Kaempferol inhibits angiogenic ability by targeting VEGF receptor-2 and downregulating the PI3K/AKT, MEK and ERK pathways in VEGF-stimulated human umbilical vein endothelial cells

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Abstract. Anti-angiogenesis is one of the most general clinical obstacles in cancer chemotherapy. Kaempferol is a flavonoid phytochemical found in many fruits and vegetables. Our previous study revealed that kaempferol triggered apoptosis in human umbilical vein endothelial cells (HUVECs) by ROS-mediated p53/ATM/death receptor signaling. However, the anti-angiogenic potential of kaempferol remains unclear and its underlying mechanism warranted further exploration in VEGF-stimulated HUVECs. In the present study, kaempferol significantly reduced VEGF-stimulated HUVEC viability. Kaempferol treatment also inhibited cell migration, invasion, and tube formation in VEGF-stimulated HUVECs. VEGF receptor-2 (VEGFR-2), and its downstream signaling cascades (such as AKT, mTOR and MEK1/2-ERK1/2) were reduced as determined by western blotting and kinase activity assay in VEGF-stimulated HUVECs after treatment with kaempferol. The present study revealed that kaempferol may possess angiogenic inhibition through regulation of VEGF/VEGFR-2 and its downstream signaling cascades (PI3K/AKT, MEK and ERK) in VEGF-stimulated endothelial cells.

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Key words: angiogenesis, kaempferol, VEGF receptor-2, AKT, MEK/ERK, human umbilical vein endothelial cells (HUVECs)

Introduction

Anti-angiogenesis is becoming a very promising goal for cancer therapy (1,2). Angiogenesis is a new passageway from pre-existing blood vessels and an essential step involved in physiological and tumor pathological processes (1,3). Tumor cell growth and metastases processes depend on the induction of a satisfactory blood support (1,4). Many chemotherapeutic agents such as paclitaxel (Taxol) inhibit tumor cell growth, proliferation and induce apoptotic cell death in cancer treatment. Furthermore, the blocking of angiogenesis provides a novel therapeutic target against tumor cells (5,6). In clinical anti-angiogenic therapy, bevacizumab (Avastin) is a monoclonal antibody for anti-vascular endothelial growth factor (VEGF) that counteracts the action of VEGF and inhibits tumor angiogenesis (7,8). Numerous phytochemicals, such as curcumin or epigallocatechin-3-gallate (EGCG) have been demonstrated to exert anti-angiogenic bioactivities in several *in vitro* and *in vivo* models (9-11). Thus, identification of phytochemicals with non-cytotoxic effects on normal cells and effective anti-angiogenic action could be of great clinical significance (11-13).

Kaempferol is a flavonoid phytochemical found in fruits and vegetables and in some traditional Chinese medicines (TCM) (14-16). Kaempferol has been reported to exert biological activities such as anti-inflammatory (17,18), antioxidant (19,20), cardioprotective (19) and antitumor (21-23). Kaempferol has been demonstrated to provide chemopreventive effects on different tumor systems including tumor initiation, promotion, and progression (24,25). Recently, our previous study revealed that kaempferol caused endoplasmic reticulum stress and mitochondria-dependent apoptosis in human osteosarcoma U-2 OS cells (26) and triggered AMPK and AKT-dependent autophagic cell death in human hepatocarcinoma SK-HEP-1 cells (27). In addition, we also demonstrated that kaempferol suppressed U-2 OS cell metastasis through suppression of the ERK/p38/JNK and AP-1 signaling pathways (28). In an anti-angiogenic study, our earlier research indicated that kaempferol induced ROS-mediated p53/ATM-dependent apoptosis in human umbilical vein endothelial cells (HUVECs) (29). However, there is no available information regarding the possible major target and anti-angiogenic mechanism of kaempferol in endothelial cells. In the present study, we analyzed the anti-angiogenic effects of kaempferol on HUVECs. Our results demonstrated that kaempferol inhibited HUVEC proliferation, migration and tube formation. The molecular levels indicated that kaempferol suppressed VEGF receptor-2 (VEGFR-2) expression and its downstream signaling cascades (AKT/mTOR and MEK/ERK) in HUVECs.

