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Kaempferol suppresses proliferation but increases apoptosis and autophagy by up-regulating microRNA-340 in human lung cancer cells

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ABSTRACT

Background: Lung cancer is a leading cause of cancer-related deaths worldwide with unsatisfied prognosis. Kaempferol is a dietary flavonoid that inhibits tumorgenesis, and we aimed to uncover the underlying mechanism of kaempferol in lung cancer cells.

Methods: A549 cells were stimulated with kaempferol, and then cell proliferation, apoptosis, autophagy and expression of miR-340 were assessed. Subsequently, effects of kaempferol on protein expression of phosphatase with tensin homology (PTEN) and key kinases in the phosphatidylinositol-3-kinase (PI3K)/AKT pathways were detected by Western blot analysis. Moreover, the effects of miR-340 inhibition on kaempferol-induced alterations in A549 cells were also studied.

Results: Cell viability, proliferation and cyclinD1 expression level in A549 cells were all reduced by kaempferol. Conversely, cell apoptosis and autophagy were promoted by kaempferol. We found autophagy promoted apoptosis in kaempferol-treated A549 cells. Then, expression of miR-340 was identified to be up-regulated by kaempferol treatment. After treatments with kaempferol, PTEN level was elevated and levels of p-PI3K and p-AKT were decreased. Moreover, the alterations induced by kaempferol were abrogated by miR-340 inhibition.

Conclusion: Kaempferol inhibited proliferation but induced apoptosis and autophagy in A549 cells. Additionally, kaempferol might function through up-regulating miR-340, along with up-regulation of PTEN and inactivation of the PI3K/AKT pathway.

1. Introduction

Lung cancer is a commonly diagnosed lethal cancer, which is identified to be the leading cause of cancer-related deaths worldwide [1]. According to histological classification, lung cancer is categorized into non-small cell lung cancer (NSCLC) accounting for 85% of all lung cancers, and small-cell lung cancer (SCLC) [2]. Survival rate of patients with early stage NSCLC, who are received surgical resection, is higher than 80% [3]. However, most patients are diagnosed in advanced stages, resulting in a dismal 5-year-survival rate of less than 15% [4,5]. As reported, 1.8 million new cases and 1.6 million deaths occurred due to lung cancer in 2012 worldwide [6]. Therefore, there is a real pressure to develop more reliable and effective therapeutic strategies for treatment of lung cancer.

Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a natural dietary flavonoid that is abundant in multiple plants, such as apples, Brussels sprouts, broccoli, grapefruit and *etc.* [7]. Accumulating evidence has proven the anticarcinogenic action of kaempferol in diverse cancer types. For example, gastric cancer tumor growth is identified to be repressed both *in vitro* and *in vivo* [8]. In renal cell carcinoma, cell apoptosis and cell cycle arrest are reported to be induced by kaempferol [9]. Recently, kaempferol has been reported to suppress epithelial-to-mesenchymal transition and migration in A549 lung cancer cells [10]. Another study also reported that radiosensitization of A549 cells was enhanced by kaempferol [11]. Those literatures suggested the potential anti-tumor capacity of kaempferol in lung cancer; however, the underlying mechanisms remain unclear.

MicroRNAs (miRNAs/miRs) are single-stranded RNA that post-transriptionally regulate gene expression and thereby participate in tumor progression of diverse cancers, including lung cancer [12,13]. For example, miR-27a acts an oncogenic role in lung cancer through regulating SMAD2 and SMAD4 [14]. Conversely, miR-769-5p acts as a tumor suppressor in lung cancer through repressing proliferation, migration and invasion [15]. Currently, miR-340 is widely reported as a tumor suppressor in gastric cancer [16], NSCLC [17] and glioblastoma [18] through inhibiting cell proliferation and inducing cell apoptosis.

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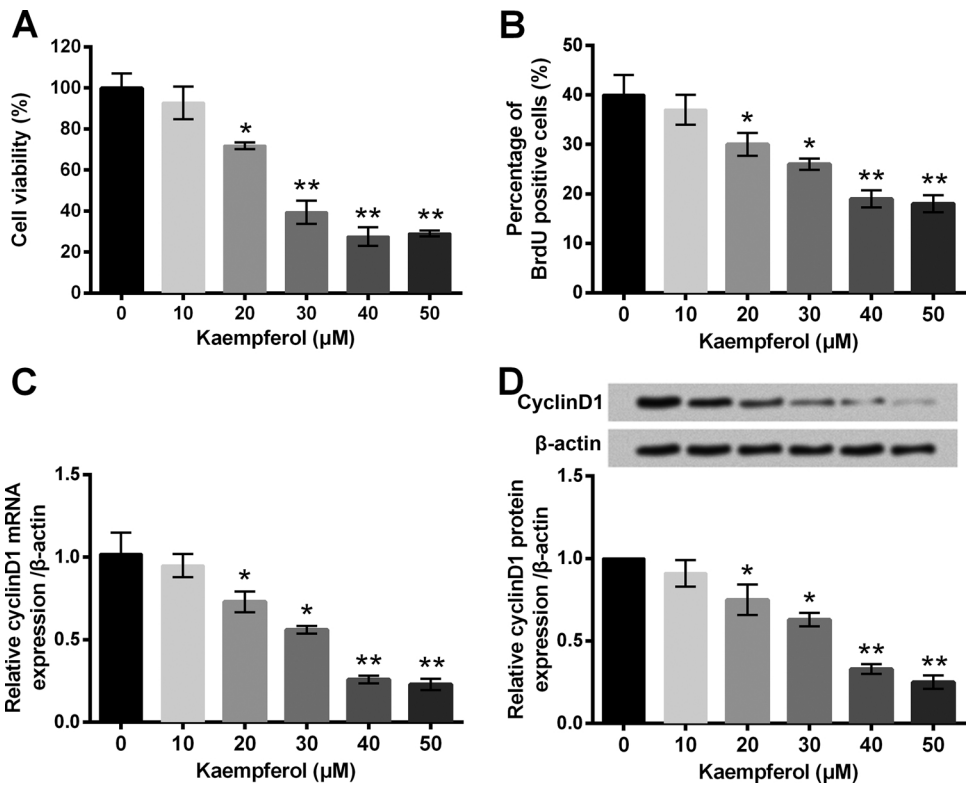

