

Kaempferol, a natural dietary flavonoid, suppresses 17 β -estradiol-induced survivin expression and causes apoptotic cell death in endometrial cancer

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Abstract. Endometrioid endometrial carcinoma, commonly known as type 1 endometrial cancer, accounts for >80% of endometrial carcinomas and is dependent on estrogen. We recently reported on the prognostic significance of the *BIRC5* survivin gene in endometrial cancer. Estradiol induces survivin expression in estrogen receptor-positive, but not in estrogen receptor-negative, cancer cells. Kaempferol, a bioflavonoid, reportedly inhibits estrogen receptor- α (ER α) in hormone receptor-positive breast cancer cells. However, whether kaempferol-mediated inhibition of ER α suppresses survivin and induces cell death in endometrial cancer remains unclarified. The present study evaluated the antitumor effects of kaempferol on endometrial cancer cells. Cell viability

assays, flow cytometry analysis, western blotting and annexin V analyses were used to analyze the antitumor effects of kaempferol. The results demonstrated that kaempferol successfully suppressed the viability of two ER-positive endometrial cancer cell lines, with IC₅₀ values of 83 and 65 μ M. In addition, kaempferol induced sub-G1 cell accumulation and apoptotic cell death ($P < 0.01$) in a dose-dependent manner. Treatment of cells with estradiol significantly induced co-expression of nuclear ER α and survivin proteins ($P < 0.001$). Further evaluation revealed that kaempferol causes apoptotic cell death largely by suppressing ER α , survivin and Bcl-2 protein. Therefore, the results of the present study suggested that targeting ER α and survivin with kaempferol may be a novel therapeutic option against endometrial carcinoma.

Introduction

Endometrial carcinoma is the most frequently diagnosed gynecologic malignancy in industrialized countries. The incidence of endometrial carcinoma is ascribed to the rising prevalence of nulliparity and metabolic diseases such as diabetes mellitus type 2 and obesity (1). Aromatization, a process in which androstenedione is converted to estrone in peripheral fat, is thought to be the main contributor to high levels of estrogen measured in obese women and significantly increases the risk of developing endometrial cancer (2). Almost 80% of diagnosed endometrial carcinomas are endometrioid adenocarcinomas (also known as type I cancers), which are strongly dependent on the stimulatory effects of estrogen (3,4). A large proportion of these tumors are diagnosed in their early stages, and hysterectomies are the primary curative treatment modality (5). Although surgical management offers a favorable prognosis, advanced stage or recurrent disease responds poorly to standard therapies and thus has a poor prognosis (4). High-dose medroxyprogesterone acetate (MPA) has been approved for the treatment of type I endometrial carcinoma. However, up to 30% of patients with endometrial

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Abbreviations: Akt, protein kinase B; Bcl-2, B-cell lymphoma 2; CHIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; E2, 17 β -estradiol; EMEM, Eagle's minimum essential medium; ER α , estrogen receptor- α ; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IAP, inhibitor of apoptosis; IC₅₀, half maximal inhibitory concentration; *MDR1*, multidrug resistance gene-1; MPA, medroxyprogesterone acetate; NgBR, Nogo-B receptor; PARP, poly adenosine diphosphate-ribose polymerase; PGC, proliferator-activated receptor γ co-activator; PI, propidium iodide; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PTEN, phosphatase and tensin homolog; XIAP, X-chromosome linked inhibitor of apoptosis protein

Key words: kaempferol, 17 β -estradiol, estrogen receptor, survivin, endometrial cancer

hyperplasia and endometrioid carcinoma are resistant to progestin therapy (6).