Materials and methods

Chemicals and reagents. Kaempferol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), vascular endothelial growth factor (VEGF), the other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Medium 200, Low Serum Growth Supplement (LSGS) and Trypsin-EDTA were obtained from Thermo Fisher Scientific, Inc. (Carlsbad, CA, USA). The primary antibodies [VEGFR-2 (cat. no. sc-504), PI3K (cat. no. sc-1637), p-AKT (Ser473) (cat. no. sc-7985-R), AKT (cat. no. sc-1618), p-mTOR (Ser2448) (cat. no. sc-101738), mTOR (cat. no. sc-8319), p-MEK1/2 (Ser218/Ser222) (cat. no. sc-7995), MEK1/2 (cat. no. sc-436), p-ERK (Thr202/Tyr204) (cat. no. sc-16982), ERK (cat. no. sc-135900) and β-actin (cat. no. sc-47778)] and secondary antibodies against goat anti-mouse (cat. no. sc-2005)/-rabbit (cat. no. sc-2004) and mouse anti-goat (cat. no. sc-2354) immunoglobulin (IgG)-horseradish peroxidase (HRP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. HUVECs were obtained from the Bioresources Collection and Research Center (BCRC), Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured in Medium 200 and LSGS in a humidified atmosphere containing 5% CO_2 and 95% air at 37˚C. The cells were applied within the second to fifth passages, and all assays performed used the same culture media with 50 ng/ml VEGF.

Cytotoxic assay. VEGF-stimulated HUVECs ($1x10^4$ cells/100 μl/well) were seeded into 96-well microplates and then incubated with or without 50, 100, 150 and 200 μM of kaempferol for 24 h. Cell viability was detected by MTT assay as previously described (27,28). Briefly, as soon as kaempferol exposure was completed, 10 μl MTT solution (5 mg/ml) was added to each well, and the plate was incubated for an additional 3 h. The purple crystals were dissolved with the addition of 100 μl DMSO. The optical density ratio was assessed spectrophotometrically at 570 nm. The percentage of cell viability at each concentration relative to the untreated control group (% of control) was plotted.

Wound healing migration assay. HUVECs were plated into 6-well plates and incubated to 90% confluence for 24 h. A linear wound was scratched using a 200-μl pipette tip through the monolayer before cellular debris was removed. Then, VEGF-stimulated HUVECs were exposed to 50, 100, 150 and 200 μM of kaempferol for 24 h. The healing process was captured using a phase-contract microscope after the wound was introduced prior to kaempferol incubation. Cell migration was determined from the images of five random fields. The gap size was analyzed by NIH ImageJ version 1.46 for Windows between the migrating cells from the opposing wound edge, and the data were expressed as the % of the initial gap size as previously described (30,31).

Boyden chamber Transwell assay. Cell invasion ability was detected as previously described (31,32). The Transwell (Millicell Cell Culture Insert; EMD Millipore, Billerica, MA, USA) with 8-μm polycarbonate filters was used after being pre-coated with Matrigel (2 mg/ml, 20 μl; BD Biosciences, Bedford, MA, USA) for 2 h at room temperature. VEGF-stimulated HUVECs ($4x10^3$ cells/0.4 ml culture medium) were seeded onto the upper compartment prior to 50, 100, 150 and 200 μM of kaempferol treatment for 24 h. The cells were then fixed with 4% paraformaldehyde in PBS and then stained with 2% crystal violet. The invading cells were counted under a light microscope before quantification with NIH ImageJ version 1.46 for Windows.

Tube formation assay. HUVECs were placed at a density of $5x10^4$ cells/well into 24-well flat-bottomed plates after Matrigel (BD Biosciences) pre-coating at 37˚C for 30 min. The VEGF-stimulated HUVECs ($5x10^4$ cells) thereafter were treated with or without 50, 100, 150 and 200 μM of kaempferol for 24 h. After exposure, HUVEC tube or network formation was evaluated using a phase-contrast microscope as previously described (33,34).

