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Original Research Article

Kaempferol inhibits the proliferation and migration of Epstein-Barr virus-positive diffuse large B-cell lymphoma cells

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Abstract

Purpose: *To assess the effect of kaempferol on the growth, apoptosis, and motility of Epstein-Barr virus (EBV)-positive diffuse large B-cell lymphoma (DLBCL) cells, and to elucidate its mechanism of action.*
Methods: *Human DLBCL cells were used to generate EBV-positive (EBV +) cell line Pfeiffer. CCK-8, colony formation, flow cytometry (FCM), and immunoblot assays were employed to determine the effects of kaempferol on the growth and apoptosis of EBV + DLBCL cells. Cell motility was evaluated by Transwell and immunoblot assays. Immunoblot assay was further conducted to unravel the mechanism of action.*
Results: *Kaempferol inhibited the growth of EBV + DLBCL cells. It also enhanced the apoptosis of EBV + DLBCL cells, but inhibited the motility of the cells. Furthermore, kaempferol inhibited PI3K/AKT axis, thereby suppressing DLBCL.*
Conclusion: *Kaempferol inhibits the growth and motility of EBV + DLBCL cells via PI3K/AKT axis. It is therefore a potential drug for the treatment of DLBCL.*

Keywords: *Epstein-Barr virus (EBV), Diffuse large B-cell lymphoma (DLBCL), Kaempferol, Apoptosis, PI3K/AKT*

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INTRODUCTION

Epstein-Barr virus (EBV) was the first virus correlated with cancers. EBV causes a variety of cancers such as nasopharyngeal and stomach cancers [1], and is closely associated with the onset of diffuse large B-cell lymphoma (DLBCL) [2]. DLBCL is the most common type of non-Hodgkin's lymphoma, accounting for 30 - 40 % of cases in different geographical regions [3]. Although the prognosis for DLBCL has improved overall, approximately one third of patients will experience relapse/refractory disease, and remains a leading cause of morbidity and death [4]. To enhance therapy, new therapeutic agents need to be developed to improve the cure rate of DLBCL. Kaempferol is a natural flavonoid with multiple pharmacological activities, including anticancer, anti-inflammatory, and anti-apoptotic effects [5]. Kaempferol has good antibacterial activity, and also has an effect on methicillin-

resistant Staphylococcus aureus (MRSA) [6]. Studies have shown that the anticancer effect of kaempferol in A375 cells is mediated by inducing apoptosis, cell cycle arrest, and inhibition of cell motility [7]. Kaempferol enhances the sensitivity of pancreatic cancer cells to erlotinib by restraining PI3K/AKT pathway as well as EGFR [8]. It increases the apoptosis of HeLa cells through PI3K/AKT and telomerase pathways. Kaempferol also reduces OX-LDL-induced apoptosis by blocking the up-regulation of autophagy in the PI3K/Akt /mTOR pathway in endothelial cells [9]. However, the mechanism of action of kaempferol in DLBCL remains unclear. There is evidence that PI3K/Akt is vital in EBV-induced malignancies. The major EBV oncoprotein latent membrane protein (LMP) 1 and LMP2A activate PI3K/Akt axis, thereby affecting cell survival, apoptosis, and genomic instability [5]. Its downstream target protein is also known to cause cancer, and its role in DLBCL has also been demonstrated. This study aims to examine the effect of kaempferol on DLBCL, and its potential clinical significance in the management of the disease.

EXPERIMENTAL

Cell culture and drug treatment

The DLBCL cell line Pfeiffer as well as B95-8 were purchased from ATCC and maintained in DMEM (Gbico, USA) supplemented with 10 % of FBS and incubated at 37 °C in a 5 % CO_2 incubator.

Construction of EBV $^+$ DLBCL model

One milliliter of the culture supernatant of B95-8 was centrifuged at 14,000 rpm at 4 °C for 90 min. An aliquot of 900 µL supernatant is removed, and the remaining 100 µL in the bottom was diluted to a final volume of 5 ml and used to maintain Pfeiffer cells for 72 h. Kaempferol (Sigma) was added to Pfeiffer cells at a concentration of 5/10/20 µM and kept for 24 h.

CCK-8 assay

The Pfeiffer cells were placed into 96-well plates, maintained for 48 h, incubated with CCK-8 (Beyotime, China) for 4 h, and the absorbance measured spectrophotometrically by multifunctional enzyme marker (VarioskanLUX, Thermo, USA) at a wavelength of 450 nm.