Our group and others previously reported on the frequency of mutated genes within the phosphatidylinositol-4,5-bisphosphate 3-kinase/phosphatase and tensin homolog/protein kinase B (PI3K/PTEN/AKT) survival pathway (7,8). 17 β -Estradiol [E2; (17 β)-estra-1,3,5 (10)-triene-3,17-diol] induces development of the majority of endometrial carcinomas, reportedly by activating phosphoinositide-3-kinase catalytic alpha (PIK3CA) polypeptides, suppressing *PTEN*, and upregulating survivin through estrogen receptor- α (ER α) signaling (9). Survivin, the smallest member of the inhibitor of apoptosis (IAP) protein family, is upregulated almost exclusively in malignant cells and is barely detectable in adult terminally-differentiated tissues. However, survivin is highly expressed in most human malignant tumors, including colorectal, esophageal, pancreatic, gastric, and others (10-12). E2 selectively upregulates survivin in hormone receptor-positive, but not receptor-negative cancer cells (13). Higher expression levels of survivin have been detected in over 83% of clinical endometrial cancer samples (14). We recently reported that survivin was overexpressed in over 87% of sixteen endometrial carcinoma cell lines tested and demonstrated that high expression of the *BIRC5* survivin-encoding gene is an independent poor prognostic factor for endometrial carcinoma (15).

Kaempferol [3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one] is a dietary bioflavonoid with anticancer, anti-inflammatory, and anti-oxidant properties that suppresses cell proliferation in human cancers through various mechanisms, including induction of tumor suppressor p53 and inhibition of ER α (16). Further, kaempferol reportedly binds to ER α , preventing its interaction with coactivator peroxisome proliferator-activated receptor gamma coactivator (PGC)-1 alpha. Antitumor effects associated with kaempferol have been reported in various human cancers, including osteosarcoma, breast, and ovarian cancers (17-19). However, the effects of kaempferol on endometrial carcinoma cells and whether the inhibitory effects of kaempferol against ER α affect estradiol-induced survivin expression remain unclarified. Thus, we aimed to evaluate the antitumor effects of kaempferol on endometrial carcinoma cells, as well as its effect on survivin protein expression following suppression of ER α .

Materials and methods

Endometrial cancer cell lines. The estrogen receptor-positive Ishikawa cell line was kindly offered by Dr. Masato Nishida (Kasumigaura Medical Center, Ibaraki, Japan). The HEC-265 endometrial cancer cell line, also positive for estrogen receptors, and the HEC108 and HEC180 estrogen receptor-negative endometrial cancer cell lines were previously established by our co-author, Prof. Hiroyuki Kuramoto (20). The cell lines were maintained in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS) and antibiotics. Phenol red-free medium was used when estradiol was applied. Kaempferol was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and stored as a 10 mM stock solution at -20°C in the dark.

Cell viability assay. Cells were cultured in 96-well plates (2x10³ cells per well) in an appropriate medium for 24 h in a humidified incubator (37°C and 5% CO₂) to allow for attachment. The medium was then removed and fresh medium containing an increasing concentration of kaempferol was added, after which the cells were incubated for another 72 h. To perform a MTT assay, 10 μ l of Cell Count Kit-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well, and the cells were incubated for 3 h before being analyzed. At that point, a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) was used to measure the change in absorbance at 450 nm. Measurements of cells treated only with dimethyl sulfoxide (DMSO) were used for normalization. This experiment was performed at least three times.

Cell cycle analysis. Cells were cultured in 6-cm dishes (4x10⁵ cells per dish) for 24 h, after which the medium was replaced with fresh medium containing DMSO (control), 36 μ M kaempferol, or 72 μ M kaempferol and further incubated at 37°C and 5% CO₂ for 48 h. Cells were then collected and processed as previously described (21,22). Cell cycle progression was performed via fluorescence-activated cell sorting (FACS) with an Epics XL instrument (Beckman Coulter, Inc., Brea, CA, USA) and CellQuest Pro v.3.1 software (BD Biosciences, Franklin Lakes, NJ, USA). This experiment was performed at least three times.