VEGFR-2, AKT and ERK1/2 kinase assay. VEGF-stimulated HUVECs ($5x10^6$ cells/75T flask) were incubated with or without 50, 100, 150 and 200 μM of kaempferol. After incubation for 6 h, the cells were lysed, and the activity of VEGFR-2, AKT and ERK1/2 kinase was determined in accordance with the manufacturer's instructions provided in the AKT Kinase Assay kit (Nonradioactive), the p44/42 MAP Kinase Assay kit (Nonradioactive) and the HTScan VEGF Receptor 2 Kinase Assay kit, respectively (Cell Signaling Technology, Inc., Danvers, MA, USA). Consequently, the purified samples were loaded on 12% SDS-PAGE to detect targeting proteins by immunoblotting analysis as previously described (35,36).

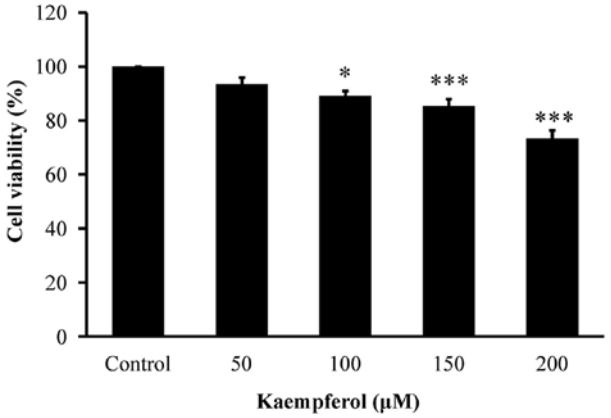

Figure 1. Kaempferol inhibits cell viability in VEGF-stimulated HUVECs. Cells were treated with 50, 100, 150 or 200 μM of kaempferol for 24 h and then viability was determined using the MTT assay. Data are plotted as the means ± SD (n=3). *P<0.05 and ***P<0.001 vs. the untreated control.

Western blot analysis. VEGF-stimulated HUVECs (5x10^{6} cells/75T flask) were exposed to 50, 100 and 200 μM of kaempferol for 6 h. After being harvested and lysed, the protein concentration was assessed with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Quantified protein lysates (40 μg) were subjected to 10-12% SDS-polyacrylamide electrophoresis (SDS-PAGE) gels to separate protein extracts as detailed by our previous studies (37,38). The primary antibodies (VEGFR-2, PI3K, p-AKT, AKT, p-mTOR, mTOR, p-MEK1/2, MEK1/2, p-ERK and ERK, at 1:1,000 dilution) were hybridized overnight at 4˚C, followed by the appropriate HRP-conjugated secondary antibodies (1:5,000 dilution) that were used before the electrochemiluminescence (ECL) reagent (Immobilon Western HRP substrate kit; Merck Millipore, Temecula, CA, USA). The densitometric quantification of each blot was carried out using NIH ImageJ 1.46 software.

Statistical analysis. The data are presented as the means ± standard deviation (SD) from at least three separate experiments. Statistical data was analyzed using Student's t-test, and statistical significance was considered to be P<0.05 and P<0.001.

Results

Kaempferol reduces HUVEC viability. First, VEGF-stimulated HUVECs after 0, 50, 100, 150 and 200 μM of kaempferol exposure for 24 h were assessed for growth inhibition and cytotoxicity. Our results indicated that kaempferol significantly decreased viable VEGF-stimulated HUVECs, and this effect was in a concentration- dependent manner (Fig. 1).

Kaempferol inhibits cell migration and invasion, as well as disrupts tube formation in VEGF-stimulated HUVECs. To explore the anti-angiogenic effects of kaempferol *in vitro*, its inhibitory influences on VEGF-induced tube formation and migration were investigated. Our data demonstrated that kaempferol concentration-dependently suppressed cell migration as determined by wound healing assay (Fig. 2A and B).

