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Original Articles

Gossypetin is a novel MKK3 and MKK6 inhibitor that suppresses esophageal cancer growth *in vitro* and *in vivo*

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ABSTRACT

Gossypetin as a hexahydroxylated flavonoid found in many flowers and Hibiscus. It exerts various pharmacological activities, including antioxidant, antibacterial and anticancer activities. However, the anticancer capacity of gossypetin has not been fully elucidated. In this study, gossypetin was found to inhibit anchorage-dependent and -independent growth of esophageal cancer cells. To identify the molecular target(s) of gossypetin, various signaling protein kinases were screened and results indicate that gossypetin strongly attenuates the MKK3/6-p38 signaling pathway by directly inhibiting MKK3 and MKK6 protein kinase activity *in vitro*. Mechanistic investigations showed that arginine-61 in MKK6 is critical for binding with gossypetin. Additionally, the inhibition of cell growth by gossypetin is dependent on the expression of MKK3 and MKK6. Gossypetin caused G2 phase cell cycle arrest and induced intrinsic apoptosis by activating caspases 3 and 7 and increasing the expression of BAX and cytochrome c. Notably, gossypetin suppressed patient-derived esophageal xenograft tumor growth in an *in vivo* mouse model. Our findings suggest that gossypetin is an MKK3 and MKK6 inhibitor that could be useful for preventing or treating esophageal cancer.

1. Introduction

Esophageal cancer is the sixth most common cause of cancer deaths and the eighth most commonly diagnosed cancer worldwide [1,2]. The two major subtypes of esophageal cancer are esophageal squamous cell carcinoma (ESCC), which localizes in the middle thoracic esophagus, and esophageal adenocarcinoma, which predominates in the distal esophagus [3]. Although advances in the treatment of esophageal cancer mainly include surgery, radiotherapy, chemotherapy and interventional therapy, the 5-year survival rate is only 13% [4]. Even though various targeted therapies have been studied for esophageal cancer, these therapies have shown only a limited response due to the high risk of recurrence, chemoresistance or late diagnosis [5–8]. Therefore, finding more effective targets of esophageal cancer is crucial [9–11].

The mitogen-activated protein kinase (MAPK) cascade signaling pathways play key roles in a number of biological processes [12]. One of the main signaling subgroups, the mitogen-activated protein kinase kinase 3/6 (MKK3/6)-p38 mitogen-activated protein kinase (p38 MAPK) axis has been implicated in cell growth, differentiation, apoptosis, motility and inflammation [13]. MKK3 and MKK6 are protein kinases that specifically phosphorylate and activate p38 MAPK through the formation of functional complexes between MEK3/6 and different p38 MAPK isoforms, but they do not phosphorylate the related JNKs or ERKs MAPKs [14]. The specific p38 isoforms are activated by the MKK3/6-catalyzed phosphorylation of a conserved Thr-Gly-Tyr (TGY) motif in their activation loop [15]. The phosphorylated TGY motif and

Abbreviations: MKK3/6, mitogen-activated protein kinase kinase 3/6; p38, mitogen-activated protein kinase; PDX model, patient-derived xenograft model; AKT, V-akt murine thymoma viral oncogene homolog; ERKs, extracellular signal-regulated kinases

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the length of the activation loop were found to differ in ERK2 and JNKs, which likely contributes to the substrate specificity of p38 [16]. MKK3 has been shown to be the major p38 activator in mesangial cells stimulated by transforming growth factor, but MKK6 appears to be the predominant isoform in thymocytes [17,18]. The p38 MAPK family consists of 4 isoforms (α, -β, -γ and δ) encoded by a separate gene with differences in substrate specificity and tissue distribution [19]. The p38 MAPK pathways are activated in response to various environmental and cellular stresses, inflammation, and other stimuli [20]. The p38 MAPK protein has dual functions in that it can either mediate cell survival or cell death depending on its isoform or the cell type [20]. Previous studies suggested a role for p38 MAPK in mediating pathways leading to apoptosis and growth [21]. Mice deficient in p38α are sensitive to KRAS (G12V)-induced lung tumorigenesis or chemically-induced liver cancer [22,23]. These observations led to the idea that p38 MAPK functions as a tumor suppressor. In contrast with tumor suppressor function, other findings suggested that activation of p38 MAPK signaling might produce an opposing activity such as anti-apoptotic or proliferative effects or enhanced cell survival [20,21]. Also, it might contribute to the epithelial-mesenchymal transition (EMT) of cells in primary tumors [24]. Interestingly, phosphorylated p38 is markedly activated in esophageal cancer patients and selective inhibitors of p38 can suppress esophageal cancer growth [25]. Therefore, developing a cancer therapeutic strategy for targeting p38 MAPK is important.

Flavonoids are found in various dietary sources and exhibit several biological activities, including antioxidant, anti-inflammatory, antiviral, and anti-cancer effects [26]. They have been considered as good candidates for cancer prevention [27]. Gossypetin (3,5,7,8,3′,4′-hexahydroxy flavone) is found in the flowers and the calyx of *Hibiscus sabdariffa* (*roselle*). Gossypetin has been reported to exert anti-mutagenic, anti-atherosclerotic, and antioxidant activities [28–30]. Recently, gossypetin was shown to exert anticancer activities through the induction of apoptosis and autophagic cell death in prostate cancer cells [31], and the inhibition of papilloma virus E6-dependent p53 degradation in cervical cancer cells [32]. However, whether gossypetin has anticancer activities in esophageal cancer and the underlying molecular mechanisms have not been elucidated. Here we provide evidence showing that gossypetin is a potent MKK3 and MKK6 inhibitor that suppresses esophageal cancer growth.

2. Materials and methods

2.1. Cell lines

Human esophageal squamous cell carcinoma (ESCC) lines, KYSE30, KYSE410, KYSE450 and KYSE510, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cytogenetically tested and authenticated before being frozen. KYSE30 cells were cultured in a 1:1 mixture of RPMI-1640 medium and Ham's F12 medium, 10% fetal bovine serum (FBS; Biological Industries, Cromwell, CT, USA) and 1% antibiotic-antimycotic. KYSE410, KYSE450 and KYSE510 cells were cultured with RPMI-1640 medium, 10% FBS and 1% antibiotic-antimycotic in a 37 °C, 5% CO_2 environment. KYSE30, KYSE410, KYSE450 and KYSE510 cells were originally generated by Dr. Yutaka Shimada [33]. Each vial of frozen cells was thawed and maintained in culture for a maximum of 8 weeks. Enough frozen vials were available for each cell line to ensure that all cell-based experiments were conducted on cells that had been tested and in culture for 8 weeks or less.

