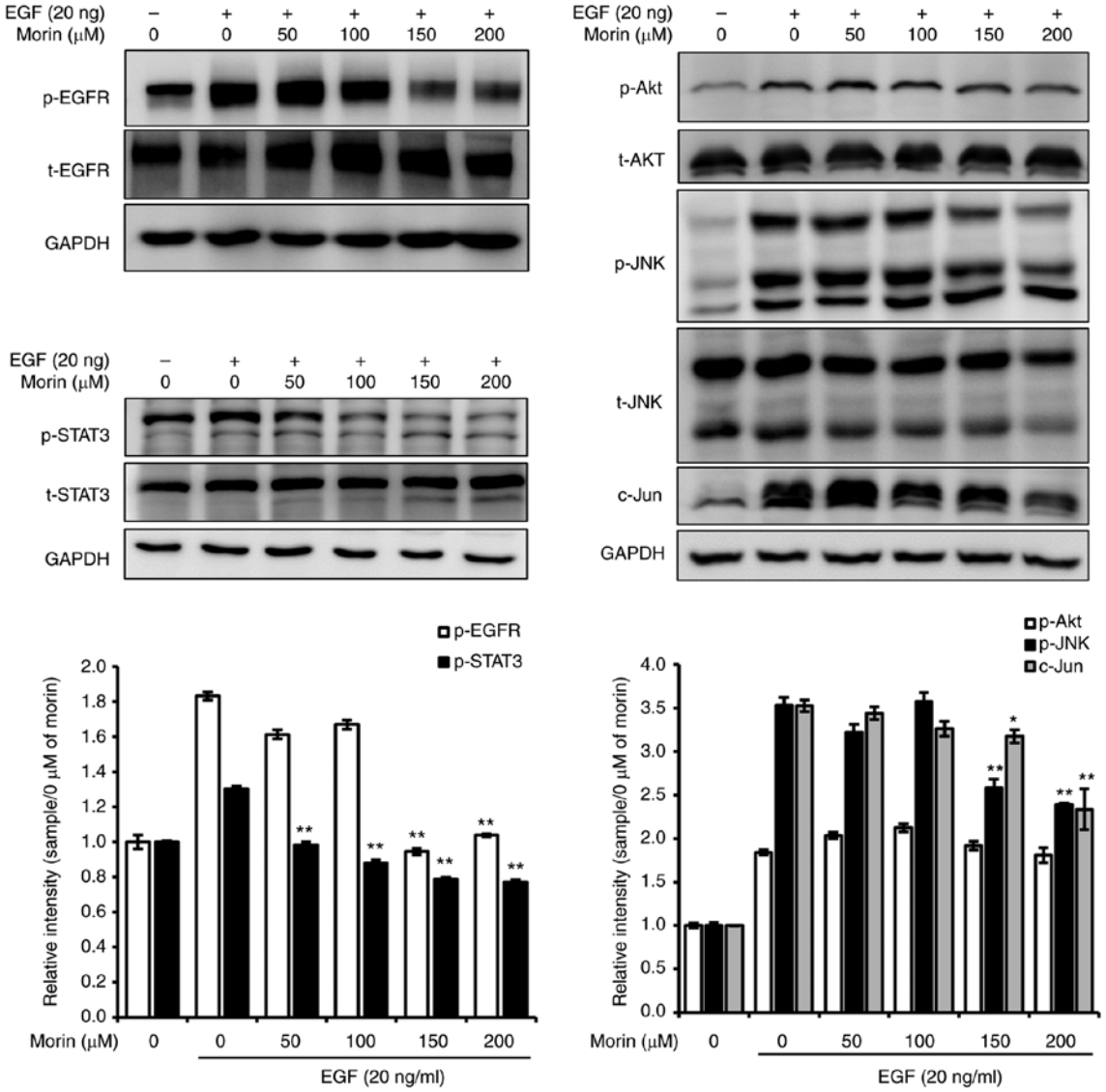

Figure 2. Effects of morin on the EGF-induced EGFR signaling pathway. Cells were seeded into 6-well plates and allowed to attach for 24 h, serum-starved for 24 h, treated with various concentrations of morin (0-200 μM) in serum-free media for 24 h, and then with EGF (20 ng) for 30 min. Cells were harvested using RIPA lysis buffer, centrifuged at 14,000 x g for 10 min, and supernatants (whole cell lysates) were collected and stored at -80˚C until required. Whole-cell lysates were subjected to 6, 8, or 10% SDS-PAGE. Bands were observed and captured using an LAS-4000 image analyzer. GAPDH was used as the internal control. The experiments were performed independently in triplicate. Band densities were normalized vs. GAPDH and data are presented as means ± SD; *P<0.05 and **P<0.01 vs. 0 μM of morin with EGF, respectively. EGFR, epidermal growth factor receptor; STAT3, signal transducer and activator of transcription 3; JNK, c-Jun N-terminal kinase; p-, phosphorylated; t-total.

