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Kaempferol suppresses proliferation and induces apoptosis and DNA damage in human gallbladder cancer cells through the CDK4/CDK6/cyclin D1 pathway

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Abstract. – OBJECTIVE: Kaempferol has been reported to play an anti-tumor role in various human cancers, while its role in gallbladder cancer (GBC) is unclear.

MATERIALS AND METHODS: We found that kaempferol significantly inhibited the growth, invasion and migration, meanwhile induced apoptosis through cells arrested at G0/G1 phase of GBC cell lines, including GBC-SD and SGC996 cells in vitro.

RESULTS: Kaempferol promoted the release of cytochrome C from the mitochondria to cytoplasm, the activation of c-caspase-3 and c-caspase-9 and increased the expression levels of pro-apoptotic factor Bax, meanwhile decreased the expression levels of anti-apoptotic factor Bcl-2. In addition, the expression levels of CDK4, CDK6 and cyclin D1, which are members of the CDK4/CDK6/cyclin D1 signaling pathway, were also decreased by kaempferol. Moreover, kaempferol could efficiently prevent tumor progression of GBC in the xenograft in vivo.

CONCLUSIONS: Our results demonstrated that kaempferol suppressed GBC progression through activation of the CDK4/CDK6/cyclin D1 signaling pathway, suggesting that it might be a potential anti-tumor agent for clinical treatment of GBC.

Key Words:
GBC, Kaempferol, Cell viability, Apoptosis.

Introduction

Gallbladder cancer (GBC) is a rare biliary tract malignancy in developed countries and is more common in some regions of developing countries in the world[1]. According to the data from Mapuche Indians from Valdivia, Chile and South America, the incidence rate of GBC is approximately 12.3/100,000 for males and 27.3/100,000 for females[2]. Symptoms of GBC may be misdiagnosed as biliary colic and over half of new diagnoses are made after laparoscopic cholecystectomy for presumed benign disease[3]. The early development of GBC has a high propensity to metastatic dissemination; thus, most patients are diagnosed at intermediate to advanced stages[4]. The lack of evident symptoms of GBC at initial stage results in difficulties in the treatment of GBC[5]. Therefore, it is necessary to explore novel biomarkers and to of

GBC. Kaempferol is a natural flavonoid compound found in some vegetables and fruits with a wide range of pharmacological activities[6-8]. Kaempferol has been identified to exert anti-cancer function in a series of biological processes in different human cancers. In gastric cancer, kaempferol can induce autophagic cell death by activating the IRE1/JNK/CHOP pathway[9]. In ovarian cancer, kaempferol can induce apoptosis and G0/G1 cell cycle arrest by inhibiting the MEK/ERK and STAT3 signal transduction pathways[10]. In pancreatic cancer, kaempferol inhibits cell growth and migration *via* blocking the EGFR-related pathway *in vitro*[11]. In liver cancer, kaempferol was identified to inhibit proliferation, invasion and migration of HepG2 cells through downregulating microRNA-21, which negatively regulated the expression of PTEN[12]. Although the anti-tumor activity of kaempferol has been studied in many human cancers, its role and mechanism in GBC remains unclear.

The process of programmed cell death, apoptosis[13,14], is a complex process involved in the activation of a group of cysteine proteases called

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"caspases"[15]. Bcl-2 and Bax are two apoptosis-related proteins. Bcl-2 is an anti-apoptosis factor[16] and Bax is a pro-apoptotic factor[17]. Cyclin D1 and Cyclin-dependent kinase (CDK) 4/6 are primary mitogens in the G1 phase that is closely involved in cell division[18]. Scholars[19,20] demonstrated that downregulation of Cyclin D1 and CDK4/6 can result in the block of G0/G1 transition, which then causes the apoptosis and inhibitory migration of cancerous cells. It has been reported that the CDK4/CDK6/cyclin D1 pathway is involved in the progression of human cancers. MiR-9 can induce cell arrest and apoptosis of oral squamous cell carcinoma *via* the CDK 4/6 pathway[21]. LEE011, a novel inhibitor of both CDK4 and 6, can inhibit cell proliferation and induce G1 arrest and cellular senescence in leukemia cells[22]. The relationship between kaempferol and cell apoptosis in GBC has not been studied.