2.2. Reagents and antibodies

Gossypetin (purity > 90% by HPLC) was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). CNBr-Sepharose 4B beads were from GE Healthcare (Piscataway, NJ, USA). Antibodies to detect phosphorylated AKTs (S473), phosphorylated GSK3β (S9), phosphorylated p38 (T180/Y182), phosphorylated MKK3/6 (S189/S207), phosphorylated ERKs (T202/Y204), phosphorylated JNKs (T193/T185), phosphorylated HER2 (Y877), phosphorylated EGFR (Y1068), total MKK3, total MKK6, total AKTs, total GSK3β, total p38, total JNKs, total HER2, total JAK1, total EGFR, CDKN1B, cleaved caspase 3, cleaved caspase 7, BAX and BCL-XL were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibodies to detect BCL2, cytochrome c and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phosphorylated JAK1 (Y1022) was from Sigma-Aldrich (St Louis, MO, USA) and COX2 was from Abcam (Chembridge science park, Chembridge, UK). The active MKK3, active MKK6, active ERK2, active AKT1, active EGFR and inactive p38α human recombinant proteins for kinase assays or binding assays were purchased from SignalChem (Richmond, BC, Canada).

2.3. Anchorage-independent cell growth

Cells (8×10^3 per well) suspended in complete growth medium (Basal Medium Eagle; BME) supplemented with FBS (10% FBS) were added to 0.3% agar with or without different concentrations of gossypetin in a top layer over a base layer of 0.6% agar with or without different concentrations of gossypetin. The cultures were maintained at 37 °C in a 5% CO_2 incubator for 2 weeks and then colonies were counted under a microscope using the Image-Pro Plus software (v.6) program (Media Cybernetics, Rockville, MD, USA).

2.4. Cell proliferation assay

Cells were seeded ($1.2–2.5 \times 10^3$ cells per well) in 96-well plates with 100 μl complete growth medium and incubated for 24 h. Cells were treated with various concentrations of gossypetin in 100 μl of complete growth medium. After incubation for 48 h, 20 μl of the MTT solution (Solarbio, Beijing, China) were added to each well. After incubation for 2 h at 37 °C in a 5% CO_2 incubator, the cell culture medium was removed. Then 150 μl of DMSO were added to each well and crystal formation was dissolved. Absorbance was measured at 570 nm using the Thermo Multiskan plate-reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. In vitro kinase assay

The kinase assay was performed according to the instructions provided by Upstate Biotechnology (Billerica, MA, USA). The active recombinant MKK3 (200 ng) or MKK6 (200 ng) protein was mixed with various doses of gossypetin in 10 × buffer and kept at room temperature for 15 min. Then, the inactive p38α recombinant protein, ATP and 1 × buffer were added and incubated at 30 °C for 30 min. The reaction was stopped by adding 10 μl protein loading buffer and the mixture was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). MKK3 or MKK6 activity was detected by an p38 phosphorylation antibody.

2.6. Pull-down assay using CNBr-gossypetin-conjugated beads

Total cell lysates (1 mg) or recombinant proteins (300 ng) were incubated with gossypetin-Sepharose 4B (or Sepharose 4B only as a control) beads (50 μl, 50% slurry) in reaction buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP40, 2 μg/ml bovine serum albumin). After incubation with gentle rocking overnight at 4 °C, the beads were washed 5 times with buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP40) and binding was visualized by Western blotting.

2.7. Computational modeling of gossypetin with MKK6

To study the binding and interaction of gossypetin with MKK6, we

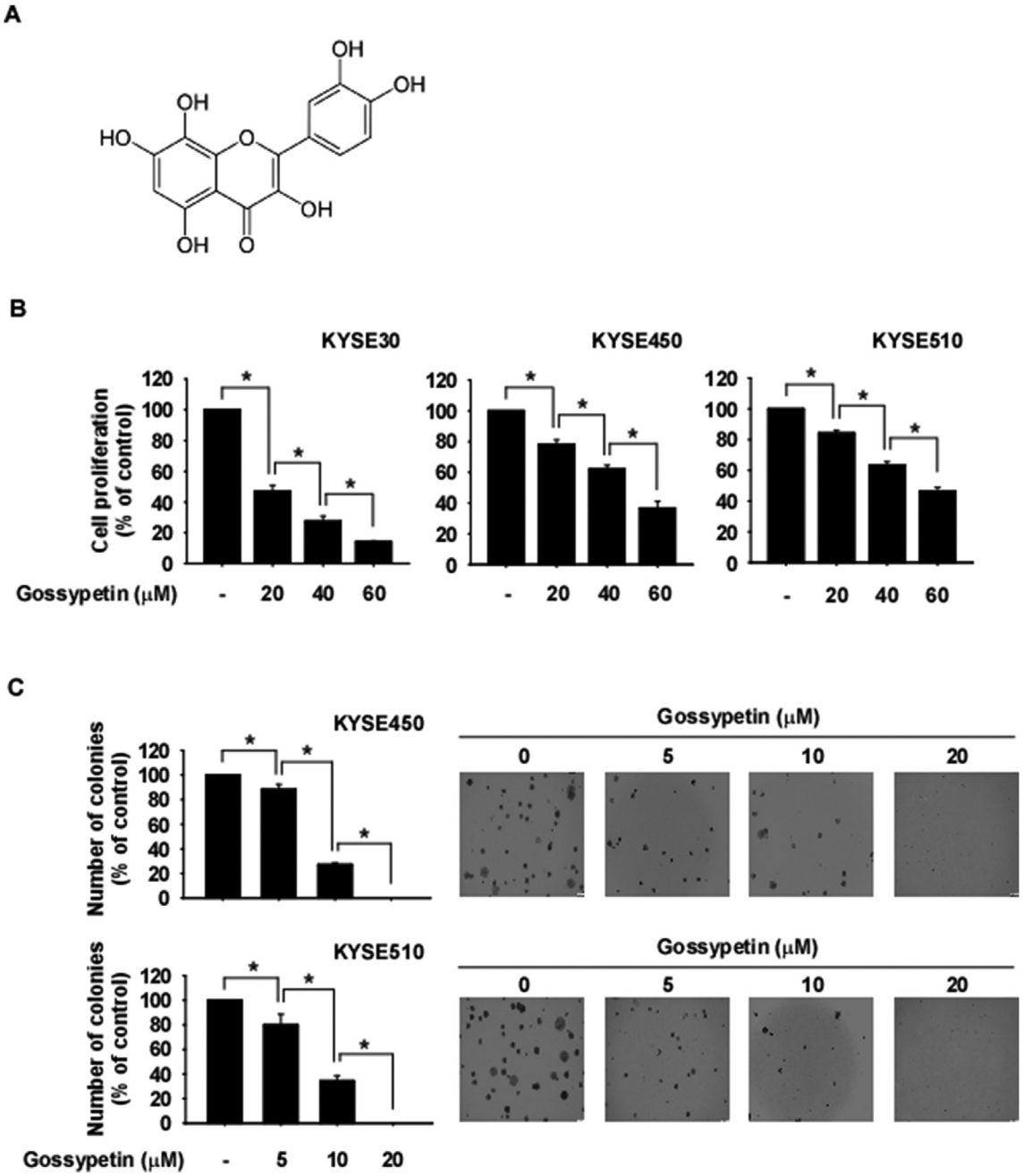

Fig. 1. Gossypetin inhibits proliferation and anchorage-independent growth of esophageal cancer cells. (A) Chemical structure of gossypetin. (B) Effect of gossypetin on esophageal cancer cell growth. Cells were treated with gossypetin at various concentrations and then incubated for 48 h. Cell growth was measured at an absorbance of 570 nm. (C) Effect of gossypetin on anchorage-independent growth of esophageal cancer cells. Cells were treated with gossypetin and incubated for 2 weeks. Colonies were counted using a microscope and the Image-Pro PLUS (v.6) computer software program. All data are shown as means ± S.D. of triplicate values from 3 independent experiments and the asterisk (*) indicates a significant ($p < 0.05$) inhibitory effect of gossypetin.