Accumulated DNA damage should lead to apoptotic cell death mediated by mitochondrial dysfunction (20,21). Therefore, we tested the effect of morin on the expression levels of Bcl-2 family members (Bcl-xL, and Bax) and on mitochondrial cytochrome *c* release by mitochondrial fractionation assay. The mitochondrial fractionation assay showed cytosolic Bcl-xL levels were diminished by morin (100-200 μM) and that cytochrome *c* had been released to the cytosol after 48 h of treatment at 50-200 μM (Fig. 4B). However, mitochondrial Bcl-xL and cytochrome *c* levels were unaffected by morin treatment. These results demonstrated that morin-induced cell death was linked with DNA damage and mitochondrial dysfunction in the HER-2-overexpressing SK-BR-3 cells.

Morin-induced cell death is associated with caspase-mediated PARP cleavage. Mitochondrial dysfunction is closely associated with caspase-dependent apoptosis (22), and thus, we assessed the effect of morin on the cleavages of caspase and PARP. As shown in Fig. 5, although significant cleavages of caspase-3, caspase-7 and PARP were not observed at 24 h, morin treatment for 48 h provoked the cleavages of caspase-3 and -7 and PARP, which suggested that morin-induced cell death was caused by activation of the caspase-dependent apoptotic signaling pathway.

Autophagy is induced after 24 h of morin treatment. We also investigated whether the observed morin-induced reduction in cell viability is associated with autophagy. Morin treatment for 24 h was found to enhance the expression levels of LC3-II in a concentration-dependent manner at 24 h, and the increased expression level was maintained at 48 h in western blot analysis (Fig. 6A) and increased cellular LC3 and p62 levels