In the present study, we found that kaempferol significantly inhibited the growth and promoted apoptosis by inducing DNA damage and inhibiting activation of the CDK4/CDK6/cyclin D1 axis *in vitro*. Moreover, our results demonstrated that kaempferol could efficiently prevent GBS progression *in vivo*, suggesting that kaempferol might be a potential anti-tumor agent for the clinical therapeutics of GBC.

Materials and Methods

Reagent

Kaempferol (purity > 98%) was obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and used for the subsequent experiments. All of the other solvents used in this study were commercially available. The structural formula of kaempferol was shown in **Supplementary Figure 1**.

Cell Culture

Human gallbladder cancer cell lines SGC996 and GBC-SD were purchased from American Type Tissue Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured with Roswell Park Memorial Institute-1640 (RPMI-1640) Me-

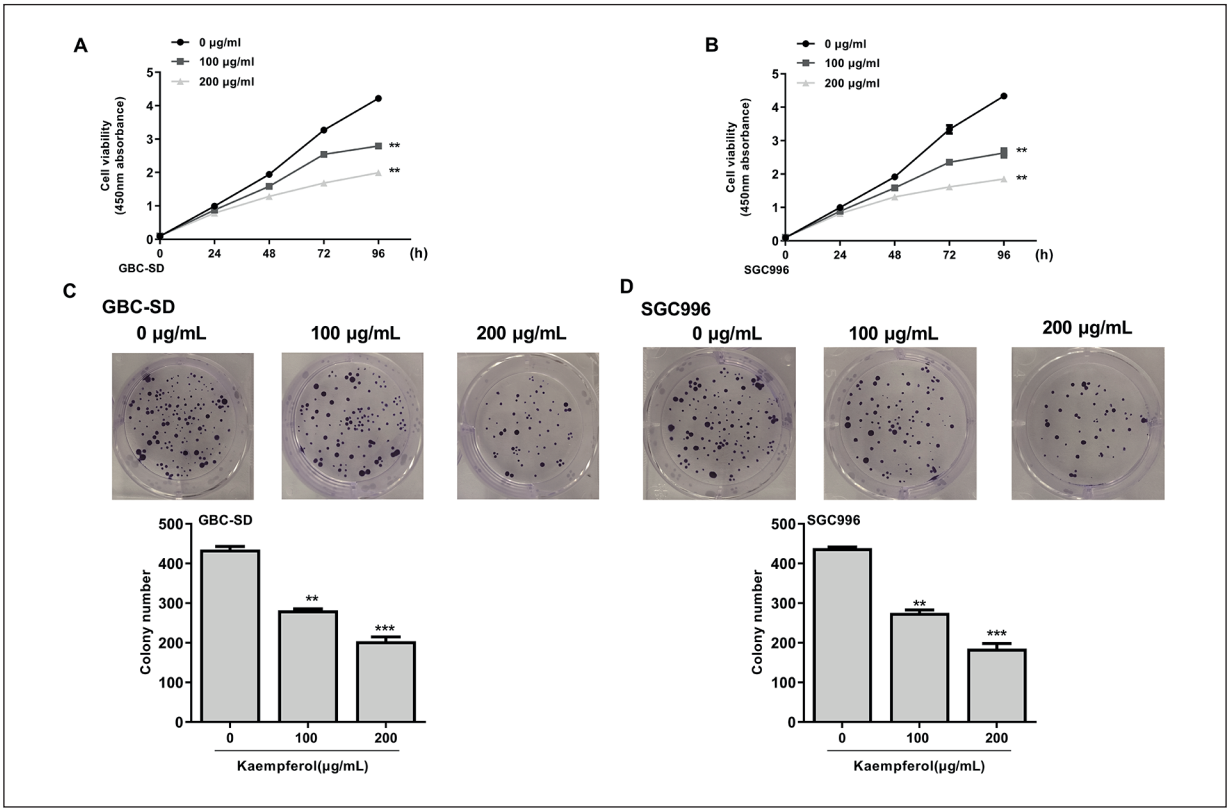

Figure 1. Effect of kaempferol on the growth of GBC-SD and SGC996 cells. **A,** and **B,** The cell viability of GBC-SD (**A**) and SGC996 cells (**B**) was evaluated by CCK-8 assay. **C,** and **D,** The colonies of GBC-SD (**C**) and SGC996 cells (D) was evaluated by colony formation assay. N = 3, * $p < 0.05$ and ** $p < 0.01$.

dium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 100 units/mL penicillin (Life Technologies, Grand Island, NY, USA) and 0.1 mg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 95% humidity and 5% CO_2.

CCK-8 Assay

Approximately 1×10^3 cells/well of GBC-SD and SGC996 cells were plated into 96-well plates and treated with different concentrations of kaempferol (0, 100 and 200 μg/mL) for different time periods. The cell viability was detected by Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Haimen, China). The absorbance at 450 nm was measured with a microplate reader (BioTek Instruments, Winooski, VT, USA).

Colony Formation Assay

Approximately 1,000 of GBC-SD and SGC996 cells were seeded into 6-well plates and treated with different concentrations of kaempferol for two weeks. Cells were then washed with fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), fixed in methanol and stained with 1% crystal violet dye (Sigma- Aldrich, St. Louis, MO, USA). The colony formation was observed and counted with a light microscope (Olympus, Tokyo, Japan).