performed *in silico* docking using the Schrödinger Suite 2016 software programs. First we downloaded the MKK6 crystal structure from the PDB Bank [34] and then prepared it under the standard procedures of the Protein Preparation Wizard (Schrödinger Suite 2016). Hydrogen atoms were added consistent with a pH of 7 and all water molecules were removed. The ATP-binding site-based receptor grid of MKK6 was generated for studying docking. The gossypetin compound was prepared for docking by default parameters using the LigPrep program. Then, the docking of gossypetin with MKK6 was accomplished with default parameters under the extra precision (XP) mode using the program Glide. Herein, we could get the best-docked representative structure.

2.8. Cell cycle analysis

Cells were plated into 60-mm culture dishes (2.5–4 × 10^4 cells/dish) and incubated for 24 h. Cells were synchronized by serum starvation for 24 h and treated with gossypetin for 48 h in 10% serum-supplemented medium. Cells were collected by trypsinization and washed with phosphate buffered saline (PBS) and then fixed in 1000 μl of 70% cold ethanol. After rehydration, cells were digested with RNase (100 μg/ml) and stained with propidium iodide (20 μg/ml). Propidium iodide staining was accomplished following the product instructions (Clontech, Palo Alto, CA, USA). The cells were analyzed by flow cytometry.

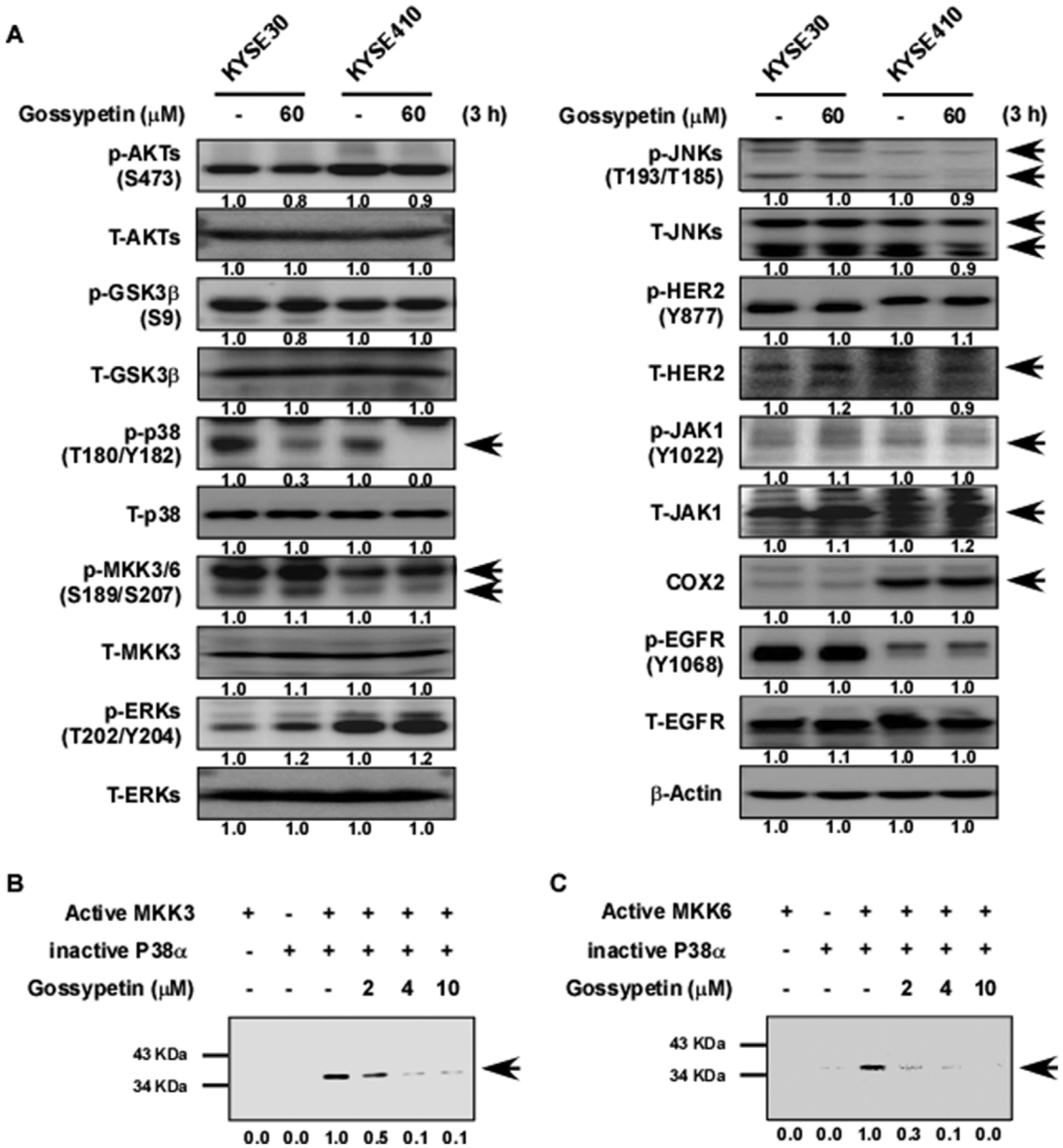

Fig. 2. Gossypetin is a potent inhibitor of MKK3 and MKK6. (A) The effect of gossypetin on various signaling pathway proteins. Cells were treated with gossypetin at 60 μM for 3 h and then signaling pathway proteins were examined by Western blotting. Gossypetin suppresses (B) MKK3 and (C) MKK6 kinase activity in a dose-dependent manner. The effect of gossypetin on MKK3 or MKK6 activity was assessed by an *in vitro* kinase assay using MKK3 (active, 400 ng) or MKK6 (active, 300 ng) and p38 (inactive, 300 ng) proteins. Effect of gossypetin on MKK3 or MKK6 activity was determined by Western blotting using a phosphorylation p38 antibody. For A-C, similar results were observed from 3 independent experiments. Band density was measured using the Image J (NIH) software program.