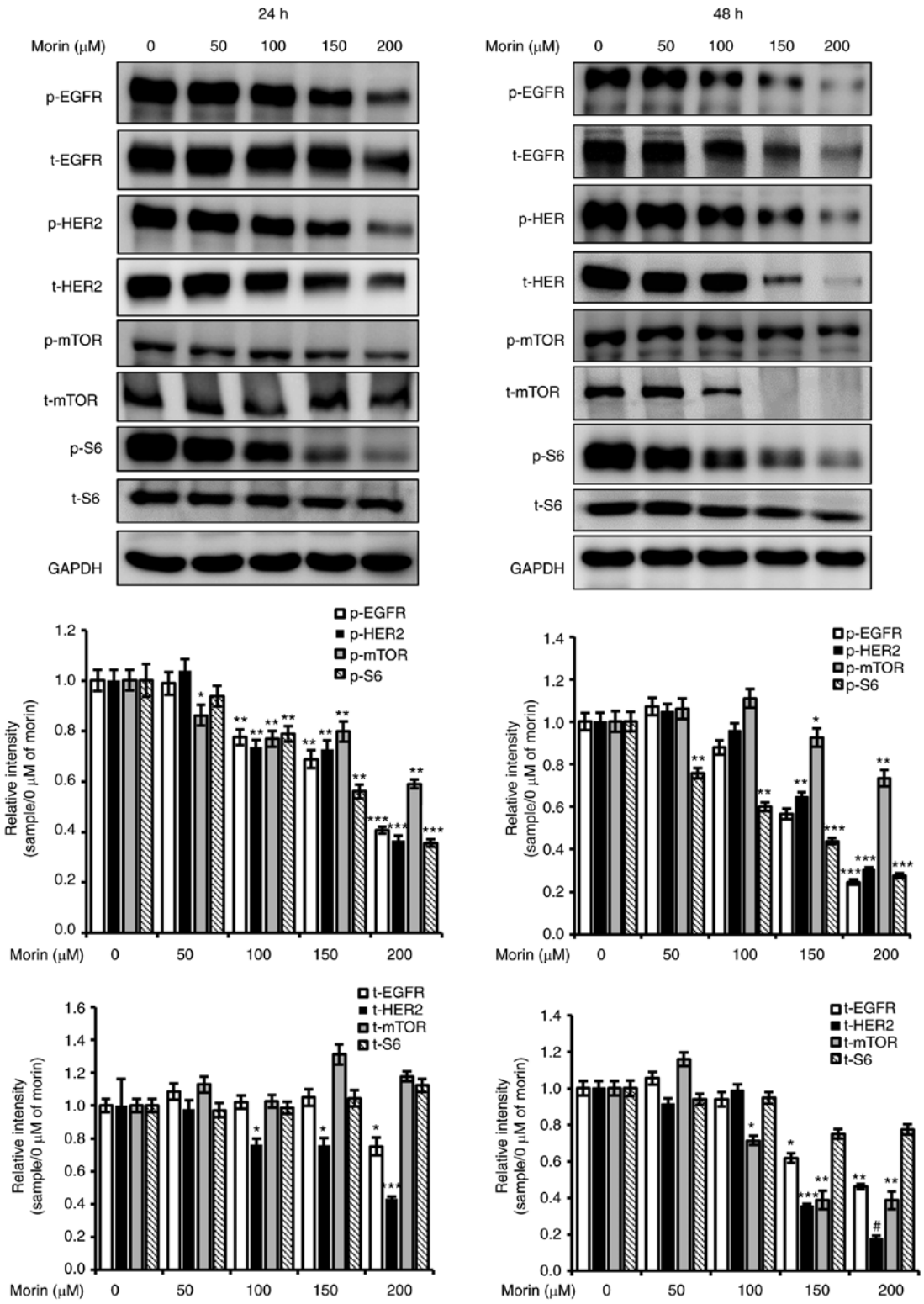

Figure 3. Effects of morin on the phosphorylation and expression of HER2 and EGFR. SK-BR-3 cells were seeded into 6-well plates and allowed to attach for 24 h. Cells were then treated with various concentrations of morin in media supplemented with 5% FBS for 24 or 48 h, harvested with RIPA lysis buffer, and centrifuged at 14,000 x g for 10 min. The supernatants (whole cell lysates) were collected and stored at -80˚C until required. Whole-cell lysates were subjected to 6 or 10% SDS-PAGE. Bands were observed and captured using an LAS-4000 image analyzer. GAPDH was used as the internal control. The experiments were performed independently in triplicate. Band densities were normalized vs. GAPDH and data are presented as means ± SD; *P<0.05, **P<0.01, ***P<0.005 and $^{\#}$P<0.001 vs. 0 μM of morin, respectively. EGFR, EGF, epidermal growth factor; mTOR, mammalian target of rapamycin; p-, phosphorylated; t-total.

at 24 and 48 h were observed by fluorescent immune cytochemistry (Fig. 6B). Furthermore, the decrease in cell viability by 200 μM of morin treatment for 48 h was recovered by autophagy inhibitor, chloroquine (Fig. S2). In addition, morin treatment for 24 increased AMPK phosphorylation (Fig. 7A) but not the expression of beclin-1 and ATG7 (Fig. 7A). These results imply that the observed morin-induced reduction in cell viability was associated with the activation of autophagy.