Colony formation assay

Pfeiffer cells (1000/well) were placed in 6-well plates as well as maintained in media (10 % FBS) for 14 days at 37 °C. The cells were fixed with PFA for 20 min and stained with 0.1 % crystal violet for 20 min, and then photographed by a fluorescence microscope (Axio A1, Zeiss, Germany).

Cell apoptosis assay

The Pfeiffer cells were fixed using 70 % ethanol. To determine cell apoptosis, the cells were stained with PI and FITC Annexin V at 4 °C and the levels of apoptosis were measured using FACSCalibur flow cytometer (BD Biosciences, Inc).

Transwell assay

The Pfeiffer cells were plated into the upper chambers with 20 % matrigel (invasion test) or without any matrigel (migration test) in DMEM (Gbico, USA) without any serum. After 24 h, the remaining cells were fixed and stained with the 0.1 % crystal violet for 15 min, and then photographed by a fluorescence microscope (Axio A1, Zeiss, Germany). The cell numbers were counted.

Immunoblot assay

Samples were separated by SDS-PAGE, and then transferred to a PVDF membrane. The proteins were blocked with 5 % milk for 1 h, and primary antibodies including Bax (Abcam, ab32503; 1:1000), Bcl-2 (Abcam, ab182858; 1:1000), cleaved caspase-3 (Abcam, ab32042; 1:1000), Akt (Abcam, ab8805; 1:1000), p-Akt (T308, Abcam, ab38449; 1:500), PI3K (Abcam, ab302958; 1:1000), p-PI3K (Abcam, ab278545; 1:500), GAPDH (Abcam, ab8245; 1:3000) were added as well as incubated at 4 ℃ overnight. The secondary antibodies were incubated for 1 h and photographed after chemiluminescence.

Statistical analysis

GraphPad 5.0 software was used for data analysis. The data are presented as mean ± SD. The data among groups were analyzed by student's t test. $P < 0.05$ was considered statistically significant.

RESULTS

Kaempferol inhibits the growth of EBV + DLBCL cells

The chemical structure of Kaempferol is shown in Figure 1 A. The results showed that EBV treatment stimulated the growth of EBV + Pfeiffer cells (Figure 1 B). Treatment with kaempferol

suppressed the growth of EBV + Pfeiffer cells ($p < 0.05$, Figure 1 B). EBV treatment increased the colony numbers of Pfeiffer cells, whereas kaempferol treatment suppressed the proliferation of the cells after EBV treatment ($p < 0.05$; Figure 1 C). Thus, kaempferol inhibited the growth of EBV + DLBCL cells.

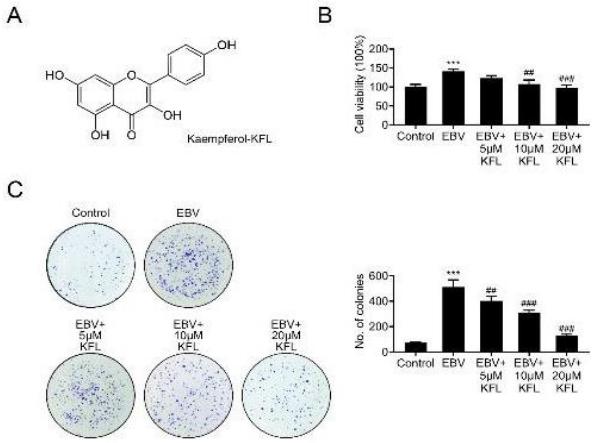

Figure 1: Kaempferol inhibits the growth of EBV + DLBCL cells. (A) Chemical structure of kaempferol; (B) Growth of Pfeiffer cells upon treatment with EBV and kaempferol at concentrations of 5, 10, and 20 uM for 24 h. (C) Proliferation of Pfeiffer cells upon the treatment with EBV and Kaempferol at the concentration of 5, 10, and 20 uM for 24 h. Data are presented as mean ± SD; ***$p < 0.001$, EBV vs control, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, EBV + kaempferol vs EBV

Kaempferol enhanced apoptosis of EBV + DLBCL cells

The results revealed that EBV treatment suppressed the apoptosis of Pfeiffer cells (Figure 2 A). However, treatment with kaempferol stimulated Pfeiffer cell apoptosis after EBV treatment ($p < 0.05$; Figure 2 A). Similarly, immunoblot assay data showed that EBV treatment increased Bcl-2 expression, but decreased Bax and cleaved caspase-3 expressions ($p < 0.05$, Figure 2 B). On the hand, treatment with kaempferol lowered the expression of Bcl-2, but increased the expression of Bax and cleaved caspase-3 in Pfeiffer cells after EBV treatment ($p < 0.05$; Figure 2 B). Thus, kaempferol increased the apoptosis of EBV + DLBCL cells.