2.9. Apoptosis assay

Cells were plated into 6-well culture dishes (5×10^4 cells/well) and incubated for 24 h. Cells were treated with gossypetin for 72 h in 10% serum-supplemented medium and collected by trypsinization and washed with PBS. Cells were stained with Annexin V (BioLegnd, San Diego, CA, USA) and propidium iodide and apoptosis was analyzed using flow cytomertry.

2.10. Patient-derived esophageal tumor xenografts (PDX)

Severe combined immunodeficiency (SCID) female mice (6–9 wk old) were maintained under "specific pathogen-free" conditions based on the guidelines established by the Zhengzhou University Institutional Animal Care and Use Committee. A human tumor specimen of esophageal cancer tissue was obtained from the affiliated Cancer Hospital in Zhengzhou University, cut into small pieces, and inoculated into the back of the neck of each mouse. Mice were divided into 2 groups of 10 animals each as follows: 1) untreated vehicle group and 2) 100 mg gossypetin/kg body weight. Gossypetin or vehicle (5% DMSO in 10% tween 80) was orally administered 5 times per week. Tumor volume was calculated from measurements of 2 diameters of the individual tumor base using the following formula: tumor volume (mm^3) = (length × width × height × 0.52). Mice were monitored until tumors reached 1.5 cm^3 total volume, at which time mice were euthanized and tumors, liver, kidney and spleen were extracted.

2.11. Hematoxylin-eosin staining and immunohistochemistry

The liver, kidney and spleen tissues from mice were embedded in

paraffin blocks and subjected to hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). Tissue sections were deparaffinized, hydrated and then permeabilized with 0.5% Triton X-100/1 × PBS for 10 min. After developing with 3, 3′-diaminobenzidine, the sections were counterstained with H&E. For IHC, sections were hybridized with the primary antibody (1:500) and a horse-radish peroxidase (HRP)-conjugated goat anti-rabbit or mouse IgG antibody was used as the secondary antibody. All sections were observed under a microscope and analyzed using the Image-Pro Plus software (v.6) program (Media Cybernetics, Rockville, MD, USA).

2.12. Statistical analysis

All quantitative results are expressed as mean values ± S.D. or ± S.E. Significant differences were compared using the Student's *t*-test or one-way analysis of variance (ANOVA). A p value of < 0.05 was considered to be statistically significant. The statistical package for social science (SPSS) for Windows (IBM, Inc. Armonk, NY, USA.) was used to calculate the *p*-value to determine statistical significance.

3. Results

3.1. Gossypetin inhibits the viability and anchorage-independent growth of esophageal cancer cells

Gossypetin is a 3,5,7,8,3′,4′-hexahydroxyflavone compound (Fig. 1A). To determine the effect of gossypetin on esophageal cancer cell growth, cells were treated with gossypetin at various doses for 48 h and growth was analyzed by the MTT assay. Results showed that anchorage-dependent esophageal cancer cell growth was significantly inhibited by gossypetin treatment in dose dependent manner (Fig. 1B). Additionally, gossypetin strongly suppressed anchorage-independent cell growth in esophageal cancer cells (Fig. 1C).

3.2. Gossypetin is a novel MKK3 and MKK6 inhibitor

To identify potential molecular targets of gossypetin, esophageal cancer cells were treated with gossypetin for 3 h and then the expression level of various signaling molecules was assessed by Western blotting. Results showed that gossypetin strongly suppressed phosphorylation of p38, but had little effect on that of other related signaling proteins (Fig. 2A). MKK3 and MKK6 are direct upstream kinases of p38. Therefore, we investigated whether gossypetin affected MKK3 or MKK6 activity by performing *in vitro* kinase assays with an active recombinant MKK3 or MKK6 protein and a substrate. The results indicated that p38 activity is strongly inhibited by gossypetin treatment in a dose-dependent manner (Fig. 2B and C) and confirmed that gossypetin directly suppresses MKK3 or MKK6 activity.

3.3. Gossypetin directly binds to MKK3 and MKK6

We created a molecular docking model of the gossypetin and MKK3 or MKK6 complex in order to determine the binding orientation of gossypetin. However, the MKK3 crystal structure is unavailable. Therefore, we performed the gossypetin-binding simulation with MKK6. To best understand how gossypetin interacts with MKK6, we docked the compound to the ATP binding pocket of MKK6 using several protocols in the Schrödinger Suite 2016. Based on the final computational docking model, we found that gossypetin formed several contacts with MKK6 at the ATP binding pocket (Fig. 3A). This indicates that gossypetin might be a potential inhibitor of MKK6. Images were generated with the UCSF Chimera program [35]. To examine the interaction between gossypetin and MKK3 or MKK6, we performed *in vitro* pull-down assays using gossypetin-conjugated Sepharose 4B beads (or Sepharose 4B as a negative control) with KYSE30 esophageal cancer cell lysates or a recombinant MKK3 or MKK6 protein. These results confirmed that gossypetin directly binds with MKK3 or MKK6, but not with AKT1, ERK2, p38 or EGFR (Fig. 3B and C and Supplemental Fig. 1A–D). Computer docking results indicated that Arg61 and Met132 on MKK6 might be involved in the binding with gossypetin. We constructed mutant MKK6 (R61K, M132A) and ectopically expressed these mutants in KYSE 450 esophageal cancer cells. Pull-down assays using the wildtype or each mutant and gossypetin-conjugated Sepharose 4B beads revealed that the R61K mutant of MKK6 showed the most reduced binding affinity with gossypetin (Fig. 3D), suggesting that this site is important for binding. Additionally, we also investigated similar amino acids of MKK3 at Arg72, Asn185, and Asp208 that are conserved in MKK6 in the binding with gossypetin. Mutant MKK3 (R72K, N185A, D208E) plasmids were transfected in KYSE 450 esophageal cancer cells and the results of pull-down assays indicated that these mutants of MKK3 did not affect the binding affinity with gossypetin (Supplemental Fig. 2).

3.4. Gossypetin induces G2 phase cell cycle arrest

To determine the effect of gossypetin on cell cycle, we performed flow cytometry analysis with propidium iodide (PI) staining. Cells were synchronized by serum starvation for 24 h and cell cycle was released with serum with or without gossypetin for 48 h. The results showed that gossypetin reduces S phase and induces G2 phase cell cycle arrest in a dose-dependent manner (Fig. 4A). We next determined whether gossypetin affects the expression of cell cycle marker proteins. After serum starvation for 24 h, cells were treated with different doses of gossypetin for 48 h and cell cycle marker proteins were analyzed by Western blotting. Results indicated that gossypetin strongly increased the expression of the CDKN1B protein (Fig. 4B).