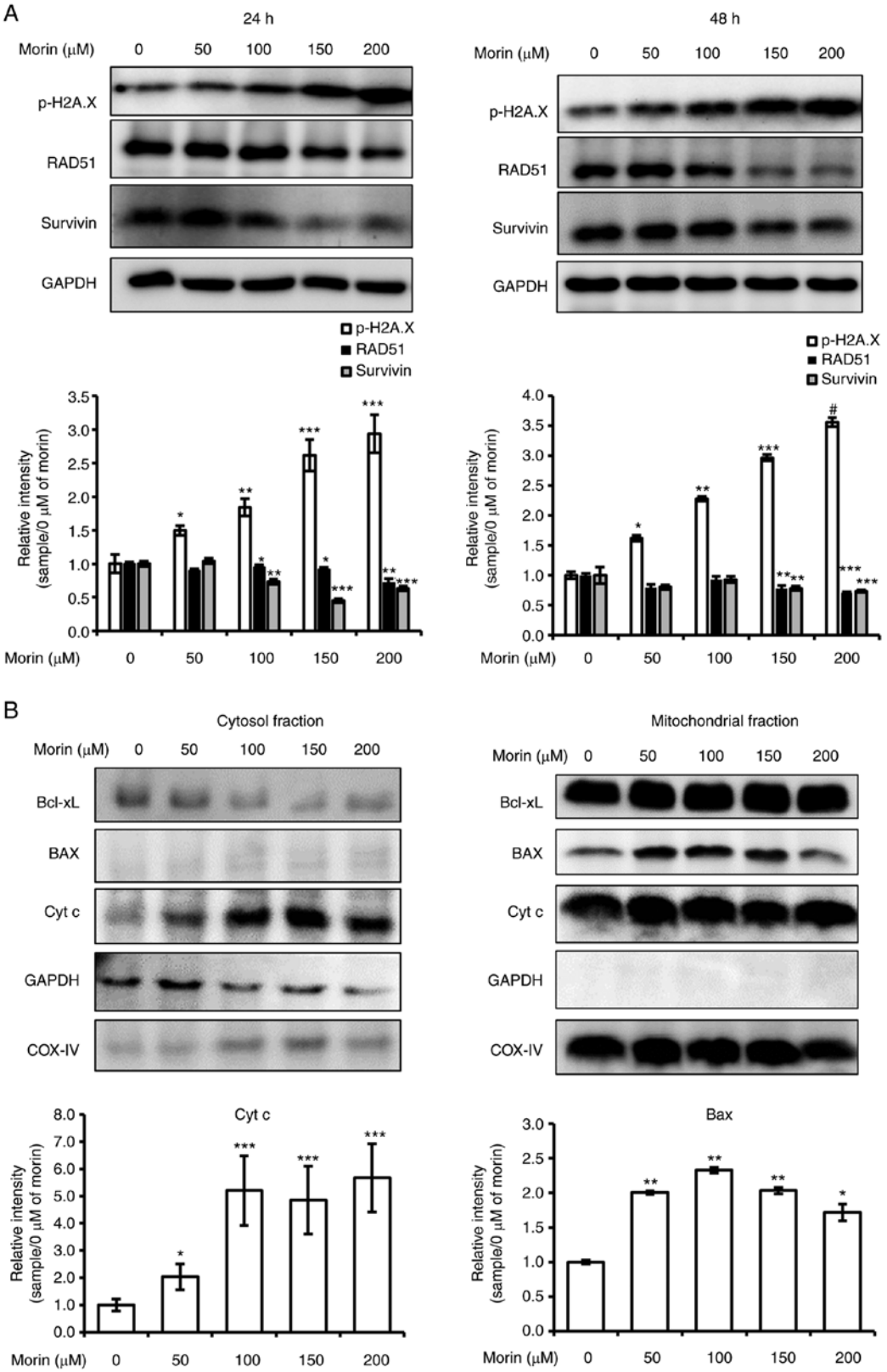

Figure 4. Effects of morin on DNA damage and mitochondrial Bcl-xL, BAX, and cytochrome *c* levels. (A and B) SK-BR-3 cells were seeded into 6-well plates, allowed to attach for 24 h, and treated with various concentrations of morin (0-200 μM) in media supplemented with 5% FBS for 24 or 48 h. (A) Cells were harvested with RIPA lysis buffer, centrifuged at 14,000 x g for 10 min, and supernatants (whole cell lysates) were collected and stored at -80˚C until required. Whole-cell lysates were subjected by 10 or 15% SDS-PAGE. Bands were observed and captured using an LAS-4000 image analyzer, and GAPDH was used as the internal control. The experiments were performed independently in triplicate. Band densities were normalized vs. GAPDH and data are presented as means ± SD; *P<0.05, **P<0.01, ***P<0.005 and $^{\#}$P<0.001 vs. 0 μM of morin, respectively. (B) Cells were harvested with a scraper and collected by centrifugation at 800 x g for 5 min and separated into mitochondrial and cytosolic fractions. All fractions were subjected to 10 or 15% SDS-PAGE. Bands were observed and captured using an LAS-4000 image analyzer. COX-IV and GAPDH were used as internal controls for mitochondrial and cytosolic fractions, respectively. The experiments were performed independently in triplicate. Band densities were normalized vs. COX-IV or GAPDH, and results are presented as means ± SD; *P<0.05, **P<0.01 and ***P<0.005 vs. 0 μM of morin, respectively. Bcl-xL, B-cell lymphoma-extra large; Bax, Bcl-2 associated X protein; Cyt c, cytochrome *c*; RAD51, DNA repair protein RAD51 homolog 1; p-, phosphorylated.