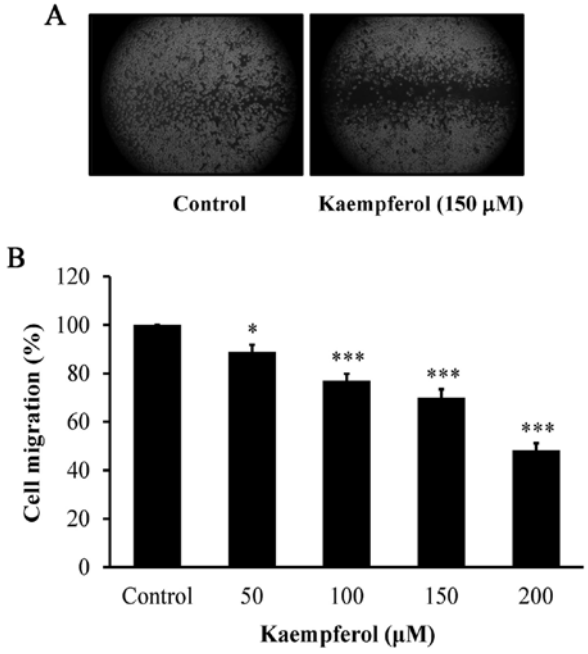

Figure 2. Kaempferol suppresses migratory potential of VEGF-stimulated HUVECs *in vitro*. (A) Images of the wound healing assay (x100, magnification) were performed after confluent monolayers were wounded. The wounded monolayer was incubated with 50, 100, 150 and 200 μM of kaempferol for 24 h. (B) Migration distances were quantified with ImageJ software. Each bar represents the mean ± SD in triplicate. *P<0.05 and ***P<0.001 vs. the VEGF-treated only control.

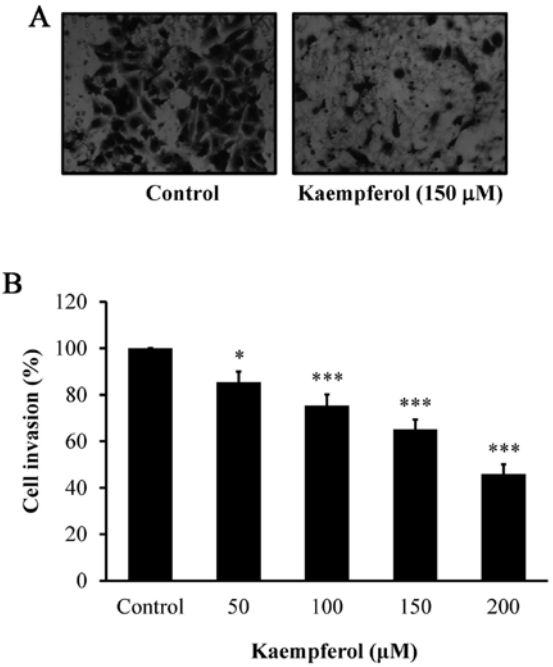

Figure 3. Kaempferol reduces VEGF-stimulated HUVEC invasion *in vitro*. (A) Effects of kaempferol treatment on the invasive ability of VEGF-stimulated HUVECs were analyzed by a Boyden chamber assay. (B) The number of invading cells was assessed as mentioned in Materials and methods. Each bar is representative of the mean ± SD of three independent samples for each treatment. *P<0.05 and ***P<0.001 vs. the VEGF-treated only control.

Kaempferol also significantly suppressed cell invasion (Fig. 3A and B) in a concentration-dependent manner.