3.5. Gossypetin induces apoptosis of esophageal cancer cells

To investigate whether gossypetin affects apoptosis of esophageal cancer cells, we performed examined the viability of gossypetin-treated or –untreated cells by counting cells found in the suspended (dead) or attached (live) fraction after treatment for 72 h with different doses of gossypetin. The results showed that the number of suspended cells was significantly increased in gossypetin-treated cells compared with control (Fig. 5A, *left panel*). In contrast, the number of attached cells was significantly decreased in gossypetin-treated cells (Fig. 5A, right panel). To understand whether the elevated cell death was due to an increase in apoptosis, cells were treated with gossypetin for 72 h and annexin V expression was analyzed. Results showed that gossypetin-treated cells in early and late apoptosis were significantly increased compared to control cells (Fig. 5B and C). To confirm the effect of gossypetin on apoptotic signaling pathways, expression of pro-apoptotic and anti-apoptotic marker proteins was investigated. Results showed that cleaved caspase 3, 7, BAX, and cytochrome c were strongly induced by gossypetin treatment and anti-apoptotic BCL2 and BCL-XL were markedly reduced (Fig. 5D). The results suggested that gossypetin might induce the signaling of intrinsic cellular apoptosis.

3.6. Gossypetin inhibits patient-derived esophageal xenograft tumor growth in vivo

To study the anti-tumor activity of gossypetin *in vivo*, patient-derived esophageal tumor tissues were inoculated into the back of the neck of athymic nude mice. Mice were orally administrated gossypetin (100 mg/kg) or vehicle 5 times a week over a period of 21 days. Results showed that gossypetin significantly decreased the volume of esophageal tumor growth by over 60% relative to the vehicle-treated group (Fig. 6A; $p < 0.05$). Additionally, mice tolerated treatment with gossypetin without significant loss of body weight similar to the vehicle-treated group (Fig. 6B). We then examined the effects of gossypetin on the Ki67 tumor proliferation marker by using immunohistochemistry.

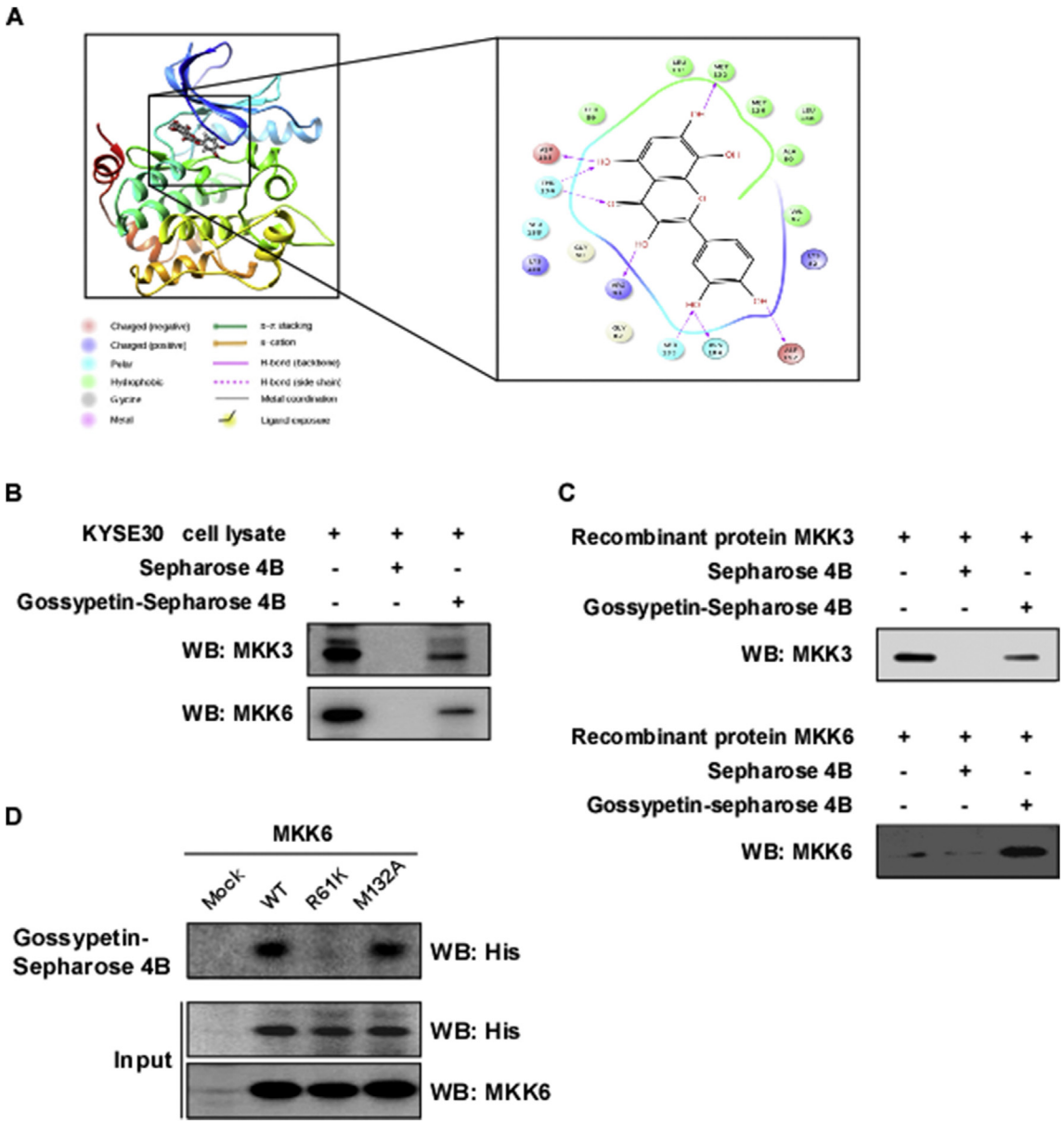

Fig. 3. Gossypetin binds to MKK3 and MKK6. Modeling of gossypetin binding with MKK6. (A, *left panel*) Gossypetin binding with MKK6 at the ATP binding pocket. (A, *right panel*) Ligand Interaction Diagram (LID) of the binding. The MKK6 structure is shown as ribbon representation and gossypetin is shown as stick. LID legend is shown below. Gossypetin directly binds to MKK3 and MKK6 in (B) esophageal cancer cell lysates, (C) recombinant proteins or (D) cells ectopically expressing MKK6 (WT, mutant R61K, or M132A) were incubated with gossypetin-conjugated Sepharose 4B beads or with Sepharose 4B beads alone. The pulled down proteins were analyzed by Western blotting. For B-D, similar results were obtained from 3 independent experiments.

The expression of Ki67 was significantly decreased by gossypetin (Fig. 6C). Furthermore, to evaluate the potential toxic effects of gossypetin on tissue morphology, the liver, kidney and spleen tissues were stained with hematoxylin and eosin (H&E). Results indicated no obvious morphological differences between tissues from treated or untreated mice (Supplemental Fig. 3A–C). To confirm these results from the *in vivo* PDX model, we investigated the effect of gossypetin on MKK3 and MKK6 and their downstream signaling targets by Western blotting analysis of PDX esophageal tumor samples. The phosphorylation of p38, the direct downstream protein of MKK3/6 was strongly inhibited in the gossypetin-treated group (Fig. 6D). This finding suggested that gossypetin suppresses patient-derived esophageal tumor growth through inhibition of MKK3/6 *in vivo*.