Apoptosis evaluation. Cells were plated in 60-mm dishes (4x10⁵ cells per dish) and incubated for 24 h. The medium was then replaced with fresh medium containing DMSO (control), 36 μ M kaempferol, or 72 μ M kaempferol and incubated at 37°C and 5% CO₂ for another 48 h. Estradiol (10 nM final concentration) was added to the cells 3 h prior to harvesting. Cells were collected and processed as discussed elsewhere (23). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-positive cells are expressed as a percentage of apoptotic cells, as determined via flow cytometry. The experiment was repeated at least three times. Western blotting. Western blots (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used to analyze protein samples extracted from cells that were treated with DMSO (control), 36 μ M kaempferol, or 72 μ M kaempferol for 48 h and then 10 nM of E2 for 3 h. A ProteoExtract subcellular proteome extraction kit (Calbiochem; EMD Biosciences, Inc., Merck KGaA, Darmstadt, Germany) was used to differentially extract proteins according to their subcellular (i.e., membrane, cytosol, or nuclear) localization. Primary antibodies for the following compounds were used to probe the membranes: ER α (D-12), p53 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), survivin (71G4B7), B-cell lymphoma 2 (Bcl-2) protein, and cleaved poly adenosine diphosphate-ribose polymerase (PARP; Cell Signaling Technology, Inc., Danvers, MA, USA). The antibody-detecting housekeeping protein β -actin (Sigma-Aldrich; Merck KGaA) was used as a loading control. All antibodies were used in compliance with manufacturers' recommendations after protein bands were enhanced with ECL Select Solutions A and B (GE Healthcare Life Sciences, Piscataway, NJ, USA).

Statistical analysis. Statistical significance of differences between groups was evaluated with one-way analysis of