Kaempferol inhibited the migration and invasion of EBV + DLBCL cells

Cell motility is vital in the progression of DLBCL. Transwell assay results showed that EBV treatment stimulated motility of Pfeiffer cells ($p < 0.05$; Figure 3 A and B). However, kaempferol treatment decreased both the migration and invasion of Pfeiffer cells after EBV treatment, suggesting the suppression of motility in EBV + DLBCL cells ($p < 0.05$; Figure 3 A and B). Thus, kaempferol inhibits the migration and invasion of EBV + DLBCL cells.

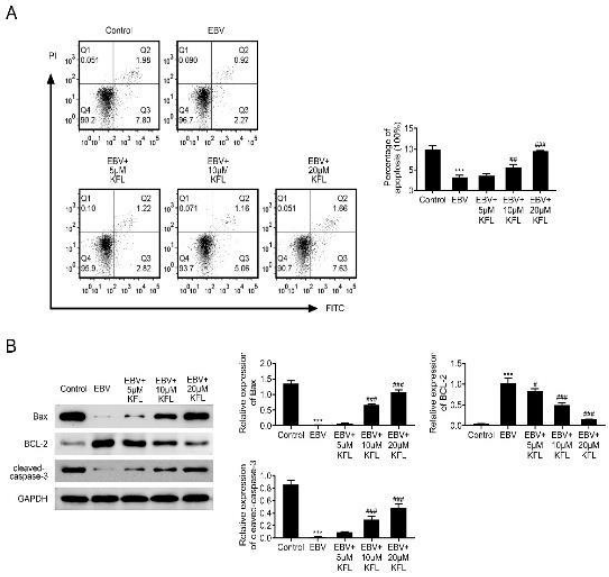

Figure 2: Kaempferol enhances apoptosis of EBV $^{+}$ DLBCL cells. (A) Apoptosis of Pfeiffer cells after treatment with EBV and kaempferol at concentrations of 5, 10, and 20 uM for 24 h. (B) Expression of Bax, Bcl-2, and cleaved caspase 3 in Pfeiffer cells upon the treatment with EBV and kaempferol at concentrations of 5, 10, and 20 uM for 24 h. Data are presented as mean ± SD; ***$p < 0.001$, EBV vs control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, EBV + kaempferol vs EBV

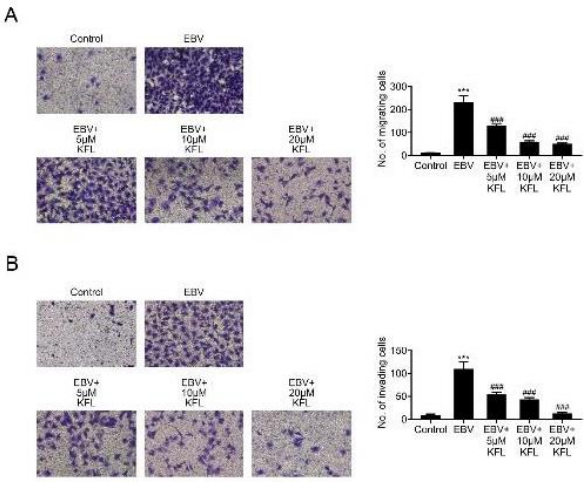

Figure 3: Kaempferol inhibits the migration and invasion of EBV $^{+}$ DLBCL cells. (A) Migration of Pfeiffer cells upon treatment with EBV and kaempferol at concentrations of 5, 10, and 20 uM for 24 h. (B) Invasion of Pfeiffer cells upon treatment with EBV and kaempferol at concentrations of 5, 10, and 20 uM for 24 h. Data are presented as mean ± SD; ***$p < 0.001$, EBV vs control, $^{\#\#\#}p < 0.001$, EBV + kaempferol vs EBV

Kaempferol inhibits PI3K/AKT axis in EBV + DLBCL cells

EBV treatment increased the phosphorylation of PI3K and AKT in Pfeiffer cells ($p < 0.05$; Figure 4). However, Kaempferol treatment suppressed PI3K levels and AKT phosphorylation in Pfeiffer cells after EBV treatment ($p < 0.05$; Figure 4), indicating the regulatory effect of kaempferol on PI3K/AKT pathway in EBV$^+$ DLBCL cells. Therefore, kaempferol inhibited PI3K/AKT axis in EBV + DLBCL cells.