4. Discussion

Dietary intake of flavonoids have been reported to reduce the risk of various cancers, including esophageal cancer [36–38]. In the present study, the flavonoid gossypetin significantly inhibited anchorage-dependent and -independent esophageal cancer cell growth and also patient-derived esophageal tumor growth *in vivo* (Figs. 1 and 6). This is the first study reporting the chemopreventive properties of gossypetin in esophageal cancer. The findings might provide useful insights and supporting evidence for cancer chemoprevention, especially in regions with high incidence and mortality of esophageal cancer.

Based on the results of cell-based and *in vitro* assays, we identified the molecular targets of gossypetin as MKK3 and MKK6 (Figs. 2 and 3). MKK3 and MKK6 are protein kinases that can specifically phosphorylate and activate p38α. Several reports suggest that the MKK3/6-p38α signaling pathway plays a tumor suppressor role or oncogene [39–42].

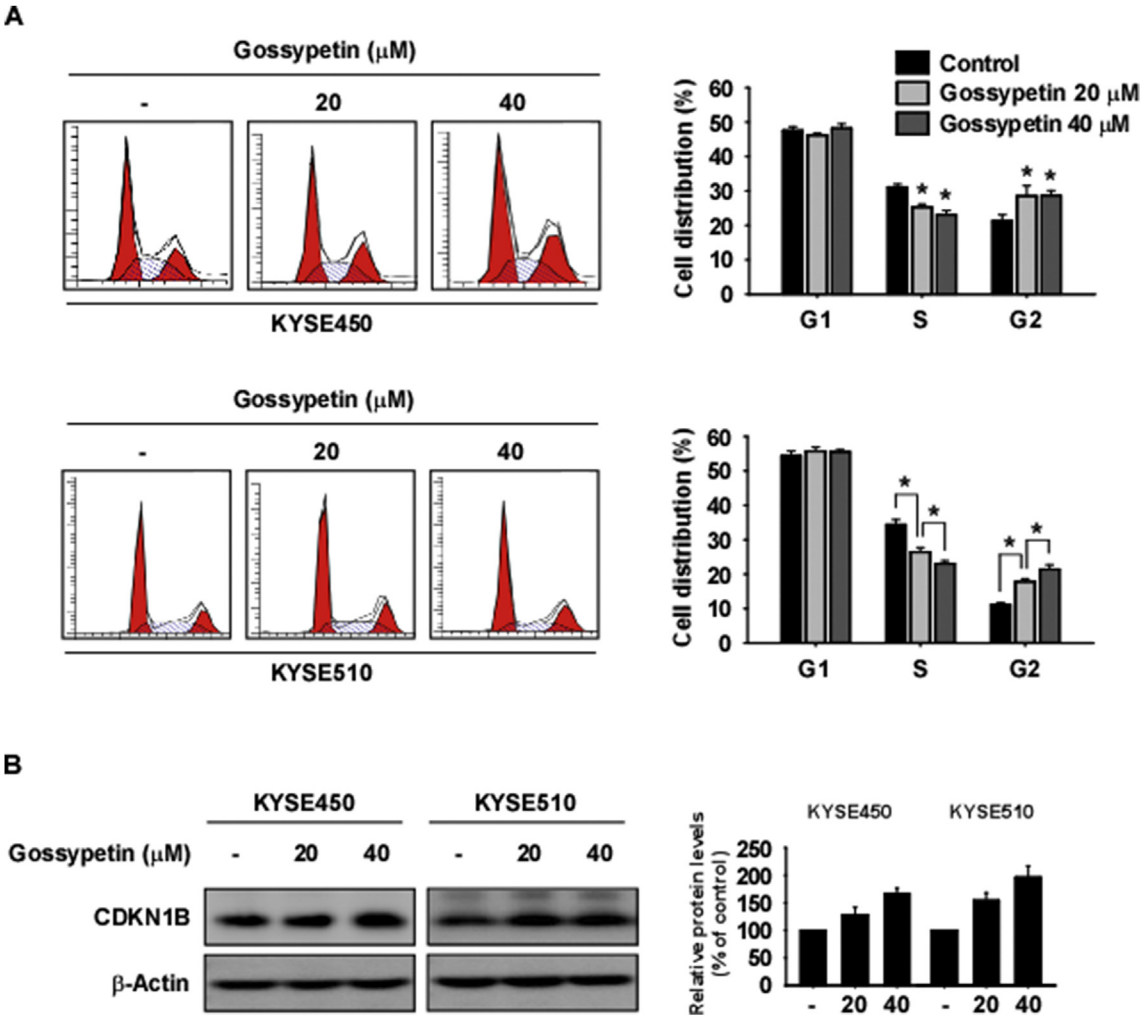

Fig. 4. Gossypetin induces G2 phase arrest. (A) The effect of gossypetin on cell cycle was determined in KYSE30 and KYSE510 esophageal cancer cells. Cells were synchronized by serum starvation for 24 h and treated with gossypetin for 48 h in 10% serum-supplemented medium. Cells were stained with propidium iodide (PI) and cell cycle was analyzed by Fluorescence Activated Cell Sorting (FACS). For A, data are represented as mean values ± S.D. of triplicate values from 3 independent experiments and the asterisk (*) indicates a significant ($p < 0.05$) difference compared to untreated control. (B) Effect of gossypetin on the expression of the CDKN1B protein. The expression of CDKN1B was analyzed by Western blotting and band density was measured using the Image J (NIH) software program. For B, the 3 independent results of the expression of CDKN1B protein are shown as a graph.

P38 is activated in esophageal cancer patients and SB203580, a specific p38 inhibitor, suppresses esophageal cancer cell growth [25]. Therefore, our results strongly suggest that gossypetin inhibits esophageal cancer growth by inhibiting the MKK3/6-p38 signaling pathway. This is the first report of a natural compound inhibitor of MKK3/6 in esophageal cancer. Additionally, we investigated whether the inhibition of cell growth by gossypetin is dependent on the expression of MKK3 and MKK6. Cells expressing siMKK3 and siMKK6 or overexpressing MKK3 and MKK6 were established. Cells were treated with gossypetin and incubated for 48 h and proliferation was determined by MTT assay. The results indicated that cells expressing siMKK3 and siMKK6 were resistant to gossypetin's inhibitory effect on proliferation compared to cells expressing sicontrol (Supplemental Fig. 4A and B). In contrast, cells overexpressing MKK3 and MKK6 were sensitive to gossypetin (Supplemental Fig. 4C and D). These results showed that the effect of gossypetin on the growth of cancer cells is dependent on MKK3 and MKK6 expression. Therefore, we suggest that MKK3 and MKK6 are the main target proteins of gossypetin in esophageal cancer cells.