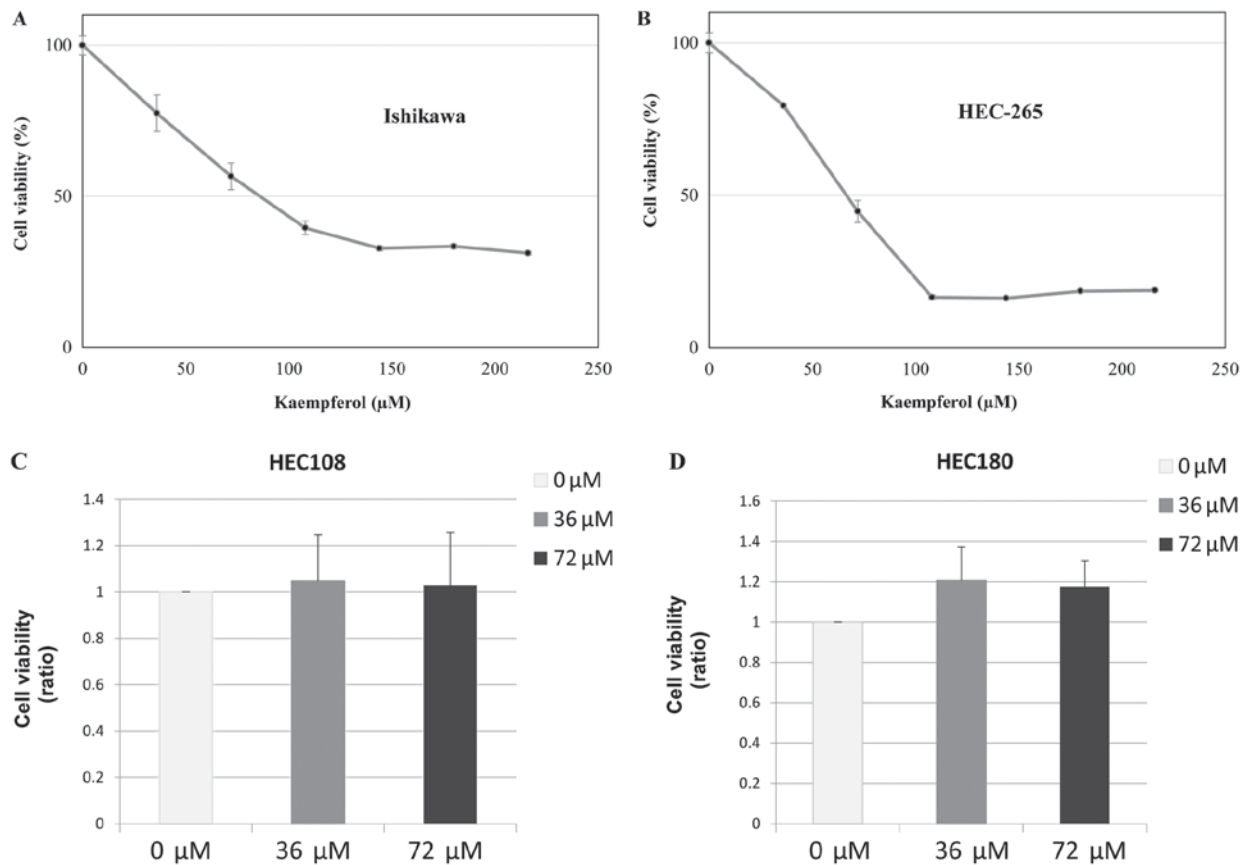


Figure 1. Kaempferol suppresses growth of Ishikawa and HEC-265 estrogen receptor-positive endometrial cancer cells. Growth inhibition in (A) Ishikawa and (B) HEC265 endometrial cancer cells, with an IC_{50} of 83 and 65 μM , respectively. Kaempferol did not suppress the growth of HEC108 or HEC180 estrogen receptor-negative endometrial cancer cells. Cell viability in (C) HEC108 and (D) HEC180 endometrial cancer. IC_{50} , half-maximal inhibitory concentration.

variance with Tukey's post hoc test using JMP Pro. v.12 (SAS Institute, Inc., Cary, NC, USA) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) software. ImageJ v.1.48 software (NIH, Bethesda, Maryland, USA) was used to quantify protein expression bands. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Kaempferol suppresses viability of Ishikawa and HEC-265 cells. Cell viability assays were performed to assess the growth inhibitory effects of kaempferol against Ishikawa and HEC-265 estrogen receptor-positive endometrial cancer cells. Kaempferol successfully suppressed the growth of Ishikawa and HEC-265 cells, with IC_{50} values of 83 and 65 μM , respectively (Fig. 1A and B). We further examined the anti-tumor effects of kaempferol in HEC108 and HEC180 estrogen receptor-negative endometrial cancer cells. Kaempferol did not suppress the growth of HEC108 and HEC180 cells (Fig. 1C and D).

Kaempferol induces sub-G1 and G2/M phases of the cell cycle. To understand the mechanisms by which kaempferol suppresses cell growth in endometrial carcinoma, cells treated with varying concentrations of kaempferol were incubated for 48 h and then evaluated via FACS for cell cycle progression. We found that kaempferol induced cell accumulation in the

sub-G1 and G2/M phases in Ishikawa and HEC-265 endometrial cancer cells, while significantly suppressing the G1 phase ($P < 0.01$; Fig. 2A and B).

Kaempferol induces apoptotic cell death in endometrial cancer by suppressing $ER\alpha$, survivin and Bcl-2. We evaluated the cytotoxicity of kaempferol against endometrial cancer cells and possible mechanisms by analyzing the induction of apoptosis using an annexin V-FITC/PI assay. We found that kaempferol significantly ($P < 0.01$) induced apoptotic cell death in Ishikawa and HEC-265 cells (Fig. 3A). Further analysis via western blot revealed that kaempferol induced apoptosis largely by suppressing $ER\alpha$ and the antiapoptotic proteins survivin and Bcl-2 and by inducing p53 and PARP cleavage (Fig. 3B).

E2 significantly induces nuclear co-expression of $ER\alpha$ and survivin in endometrial cancer cells. To investigate the site of action of E2 and its role in $ER\alpha$ and survivin activity in subcellular compartments, subcellular protein samples were extracted from Ishikawa and HEC-265 cells treated with E2 and/or kaempferol and analyzed via western blot. E2 significantly induced co-expression of nuclear $ER\alpha$ and survivin (Fig. 4A). This result suggests that E2 primarily induces nuclear $ER\alpha$ and survivin, consequently preventing apoptosis. Treatment of cells with kaempferol inhibited estradiol-induced upregulation of survivin and led to apoptotic cell death (Fig. 4B).