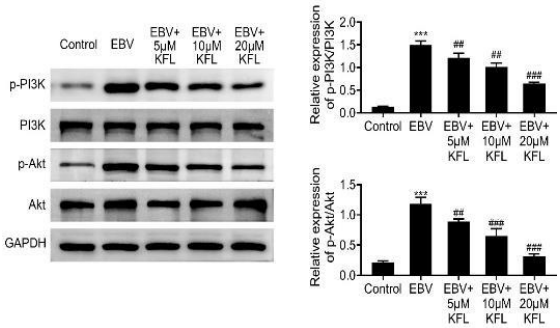

Figure 4: Kaempferol inhibits the PI3K/AKT axis in EBV + DLBCL cells. Expression and phosphorylation levels of PI3K and AKT in Pfeiffer cells upon treatment with EBV and kaempferol at concentrations of 5, 10, and 20 uM for 24 h. Data are presented as mean ± SD, $^{***}p < 0.001$, EBV vs control, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, EBV + kaempferol vs EBV

DISCUSSION

DLBCL is a heterogeneous malignancy of the lymphatic system [10]. Treatment options for DLBCL are varied, including chemotherapy, radiation and immunotherapy. After treatment with standard R-CHOP, the survival period of more than 60 % of patients is significantly extended, but there is still disease recurrence or refractory condition in 30 – 40 % of patients for whom prognosis is poor [11]. Following further research on the heterogeneity and molecular mechanism of DLBCL, some novel molecularly-targeted drugs have been generated. In addition, new chemotherapy schemes and new drugs are emerging, bringing new hope for individualized precision treatment of relapsed and refractory DLBCL [6].

Epstein-Barr virus (EBV) is an important pathogenic factor of lymphoma, and is closely related to the clinical outcome of DLBCL [12]. In the present study, kaempferol inhibited the proliferation and migration of EBV-positive DLBCL cells, and hence it can be used as a drug to combat DLBCL. The results also showed that kaempferol suppresses the growth of EBV$^+$ DLBCL cells, stimulates apoptosis, and inhibits the motility of the cells. Thus, kaempferol acts as a DLBCL drug; it is a flavonoid widely found in leaves used in a variety of plant-derived foods and traditional medicines [9]. Studies have demonstrated that kaempferol has anti-cancer, fertility inhibition, anti-epilepsy, anti-inflammatory, antioxidant, anti-ulcer, cough suppressant and other properties [13]. It also significantly inhibits NADPH oxidase activity, and reduced reactive oxygen species (ROS) by directly binding to NADPH oxidase [5]. Kaempferol prevents Ang II-induced SA node cell death by reducing CAMKII oxidation.

Prospective studies have shown that the consumption of kaempferol significantly reduces the risk of ovarian cancer; 24 h of treatment with kaempferol suppressed the proliferation of cells [14]. In the present study, its anti-growth effects on DLBCL cells was found. Kaempferol also has anti-inflammatory effects, as it inhibits the expression of IL-4 and cyclooxygenase 2 by inhibiting Src kinase, and down-regulates the NF-κB pathway [15]. It inhibits angiogenesis and induces apoptosis of ovarian cancer cells.

In the present study, kaempferol suppressed the growth of DLBCL cells by targeting PI3K/AKT axis. The functions of PI3K/AKT axis include promoting cell metabolism, growth, survival and participation in the occurrence of cancer, chemotherapy resistance, and the regulation of angiogenesis [16]. Approved PI3K/Akt inhibitors, copanlisib, capivasertib, and ipatasertib are particularly effective against follicular lymphoma, ovarian cancer, breast cancer, and prostate cancer. DLBCL is often associated with the continued activation of PI3K/AKT axis [17]. NVP-BEZ235 is an inhibitor of PI3K that induces the apoptosis of DLBCL cells by inhibiting the expression of MCL-1, and inducing dephosphorylation of 4EBP1 [18]. On the other hand, kaempferol inhibits DLBCL by inhibiting PI3K/AKT axis.

CONCLUSION

Kaempferol blocks the growth of EBV$^+$ DLBCL cells, stimulates apoptosis, and restrains the motility of EBV$^+$ DLBCL cells by targeting PI3K/AKT axis. Therefore, it has a potential to be developed as a therapeutic agent for the management of DLBCL.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Suli Lu, Zhen Wang and Dae-jung Yang designed the study and carried them out, supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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