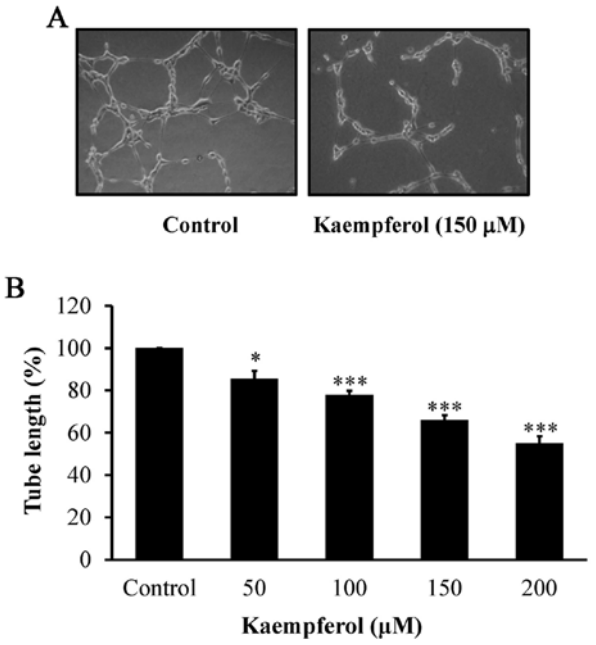

Figure 4. Kaempferol inhibits capillary-structure formation of VEGF-stimulated HUVECs. (A) The inhibitory effect of kaempferol on capillary tube formation in VEGF-stimulated HUVECs with Matrigel was assessed. (B) The level of tube length was detected as mentioned in Materials and methods. Each bar is representative of the mean ± SD of three independent samples for each treatment. *P<0.05 and ***P<0.001 vs. the VEGF-treated only control.

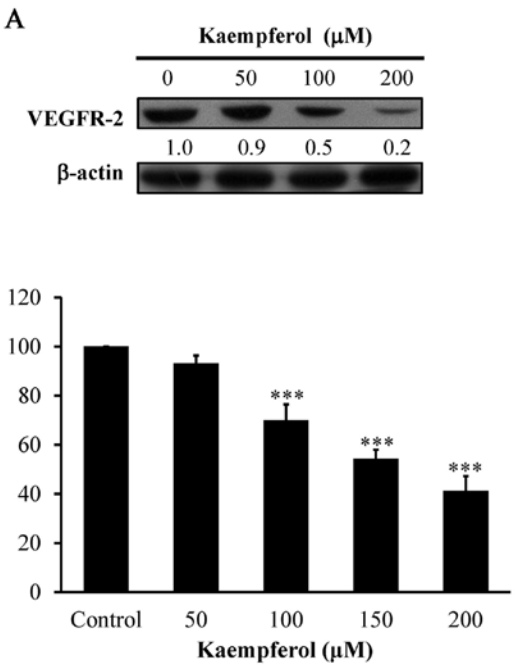

Figure 5. Kaempferol suppresses VEGFR-2 signaling in VEGF-stimulated HUVECs. (A) Cells were treated with 50, 100 and 200 μM of kaempferol for 6 h. Cells were then harvested, and the protein expression levels of associated VEGFR-2 were illustrated by western blot analysis. β-actin was employed as an internal control. (B) Cells were treated for 6 h and VEGFR-2 kinase activity was assessed as described in Materials and methods. The quantification of the data was performed, and the results represent the means ± SD (n=3). ***P<0.001 vs. the control.

To detect tube formation by endothelial cells, kaempferol at 50, 100, 150 and 200 μM was added for 24 h. The results revealed that kaempferol markedly disrupted the tube-like structures and network formation (Fig. 4A and B), and this effect was concentration-dependent. Therefore, we determined that kaempferol exhibits anti-angiogenic effects on VEGF-stimulated HUVECs *in vitro*.