Computational techniques and structural-based molecular docking have been utilized in pharmaceutical research [43]. Interestingly, molecular docking studies revealed that the key interacting residues of target proteins with natural compounds could be identified using computational techniques [44,45]. In this study, we performed computational modeling to simulate the binding mode of gossypetin with MKK6. Gossypetin was computationally predicted to possess several binding orientations within the ATP binding site of MKK6 (Fig. 3A). Additionally, the results of an *in vitro* binding assay showed that the Arg61 residue of MKK6 appears most important for the binding (Fig. 3D). Although we constructed various mutants of MKK3 and analyzed by pull-down assay, we could not identify the residue of MKK3 responsible for the binding (Supplemental Fig. 2). The reason is that the MKK3 crystal structure is unavailable. Therefore, we will investigate the sequence based-computer modeling of MKK3 and gossypetin and analyze the predicted binding residues.

Flavonoids reportedly reduce the risk of cancer potentially by inducing apoptosis, cell cycle arrest, and interfering with other signaling pathways [46]. Additionally, FR167653 is a p38 inhibitor that strongly induced apoptosis in colon cancer through caspase activation [47]. Therefore, we also investigated the underlying mechanisms of gossypetin inhibition of esophageal cancer growth and found that gossypetin strongly induced apoptosis by increasing cleaved caspase 3, caspase 7, BAX, and cytochrome c and decreasing BCL2 and BCL-XL expression (Fig. 5D). These apoptosis-related proteins are all important proteins that participate in apoptosis mediated by the mitochondrial pathway

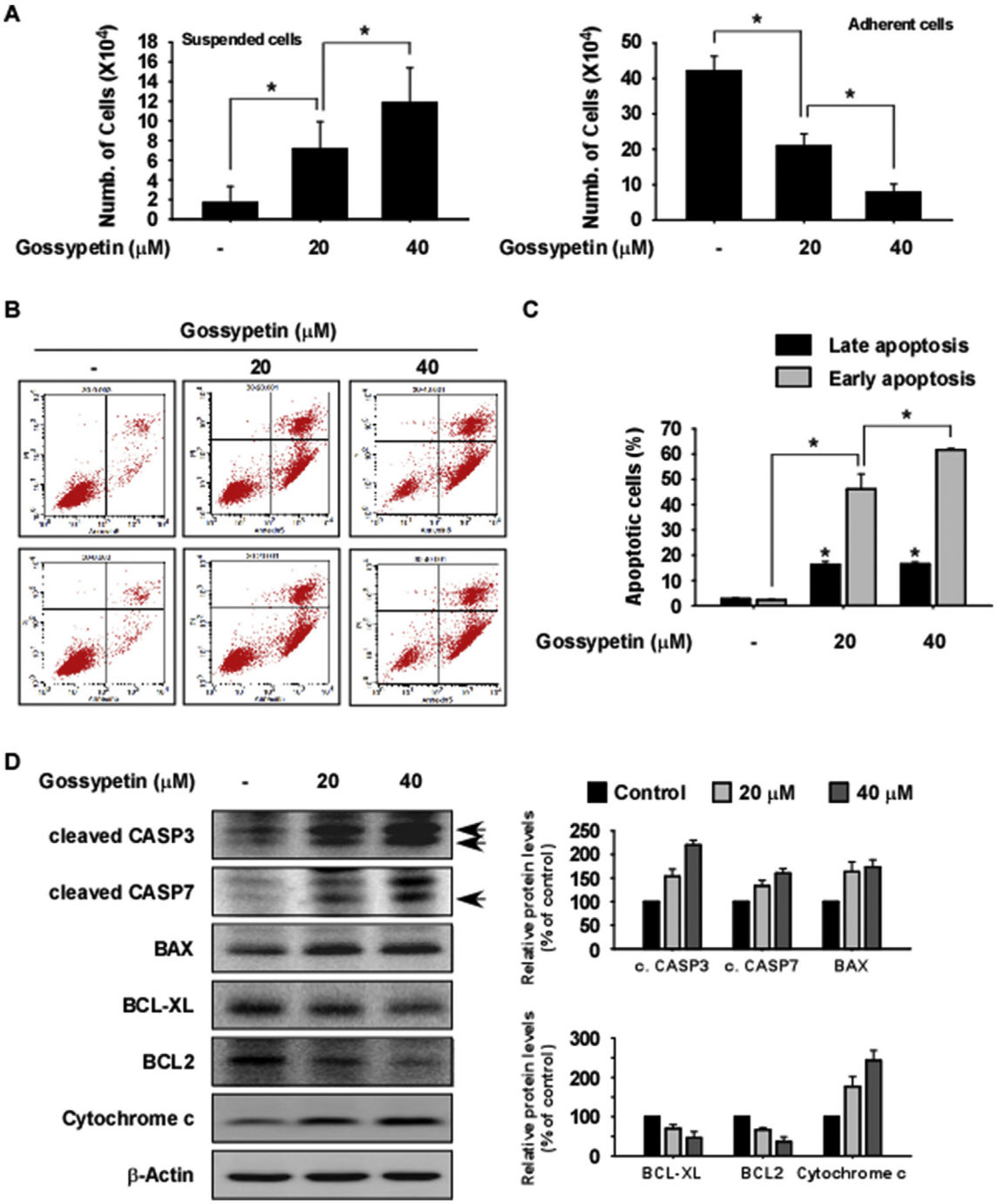

Fig. 5. Gossypetin induces esophageal cancer cell death through apoptosis. (A) Gossypetin induces cell death. Cells were seeded in a 6-well plate and treated with gossypetin at the indicated doses for 72 h. The number of suspended or attached cells was counted using a hematocytometer. (B–C) Gossypetin induces apoptosis. Cells were seeded with gossypetin in 10% FBS-supplemented medium and then incubated for 72 h. Cells were stained with annexin V and propidium iodide (PI) and apoptosis was determined by Fluorescence Activated Cell Sorting (FACS). For A-C, data are represented as means ± S.D. of triplicate values from 3 independent experiments and the asterisk (*) indicates a significant ($p < 0.05$) difference compared to untreated control. (D) Gossypetin strongly induces apoptotic-marker proteins. Cells were treated with gossypetin for 72 h and the protein levels of cleaved caspase 3, cleaved caspase 7, BAX, BCL-XL, BCL2, and cytochrome c were determined by Western blotting using β-actin as the loading control. Band density was measured using the Image J (NIH) software program. The 3 independent results of the expression of apoptotic-marker proteins are shown as a graph.

[48]. Therefore, we suggest that gossypetin can induce the mitochondrial-dependent pathway of intrinsic apoptosis in esophageal cancer cells.