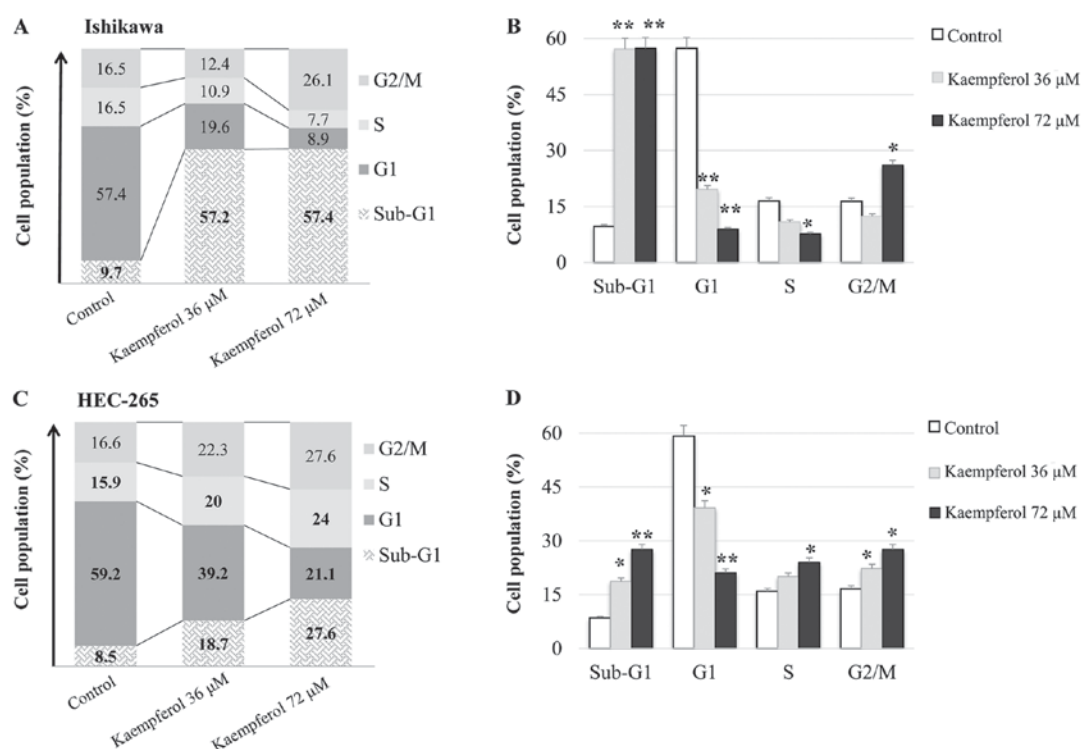


Figure 2. Effects of kaempferol on Ishikawa and HEC-265 cell cycle progression. Kaempferol induction of sub-G1 and G2/M phases as a measure of the cell population percentage in (A and B) Ishikawa and (C and D) HEC-265 endometrial cancer cells. * $P < 0.05$ and ** $P < 0.01$ vs. control. Kaemp, kaempferol.