Fig. 1. Kaempferol inhibited A549 cell proliferation.
A549 cells were stimulated with diverse concentrations of kaempferol (0, 10, 20, 30, 40 and 50 μM). **A.** Cell viability by CCK-8 assay. **B.** Cell proliferation by BrdU assay. mRNA **(C)** and protein **(D)** expression of cyclinD1 was measured by RT-qPCR and Western blot analysis, respectively. Data are presented as the mean ± SD (n = 3). *, $P < 0.05$; **, $P < 0.01$.

Shi et al. have attributed the anti-tumor effects of miR-340 to the association with enhancer of zeste homolog 2 (EZH2) [19]. Similarly, a previous study has proven that kaempferol suppresses migration of vascular smooth muscle cells through regulating miR-21 [20]. Moreover, Ginkgo biloba extract which contains kaempferol has been proposed to inhibit colorectal cancer through associating with EZH2 [21]. Therefore, we hypothesized that there might be a correlation between kaempferol and miR-340 in lung cancer cells.

In our study, the roles of kaempferol in proliferation, apoptosis and autophagy of human A549 lung cancer epithelial cells were tested. Furthermore, the interaction between miR-340 and kaempferol as well as the underlying mechanisms was also investigated.

2. Materials and methods

2.1. Cell culture and treatments

Human lung cancer epithelial cell line A549 was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in ATCC-formulated F-12 K medium containing 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY, USA). Cell growth was performed in a humidified incubator, which was filled up with an atmosphere of 5% CO_2 and 95% air, at 37 °C. Kaempferol, purchased from Sigma-Aldrich (St. Louis, MO, USA), was dissolved in dimethyl sulphoxide (DMSO) to make stock solution (50 mM). For kaempferol stimulation, cells were incubated in FBS-free medium containing 10, 20, 30, 40 or 50 μM kaempferol, and the final concentration of DMSO in culture medium was 0.1%. To inhibit autophagy, cells were treated with 5 mM 3-methyladenine (3-MA; Sigma-Aldrich) for 1 h prior to kaempferol stimulation. Cells treated with an equal amount of DMSO were acted as control.

2.2. Cell viability assay

Cell viability was assessed by using Cell Counting Kit-8 (CCK-8) assay. In brief, 5×10^3 cells (transfected or untransfected) were seeded into each well of 96-well plates and were maintained at 37 °C. After stimulation with diverse concentrations of kaempferol, 10 μl CCK-8 solution (Dojindo Molecular Technologies, Gaithersburg, MD, USA) was added into each well, and then the 96-well plates were incubated at 37 °C for 1 h. Data of absorbance at 450 nm were collected using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

2.3. Proliferation assay

Cell proliferation was tested by using Bromodeoxyuridine (BrdU) incorporation assay. In brief, 2×10^3 cells (transfected or untransfected) were seeded into each well of 96-well plates and were maintained at 37 °C overnight. After stimulation with diverse concentrations of kaempferol, 20 μl BrdU from the BrdU Cell Proliferation ELISA Kit (Abcam, Cambridge, UK) was added into each well, followed by incubation at 37 °C for 3 h. Then, cells were fixed with Fixing Solution and were incubated with anti-BrdU and peroxidase-conjugated goat anti-mouse IgG in turns according to the manufacturer's instructions. Subsequently, 100 μl of TMB Peroxidase substrate was added into each well, followed by incubation at room temperature in the dark for 30 min. After addition of Stop Solution, absorbance was read at a dual wavelength of 450/550 nm. The color of positive wells was bright yellow.

2.4. Apoptosis assay

Cell apoptosis was measured by using double-staining with fluorescein isothiocynate (FITC)-conjugated Annexin V and propidium iodide (PI). In brief, after treatment with diverse concentrations of Kaempferol, 1×10^5 cells (transfected or untransfected) were collected, washed and resuspended in binding buffer. Then, cells were stained with Annexin V-FITC and PI from the Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China) according to the manufacturer's instructions. Treated cells were subjected into an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA), and the data were analyzed by using the FlowJo software (Tree Star, San Carlos, CA,