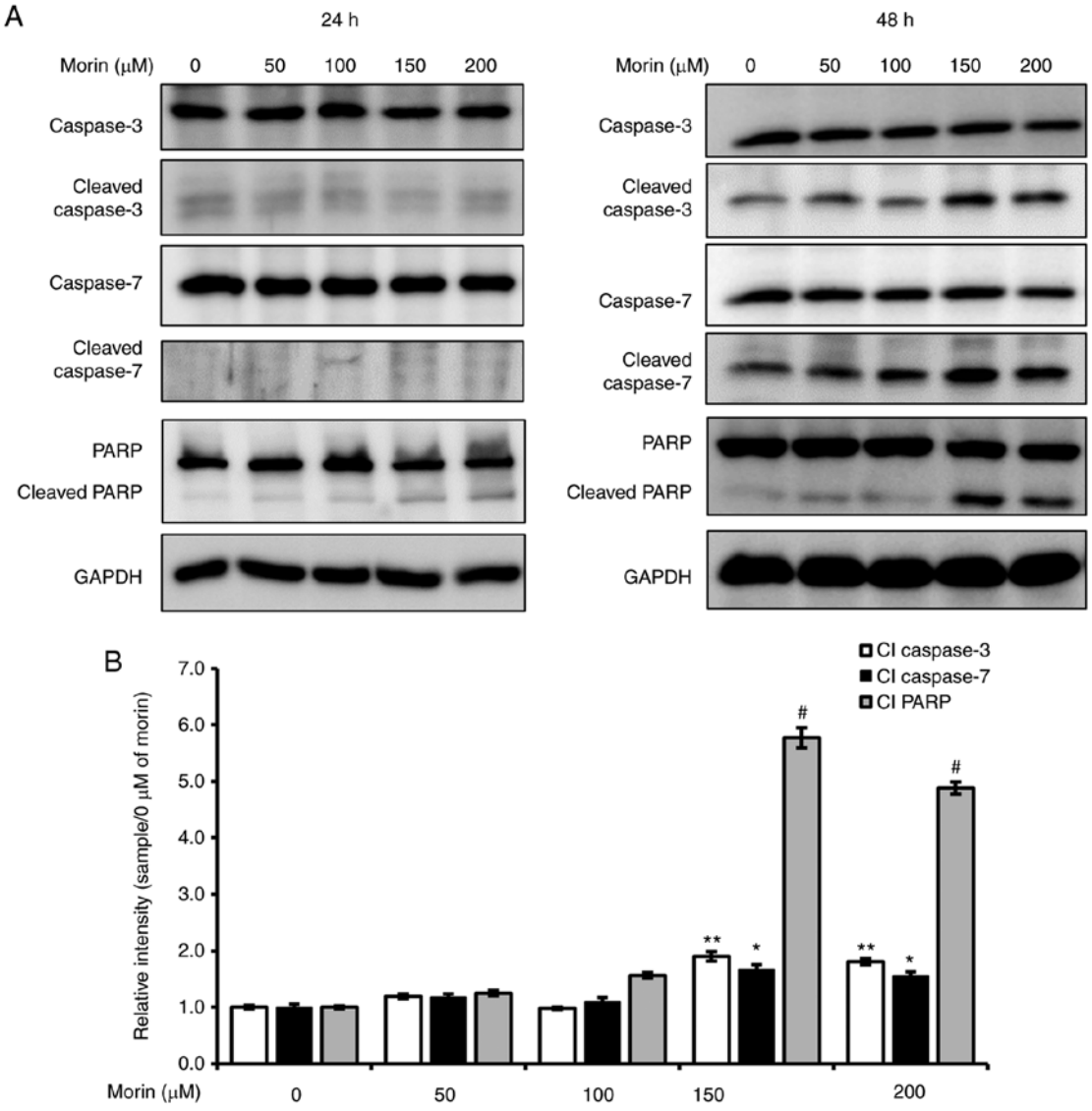

Figure 5. Effects of morin on caspases and PARP cleavage. SK-BR-3 cells were seeded into 6-well plates, allowed to attach for 24 h, and treated with various concentrations of morin (0-200 μM) in medium supplemented with 5% FBS for 24 or 48 h. Cells were harvested with RIPA lysis buffer and centrifuged at 14,000 x g for 10 min. Supernatants (whole cell lysates) were stored at -80˚C until required. Whole-cell lysates were subjected by 8 or 15% SDS-PAGE, and bands were observed and captured using an LAS-4000 image analyzer. GAPDH was used as the internal control. (B) Relative intensities of cleaved form level of caspase-3, caspase-7 and PARP vs./0 μM of morin at 48 h. The experiments were performed independently in triplicate. Band densities were normalized vs. GAPDH and data are presented as means ± SD; *, **and indicate *P<0.05, **P<0.01 and $^{\#}$P<0.001 vs. 0 μM of morin, respectively. PARP, poly(ADP-ribose) polymerase; Cl, cleaved form.

Morin-induced cell death is associated with increases in the phosphorylation of JNK and p38. MAPKs are key regulators of cell survival, proliferation, growth, and death (23,24). Therefore, we studied the effect of morin on the phosphorylation of MAPKs (ERK, JNK, and p38). The phosphorylation levels of JNK and p38 were concentration-dependently increased after 24 or 48 h of morin treatment but that of ERK were unchanged (Fig. 7B). This result suggests that increased phosphorylation levels of JNK and p38 are important in the cell death signaling pathway induced by morin.

Discussion

As mentioned above, metastasis is important in determining therapeutic efficacy and prognosis in cancer (8), and many investigators have tried to identify potent antimetastatic agents among natural products (25-27). In a previous study, we found that morin reduced 12-O-tetradecanoylphorbol-13-acetate-induced metastatic potential in HR-positive human breast cancer MCF-7 cells (15). The present investigation demonstrated that EGF-induced metastatic potential in epidermal growth factor receptor 2 (HER2)-overexpressing breast cancer SK-BR-3 cells was also diminished by morin treatment due to suppressions of cell migration and MMP-9 activity). These results demonstrated that natural substances provide a source of agents that can suppress metastasis, and showed that morin should be considered a potent antimetastatic agent in hormone receptor (HR)-positive and HER2-overexpressing breast cancer. The metastatic potential in HER2-overexpressing breast cancer was found to be regulated by the EGFR/STAT3 and MAPK signaling pathways (28-30). In the present study, morin was found to inhibit the EGF-induced phosphorylation of EGFR and STAT3 and downregulated EGF-induced