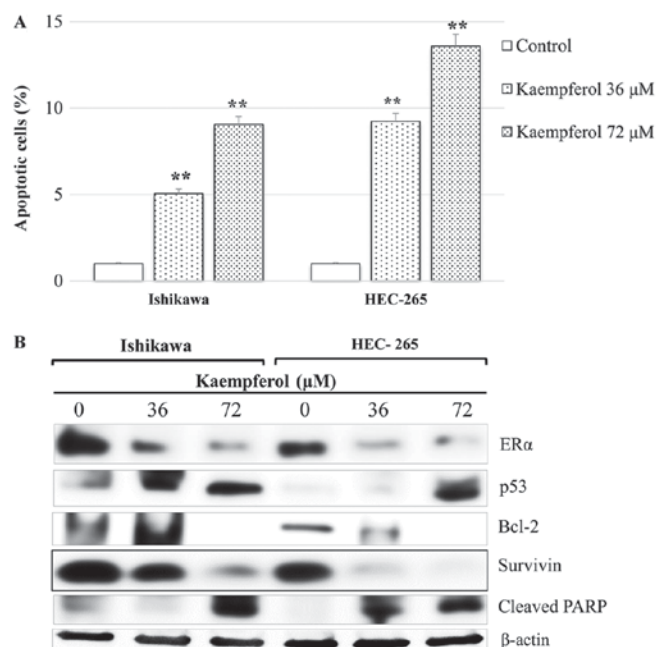


Figure 3. Induction of apoptosis by kaempferol. (A) Significant induction of apoptosis in Ishikawa and HEC-265 endometrial cancer cell lines. (B) Kaempferol suppressed ERα, Bcl-2 and survivin, and induced p53 and cleaved PARP expression. ** $P < 0.01$ vs. control. PARP, poly adenosine diphosphate-ribose polymerase; ERα, estrogen receptor α; Bcl-2, B-cell lymphoma 2.

Discussion

Targeting ERα in endometrial cancer using kaempferol may be a feasible therapeutic strategy, as ERα-mediated oncogenic

effects, including upregulation of Nogo-B receptor (NgBR) and survivin, as well as activation of the PI3K/mTOR/Akt pathway have been established (13,24). Moreover, ERα is known to interact with survivin and p53. Studies have demonstrated that ERα interacts *in vivo* with p53 bound to promoters of survivin and multidrug resistance gene-1 (*MDR1*), both of which are p53-transcriptional repression targets (24,25). Additionally, ERα can directly bind to p53, which plays an important role in mediating apoptosis and leads to downregulation of p53-mediated transcriptional activation.

Survivin interacts with p53 such that wild-type p53 suppresses the expression of survivin by blocking transcription of the *BIRC5* survivin gene (26). Furthermore, the resistance of cancer cells to apoptosis is attributable to upregulation of survivin via its promoter, which results from loss of the p53 tumor suppressor. Survivin inhibits apoptosis through several mechanisms, including direct binding and inhibition of caspases-3 and -9, synergizing with the X-chromosome-linked inhibitor of apoptosis protein (XIAP), binding to the pro-apoptotic protein secondary mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (SMAC/DIABLO), and by preventing-activation of the procaspases (26,27). Steroids are known to trigger translocation of cytoplasmic ERs to the nucleus (28); however, there is limited knowledge about the mechanisms involved and the resulting effects.

The Wnt signaling pathway plays an important role in stem cells and is responsible for regulation of survivin expression. Thus, survivin antagonists may affect cancer stem cells (29). Although there are various apoptosis-based cancer therapies (30), targeting survivin provides several advantages. Suppressing survivin compromises not only the anti-apoptotic cascade, but