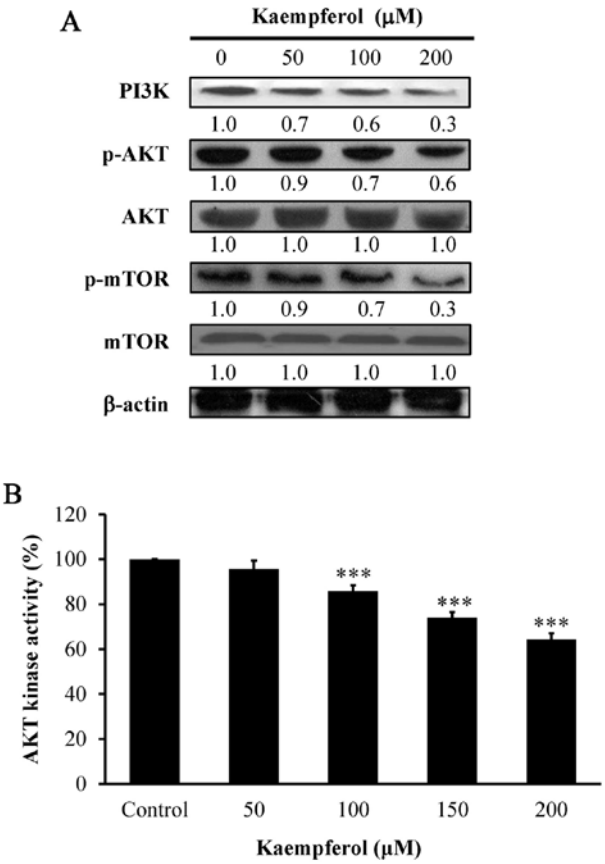

Figure 6. Kaempferol alters abundance of PI3K/AKT/mTOR signaling and inhibits AKT kinase activity in HUVECs. (A) Cells were exposed to 50, 100 and 200 μM of kaempferol for 6 h. Cells were then harvested and lysed before the levels of PI3K, p-AKT (Ser473), AKT, p-mTOR (Ser2448) and mTOR were detected by western blot analysis. β-actin was employed as an internal control. (B) Cells were incubated with kaempferol for 6 h, and AKT kinase activity was assessed. The results represent the the means ± SD (n=3). ***P<0.001 vs. the control.

Kaempferol suppresses VEGFR-2 signaling in VEGF-stimulated HUVECs. To clarify whether the angiogenic suppression requires VEGFR-2 signaling in kaempferol-treated HUVECs, the level of VEGFR-2 was detected. The protein level of VEGFR-2 (Fig. 5A) and kinase activity (Fig. 5B) were markedly suppressed by kaempferol exposure in a concentration-dependent manner. Our data demonstrated that kaempferol-inhibited angiogenesis may be involved in VEGFR-2 signaling in VEGF-stimulated HUVECs.

Kaempferol alters abundance of PI3K/AKT/mTOR signaling in VEGF-stimulated HUVECs. To determine the major pathway involved in the anti-angiogenic effect of kaempferol, we detected PI3K/AKT/mTOR signaling after kaempferol treatment at 6 h. Our results indicated that the protein levels of PI3K, and phosphorylation of both AKT and mTOR were significantly decreased in a concentration-dependent manner (Fig. 6A). The results revealed that PI3K/AKT/mTOR