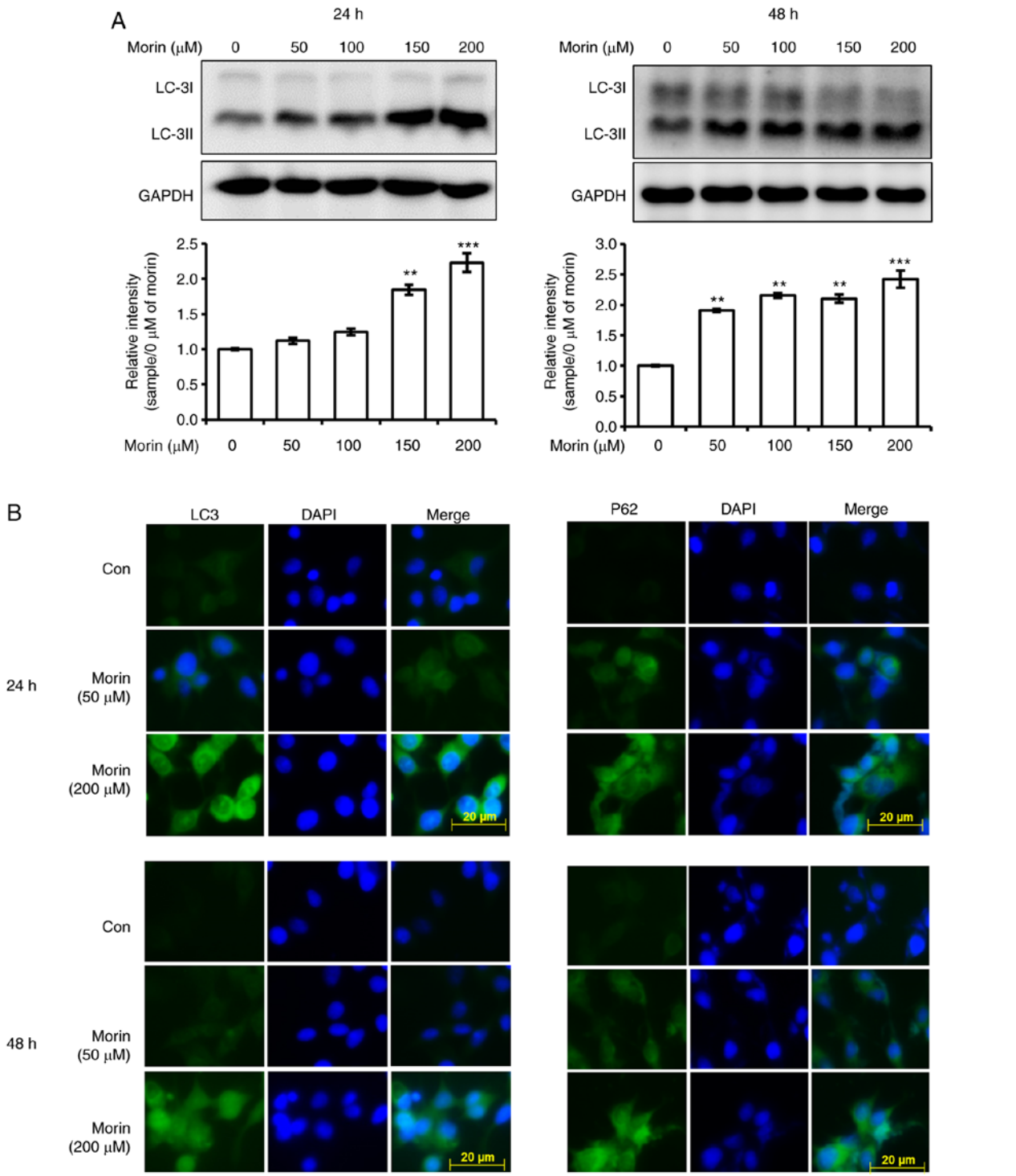

Figure 6. Effects of morin on autophagy. (A) SK-BR-3 cells were seeded into 6-well plates, allowed to attach for 24 h, and treated with various concentrations of morin (0-200 μM) in media supplemented with 5% FBS for 24 or 48 h. Cells were harvested using RIPA lysis buffer and centrifuged at 14,000 x g for 10 min. Supernatants (whole cell lysates) were collected and stored at -80˚C until required. Whole-cell lysates were subjected to 15% SDS-PAGE. Bands were observed and captured using an LAS-4000 image analyzer. GAPDH was used as the internal control. The experiments were performed independently in triplicate. Band densities were normalized vs. GAPDH and data are presented as means ± SD; **P<0.01 and ***P<0.005 vs. 0 μM of morin, respectively. (B) Cells were attached to collagen-coated coverslips for 24 h and treated with various concentrations (0, 50 and 200 μM) of morin in media supplemented with 5% FBS for 24 or 48 h. Cells were then fixed and antibody reactions were performed in the dark. Fluorescent-stained cells were observed and photographed under a fluorescence microscope. LC-3, microtubule-associated protein 1A/1B-light chain 3.

JNK phosphorylation and c-Jun expression. These results indicate that the induction of metastatic potential in SK-BR-3 cells by EGF is suppressed by morin via suppression of the EGFR/STAT3 and JNK/c-Jun signaling pathways. However, further investigations in cellular and animal models are necessary to clarify the antimetastatic potential of morin in breast cancer as metastasis is a complex process that cannot be explained simply by the migration assay and the analysis of MMP-9 activity and is regulated by various signaling pathways.