Mouse xenograft models are extensively used in the process of drug screening. Currently, patient-derived xenograft (PDX) models have been created by the transfer of primary tumor tissues directly from human patients into immunodeficient mice [49]. PDX models are believed to be superior to traditional cell line xenograft models to predict responsiveness to anticancer agents because they retain more histologic and genetic characteristics of the patient tumors [50]. Thus, PDX models could also be better predictors of clinical outcomes. In this study, we established ESCC PDX models in nude mice by subcutaneous inoculation of tumor tissues from a cancer patient. We found that gossypetin inhibited patient-derived esophageal xenograft tumor

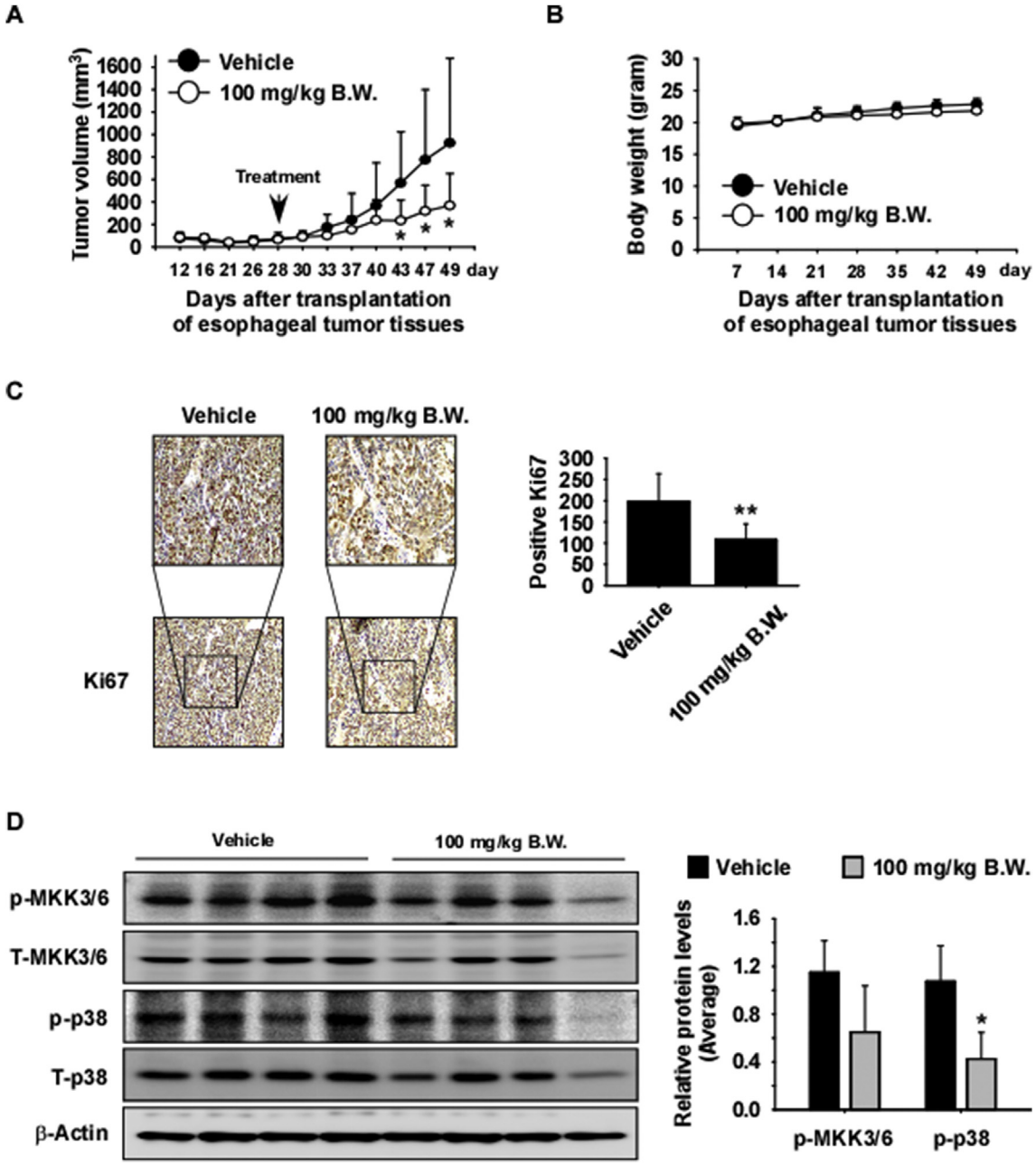

Fig. 6. Gossypetin suppresses esophageal cancer patient-derived xenograft tumor growth *in vivo*. Mice were divided into 2 groups for assessing the effect of gossypetin on esophageal cancer patient-derived xenograft (PDX) tumor growth. Groups are as follows: 1) vehicle group and 2) 100 mg/kg B.W. of gossypetin. Mice were orally administered gossypetin or vehicle 5 times a week for 21 days. (A) Gossypetin inhibits esophageal tumor growth. (B) Gossypetin has no effect on mouse body weight. Body weights from treated or untreated groups of mice were obtained once a week. For A-B, data are shown as means ± S.E. of values obtained from the experiment. The asterisk (*) indicates a significant difference between tumors from untreated or treated mice as determined by *t*-test ($p < 0.05$). (C) Immunohistochemical analysis of tumor tissues. Treated or untreated groups of tumor tissues were stained with anti-Ki67. The number of Ki67-stained cells (*right panel*) was counted from immunohistochemistry results (N = 10; **; $p < 0.01$). (D) Gossypetin inhibits MKK3/6 and p38 protein expression in esophageal tumor tissues. Tumor tissues from each group were immunoblotted with antibodies to detect MKK3/6, p38, phosphorylated MKK3/6, p-p38, and β-actin. β-Actin was used to verify equal protein loading. Band density was measured using the Image J (NIH) software program. The results of the average expression of phosphorylated MKK3/6 and -p38 proteins are shown as a graph.

growth *in vivo* by attenuating the phosphorylation of p38. The expression of Ki67 was also substantially decreased in the gossypetin treated group compared to untreated controls. Phosphorylation of p38 is reported to have a positive correlation with Ki67 expression in cancer [51]. This suggests that gossypetin can inhibit esophageal tumor growth *in vivo* by targeting the MKK3/6/p38 signaling pathway. Therefore, gossypetin might prevent esophageal cancer among healthy individuals, especially those who are at high risk to develop this cancer.

In conclusion, our study demonstrated that gossypetin is a novel MKK3 and MKK6 inhibitor that exhibits anticancer properties in *ex vivo*, *in vitro* and *in vivo*. The results have further clarified the mechanisms of gossypetin in ESCC prevention and indicates that proper utilization of gossypetin might be a practical method in esophageal cancer chemoprevention.

Author contribution

X.X. performed the *in vitro* experiments and assisted with the cell

based and *in vivo* experiments; F.L., X.W. and X.Z. assisted with the cell based assays; Y.Z. and X.M. assisted with the *in vivo* experiments; T.W. and Q.W. performed the *in vivo* experiments; H.C performed the computer modeling; K.L. and A.M.B. supervised the *in vivo* experimental design, data analysis and manuscript editing; Z.D. supervised the overall experimental design and data analysis; D.J.K. supervised designed experiments, provided the idea and prepared the manuscript.

Conflicts of interest

None of the authors have any competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.10.016.

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