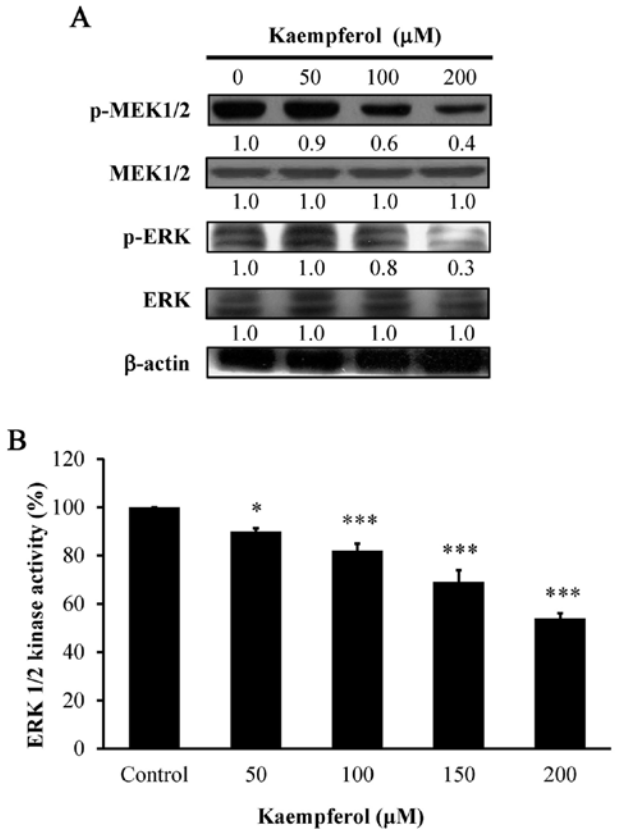

Figure 7. Kaempferol downregulates the levels of MEK and ERK signaling and suppresses ERK kinase activity in HUVECs. (A) Cells were exposed to 50, 100 and 200 μM of kaempferol for 6 h. The protein expression levels [p-MEK1/2 (Ser218/Ser222), MEK1/2, p-ERK (Thr202/Tyr204) and ERK] were revealed using immunoblotting analysis. β-actin was used as an internal control. (B) Cells were treated with kaempferol for 6 h, and ERK1/2 kinase activity was assessed. The results represent the means ± SD (n=3). *P<0.05 and ***P<0.001 vs. the control.

signaling contributed to the kaempferol-induced angiogenic effects on VEGF-stimulated HUVECs.

Kaempferol affects the protein levels of phosphorylation of MEK and ERK signaling in VEGF-stimulated HUVECs. We next aimed to clarify whether kaempferol-induced anti-angiogenesis in HUVECs was mediated mainly through phosphorylation of the MEK and ERK pathways. To demonstrate this, we further investigated the protein levels of phosphorylated MEK and phosphorylated ERK and determined that both were significantly decreased in a concentration-dependent manner after kaempferol exposure (Fig. 7A). The finding demonstrated that kaempferol attenuated angiogenesis on VEGF-stimulated HUVECs through the MEK1/2 and ERK pathways.

Kaempferol inhibits AKT and ERK kinases in VEGF-stimulated HUVECs. To determine whether AKT and ERK activities are involved in HUVECs, we assessed the kinase activities of AKT and ERK. AKT kinase (Fig. 6B) and ERK1/2 kinase (Fig. 7B) activities were concentration-dependently suppressed by kaempferol exposure. Therefore, we provide direct evidence that kaempferol inhibited angiogenic effects by blocking AKT and ERK signaling in VEGF-stimulated HUVECs.

Discussion

Flavonoids are important phytochemicals found in foods like fruits, vegetables, wine and tea (14-16,39). Notably, kaempferol is a flavonoid phytochemical, which exists in a variety of fruits and vegetables, including onions, kale, broccoli, apples, cherries, berries, tea and red wine (40,41). Kaempferol has multiple bioactivities, including antitumor effects, an antioxidant activity, and an anti-inflammatory function (17-20). In addition, kaempferol has induced apoptotic and autophagic cell death and/or cell cycle arrest in various tumor cell lines, including colon, liver, gastric and bladder cancer cells (25,27,42-47). Kim *et al* (48) first demonstrated that kaempferol modulated angiogenesis and immune-endothelial cell adhesion. Zhao *et al* (49) revealed that kaempferol from Pu-erh tea had anti-colorectal tumor cells and anti-angiogenesis effects on HUVECs. However, the target and molecular mechanism involved in the anti-angiogenic effects of kaempferol are still unknown. Notably, cell migration, invasion, tube formation and proliferation of endothelial cells are necessary processes during tumor angiogenesis (1,12,50). In the present study, we are the first to report that kaempferol at 50, 100, 150 and 200 μM inhibited VEGF-stimulated HUVEC cell proliferation (Fig. 1), inhibited cell migration (Fig. 2) and invasion (Fig. 3), and these effects were vital factors in angiogenic activity. Markedly, kaempferol inhibited tube formation (Fig. 4) in VEGF-stimulated HUVECs. Our results revealed that kaempferol triggered anti-angiogenic activity in VEGF-stimulated HUVECs, and this finding is in agreement with our previous study by our research group (29).