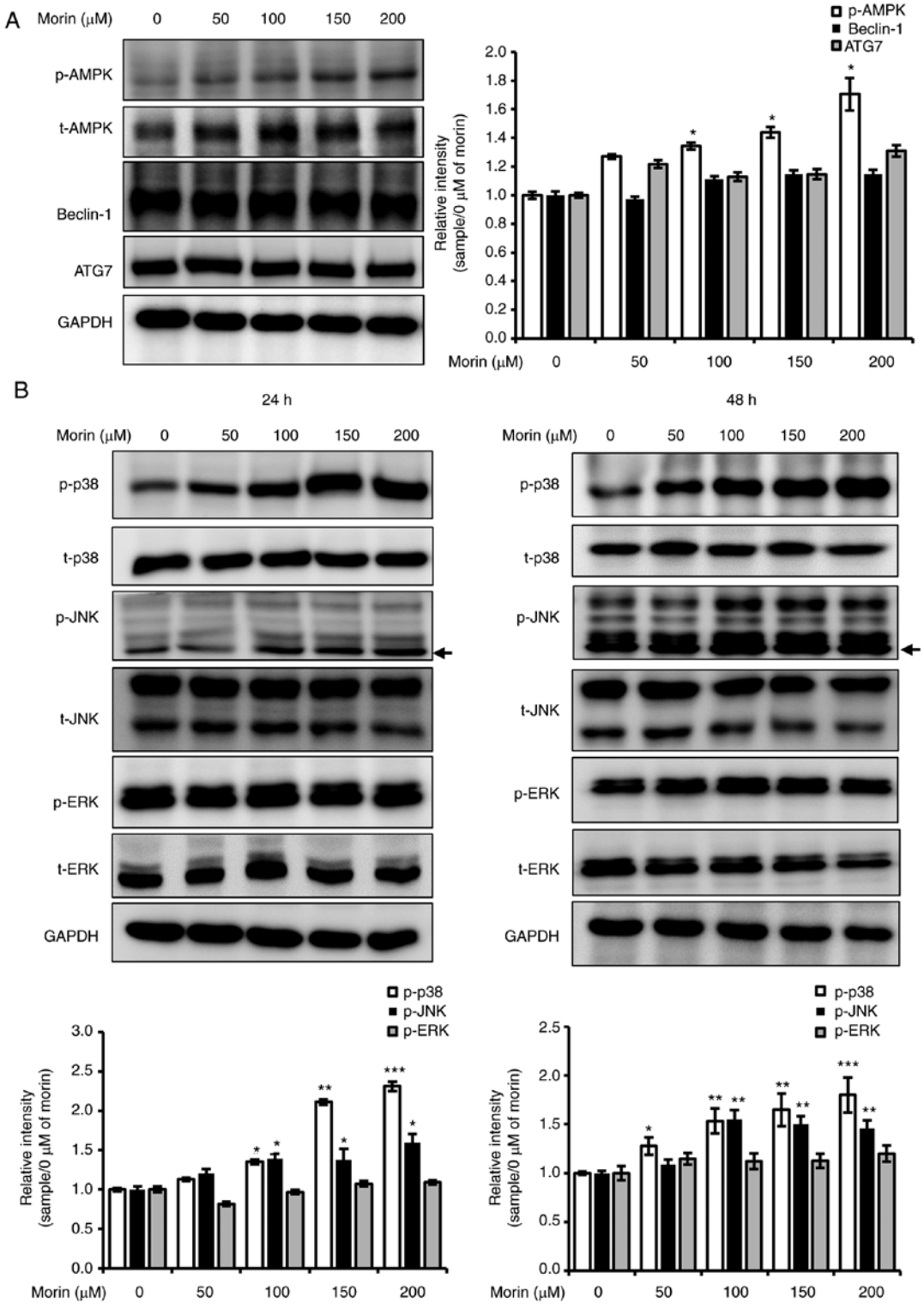

Figure 7. Effects of morin on the MAPK signaling pathway contributed to morin-induced cell death. (A) SK-BR-3 cells were seeded into 6-well plates, allowed to attach for 24 h, and treated with various concentrations of morin (0-200 μM) in media supplemented with 5% FBS for 24 h. (B) SK-BR-3 HER-2 cells were seeded into 6-well plates, allowed to attach for 24 h, and treated with various concentrations of morin (0-200 μM) in media supplemented with 5% FBS for 24 or 48 h. Arrows indicate non-specific band. (A and B) Cells were harvested with RIPA lysis buffer and centrifuged at 13,000 rpm for 10 min. Supernatants (whole cell lysates) were collected and stored at -80˚C until required. Whole-cell lysates were subjected by 8 or 10% SDS-PAGE, and bands were observed and captured using an LAS-4000 image analyzer. GAPDH was used as the internal control. The experiments were performed independently in triplicate. Band densities were normalized vs. GAPDH and data are presented as means ± SD; *P<0.05, **P<0.01 and ***P<0.005 vs. 0 μM of morin, respectively. ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p-, phosphorylated; t-, total.

HER2 and EFGR are well-known treatment targets in cancers of the breast and lung and colorectal cancer (31-34). Trastuzumab provides effective treatment for HER2-overexpressing breast cancer patients, but experience in regards to the use of trastuzumab plus cytotoxic agents to treat metastatic HER2-overexpressing breast cancer have demonstrated that trastuzumab cannot

effectively control metastasis (35). For this reason, lapatinib, a small molecule HER2 and EGFR inhibitor, is also used to treat HER2-overexpressing metastatic breast cancer in combination with trastuzumab (36). In this investigation, we found that morin inhibited cell viability and EGF-induced metastatic potential and suppressed EGFR phosphorylation in SK-BR-3 cells. Furthermore, the expression levels and phosphorylation of HER2 and EGFR were significantly reduced after 48 h of morin treatment with concomitant reductions in the phosphorylation and expression of mTOR and S6. In addition, previous studies demonstrated that morin did exhibit cytotoxicity in cancer cells but not in normal epithelial cells (16,37). Therefore, these results show that the morin-induced reductions in metastatic potential and cell viability were mediated by inhibition of the HER2/EGFR signaling pathway without cytotoxicity in normal cells.