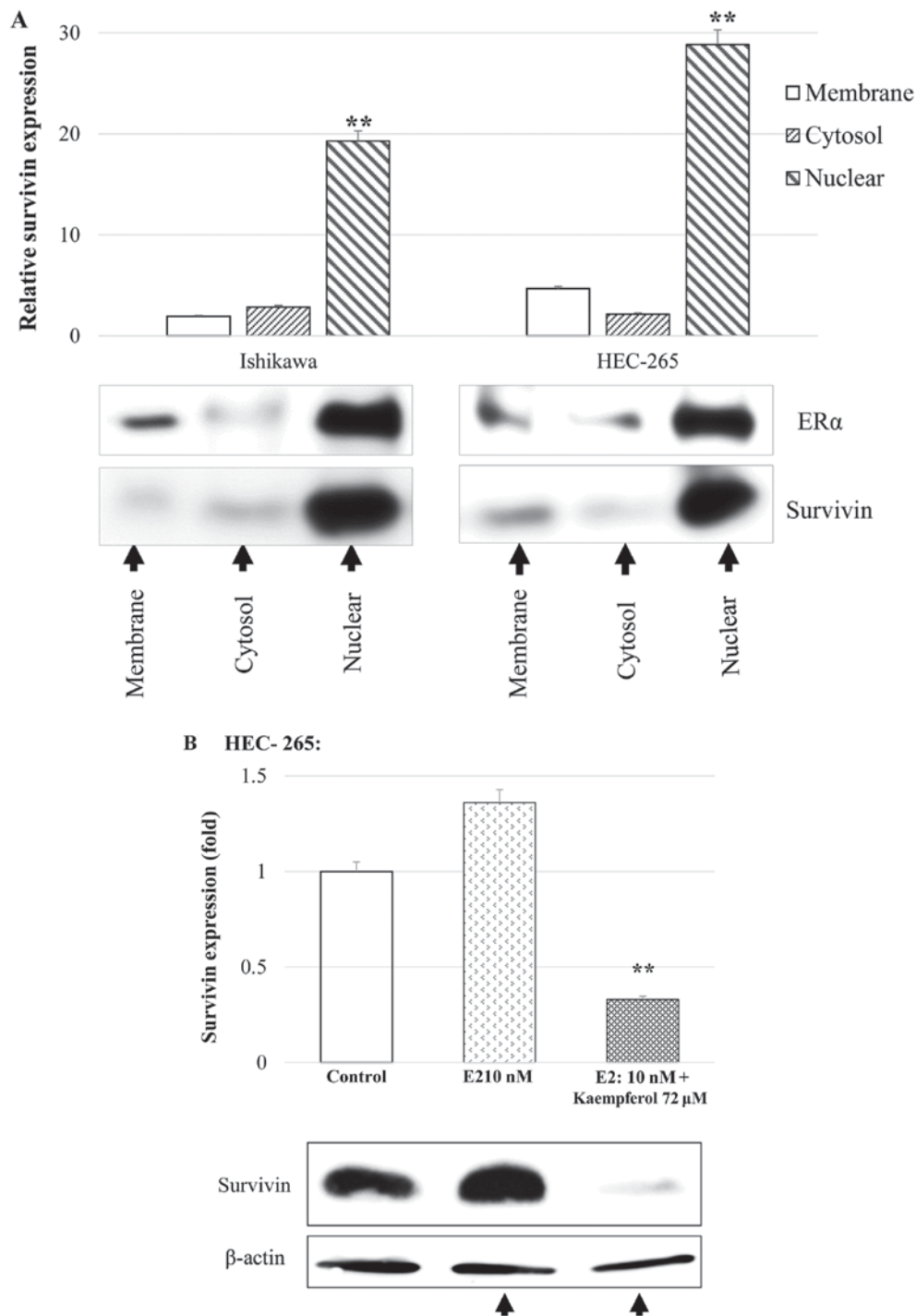


Figure 4. Effects of kaempferol on survivin protein expression. (A) Estradiol induces nuclear co-expression of ERα and survivin in Ishikawa and HEC-265 endometrial cancer cells. Arrows indicate treatment with E2 (10 nM). (B) Kaempferol inhibited E2-induced survivin expression in the HEC-265 endometrial carcinoma cell line. Arrows indicate treatment with E2 (10 nM). **P<0.01 vs. control. ERα, estrogen receptor α; E2, 17β-estradiol.

also the multiple cellular signaling networks required to maintain tumors and their microenvironments (31). Additionally, targeting survivin does not affect normal cells or tissues. For instance, a phase I clinical trial of a survivin-based vaccination has been completed using immunologic antigen-specific responses in which no side effects were reported (32,33). Thus, survivin-based therapeutics might possess more favorable toxicity profiles than other treatment options.

The findings of this study provide a novel therapeutic strategy for endometrial cancer treatment using kaempferol,

a natural dietary flavonoid. This approach offers additional merits attributable to the readily availability and affordability of kaempferol and because kaempferol effectively targets E2-induced/ERα-mediated oncogenic signaling pathways, which play a crucial role in tumorigenesis of most endometrial carcinomas. As we proposed in Fig. 5, kaempferol inhibits ERα, leading to suppression of survivin and apoptotic cell death, in addition to inducing p53, which was reported previously (34-36). Results show that kaempferol had no antitumor effects against estrogen receptor-negative endometrial cancer