It is well known that vascular endothelial growth factor (VEGF) stimulates VEGF receptor further to activate its kinase activity which is a serious step in initiated tumor angiogenesis (51). Suppression of angiogenesis through the blocking of the VEGF/VEGFR signaling pathway has developed as a potential approach in antitumor therapy (51,52). VEGFR family members include KDR (kinase insert domain-containing receptor; VEGFR-2), FLT1 (Fms-like tyrosine kinase; VEGFR-1), and FLT4 (VEGFR-3) (51). VEGFR-2 binds VEGF-A, which is expressed in vascular endothelial cells and hematopoietic stem cells (53). In the present study, we focused on VEGFR-2 and its downstream signaling in kaempferol-treated HUVECs. Our results demonstrated that kaempferol triggered anti-angiogenic activity in VEGF-stimulated HUVECs by decreasing the VEGFR-2 protein level (Fig. 5A) and kinase activity (Fig. 5B). It has been documented that Y1175 and Y1214 in human VEGFR-2 are the main auto-phosphorylation sites following VEGF binding, and the activation of several downstream pathways, including PI3K/AKT and MEK/ERK levels (54,55). Our results revealed that kaempferol also reduced VEGFR-2 downstream protein levels, including PI3K, p-AKT, p-mTOR (Fig. 6A) and p-MEK1/2, p-ERK1/2 signaling (Fig. 7A). In addition, kaempferol also reduced AKT and ERK1/2 kinase activity (Figs. 6B and 7B). Our findings revealed that the kaempferol-inhibited angiogenic effects on VEGF-stimulated HUVECs may require VEGR-2 signaling.

In conclusion, these data clearly revealed the molecular signaling pathway in VEGF-stimulated HUVECs induced by kaempferol as summarized in Fig. 8. These findings provide

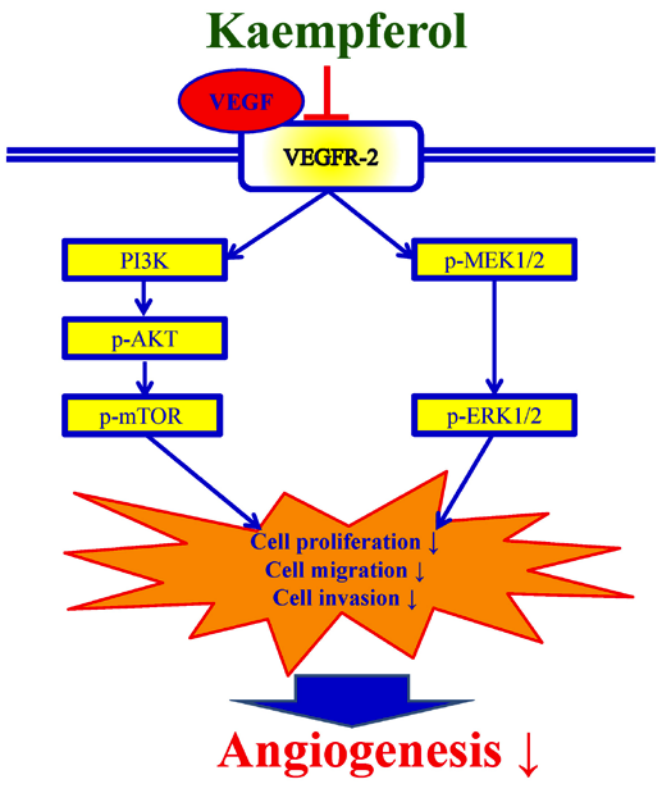

Figure 8. A model describing the molecular mechanism of kaempferol on the anti-angiogenic activity of VEGF-stimulated HUVECs.

evidence demonstrating the anti-angiogenic activity of kaempferol, and we suggest that kaempferol which is a phytochemical may act as an angiogenesis inhibitor for cancer treatment in the near future.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HKC, CTH, PCS and JSY conceived and designed the experiments. HKC, CTH, YSL and CCL performed the experiments. CYS, PSC, HYC and FJT analyzed the data. HKC, CTH, PCS, JSY and CCL wrote the paper and performed all the necessary modifications to the manuscript. All authors read, approved the final manuscript and agreed 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.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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