DNA damage is a major cause of cancer cell death whether caused by chemicals, reactive oxygen species, alkylating agents, or radiation exposure, and when DNA is damaged, the expression levels of DNA repair enzymes are increased appropriately. RAD51 is an important DNA repair protein and survivin participates in the activation of DNA repair (37-40). In fact, some authors have suggested that RAD51 and survivin should be considered selective targets for cancer therapy in glioma cells (38,39,41). Accordingly, the accumulation of DNA damage by downregulating repair enzymes appears to present a therapeutic strategy. In the present study, morin enhanced the phosphorylation of H2A.X (a DNA damage marker) and inhibited the expression of RAD51 and survivin in the SK-BR-3 cells. Furthermore, increased TUNEL-positive cells were observed in the morin-treated cells. Consequently, our results suggest morin-induced cell death is caused by an accumulation of DNA damage and inhibition of DNA repair.

DNA damage-linked cell death usually involves apoptosis, and DNA damage-mediated apoptosis is associated with mitochondrial disruption caused by cytochrome *c* release to the cytosol (42). Many investigators have shown that mitochondrial damage increases caspase activity in cancer cells (43,44), and the release of cytochrome *c* by mitochondria is known to be induced by the activation of caspases, which is a key player in apoptotic cell death (45,46). When SK-BR-3 HER-2 cells were treated with morin for 48 h, mitochondrial Bax levels were increased and cytochrome c was released to the cytosol. Furthermore, treatment with morin for 48 h induced PARP cleavage and activation of caspase-3 and -7. Our results indicate that morin-induced SK-BR-3 HER-2 cell death was mediated by caspase-dependent apoptosis.

Although the cell death mechanism initiated by morin at 48 h in the HER-2-overexpressing SK-BR-3 cells was elucidated, the effect of morin at 24 h remained unclear. The viabilities of SK-BR-3 cells were slightly reduced after treatment with morin at 150 or 200 μM, and this decrease was not associated with caspase activation, which suggested that apoptosis did not contribute to reduced cell viability at 24 h. Interestingly, LC-3II and p62 expression was enhanced by morin treatment for 24 h, and this enhancement was maintained at 48 h. These two proteins are key autophagy markers, and thus, our observations indicate that morin induced autophagy at 24 h that this was sustained at 48 h. Autophagy is a protective process associated with the formation of autophagolysosomes, which remove damaged cellular components, and autophagy has been reported to suppress apoptosis (47,48). On the other hand, some investigators have suggested that some natural substances might induce autophagy-mediated apoptosis in cancer cells (49-51). In the present investigation, morin enhanced autophagy at 24 h and this was maintained at 48 h when caspase-mediated apoptosis was active. Moreover, autophagy inhibitor, chloroquine, suppressed morin-induced cell death. Hence, our results suggest that morin-induced cell death in SK-BR-3 cells is governed by autophagy-mediated caspase-dependent apoptosis.

Beclin 1 and ATG7 play important roles during autophagosome formation and in the inductions of LC-3II and p62. Although morin promoted the expression of LC-3II and p62, it did not affect the expression levels of beclin 1 or ATG7. Some investigators have reported that apoptosis can be triggered by beclin 1-independent autophagy (52,53). In a previous study, we found that DNA damage contributes to the activation of autophagy and increased LC3-II expression but not to beclin 1 or ATG7 expression in UV-exposed HaCaT human keratinocytes (54). In the present study, morin induced DNA damage and reduced RAD51 and survivin expressions. Consequently, our results imply that morin-induced DNA damage might cause beclin 1- and AtG7-independent autophagy by inducing the expression of LC-3 II and p62.

The AMPK and mTOR signaling pathways participate in the regulation of autophagy. Puissant *et al* demonstrated that resveratrol-mediated autophagic cell death was promoted by JNK-mediated p62 expression by the AMPK signaling pathway (55). We also previously found that autophagic cell death was associated with increased JNK phosphorylation in HaCaT human keratinocytes (54), and in the present study, morin increased the phosphorylated AMPK and JNK levels, decreased mTOR phosphorylation, and increased p38 MAPK phosphorylation. Although the role of p38 MAPK in autophagy has not been fully elucidated, some investigators have shown that activation of JNK and p38 MAPK contributes to autophagy and apoptosis in H9C2 and HepG2 cells (56,57). Therefore, the present investigation demonstrated that the induction of autophagy and apoptosis by morin is mediated by the activation of AMPK, JNK, and p38 MAPK signaling pathways and the suppression of the mTOR signaling pathway without the involvement of beclin-1 or ATG7.

In summary, our results revealed that morin inhibits EGF-induced metastatic potential by suppressing the EGFR signaling pathway and induces cell death via autophagy-mediated apoptosis and HER2/EGFR1 signaling pathway inhibition. Taken together, this investigation suggests that morin offers a potential means of enhancing the efficacy of HER2-overexpressing breast cancer treatments by suppressing metastatic potential and inducing cancer cell death by targeting HER2 and EGFR. However, further investigation using animal models is needed to assess the pharmaceutical effects of morin on HER2-overexpressing breast cancer in a biological system.

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Availability of data and materials

All the original data generated for this manuscript are available upon request.

Authors' contributions

KSL ad KSN designed the experiments. KSL and MGL performed experiments. KSL, MGL and KSN analyzed the data. KSL wrote the manuscript. KSL and KSN reviewed the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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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