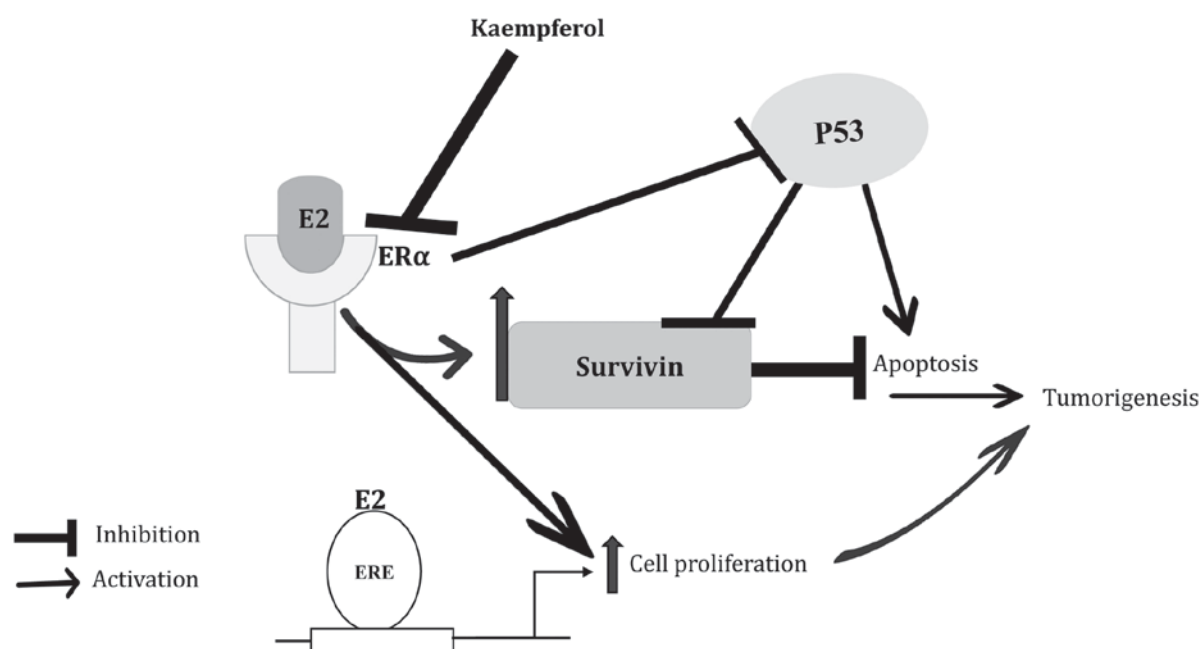


Figure 5. Proposed model for the role of kaempferol in endometrial cancer cells and possible mechanisms. E2, 17 β -estradiol; ER α , estrogen receptor- α ; ERE, estrogen responsive elements.

cells. These results further support our proposed mechanism. While other survivin antagonists have been described, kaempferol provides added benefits because ER α is upstream of survivin and can activate unrelated pathways. Furthermore, as a phytoestrogen, kaempferol may offer a more favorable toxicity profile than other survivin-based therapeutics.

This study has some limitations. First, there are no biomarkers for predicting the sensitivity of endometrial carcinoma to kaempferol. Second, *ex-vivo* experiments may be required to further clarify the antitumor effects of kaempferol in endometrial cancer. Finally, the effectiveness and tolerability of kaempferol must be assessed in patients with endometrial carcinomas in clinical trials.

The present study demonstrates the potential antitumor effects of kaempferol against endometrial carcinoma cells. Therefore, further research into its development as a novel molecularly-targeted agent against endometrial carcinoma is recommended.

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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

AHC, KS and KO conceived and designed the study. SO, TF, MT, AM, TK and YI designed the experiments. AK and MK performed the cell viability assay, and all of the other experiments were performed by AHC. AHC and KS acquired the data. The data were analyzed and interpreted by AHC, KS, KO, MT, KN, YM, OH, KK, YO and TF. AHC and KS prepared the manuscript and figures. AHC, KS, KO, YO and TF reviewed and revised the manuscript for important intellectual content. Technical and material support was provided by AK, MK, MU, TF and HK. HK and MU reviewed the manuscript for important intellectual content. MU analyzed and interpreted the data in additional experiments. HK established and provided endometrial cancer cell lines used in the study. All of the authors approved the final version of this manuscript.

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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