Transwell Assay

Transwell assay was performed as previously described[11]. For migration assay, cells were plated into serum-free Dulbecco's modified Eagle medium (DMEM, Gibco, Rockville, MD, USA) and added into the upper chamber. The mixture of DMEM with 10% FBS was added into the lower chamber. Cells were incubated for 24 h. Cells on the upper surface of the upper chamber were removed and cells on the lower surface of the upper chamber were stained with 0.1% crystal violet (Millipore, Billerica, MA, USA) for 15 min. The number of migrated cells was calculated under a microscope (Olympus, Tokyo, Japan). For invasion assay, the procedures were the same as cell migration assay except that the upper chamber was pre-coated with Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA).

Cell-Cycle Analysis

Approximately 1×10^5 cells were plated in 6-well plates and treated with indicated concentrations of kaempferol. The adherent and floating cells were washed in FBS (Invitrogen, Carlsbad, CA, USA) and fixed in ice-cold 70% ethanol at 4°C overnight. After washing, cells were incubated with 50 μg/mL propidium iodide (PI, Thermo Fisher Scientific, Waltham, MA, USA) solution supplemented with 100 μg/mL RNaseA (Qiagen, Hilden, Germany) at 37°C for 30 min in the dark. Cell cycles were examined by a FACSCalibur cytometry (Becton Dickinson, NJ, USA), and analyzed by Beckman CytoFLEX FCM (Beckman, Brea, CA, USA).

Apoptosis Analysis

The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences, Franklin Lakes NJ, USA) was used to assess cell apoptosis. In brief, transfected GBC cells were cultured for 48 h. After washing, transfected GBC cells were resuspended in the binding buffer. Afterward, Annexin V-FITC and PI were supplemented to binding buffer with transfected GBC cells and incubated for 15 min in the dark. Lastly, the FACScan flow cytometry (BD Biosciences, Franklin Lakes NJ, USA) was applied to determine the apoptosis rate of transfected GBC cells.

Western Blot

Total proteins were extracted from cultured cells and tumor tissues using radio-immunoprecipitation assay buffer (RIPA; Beyotime Institute of Biotechnology, Haimen, China). Equal amount of protein samples were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF, Millipore, Billerica, MA, USA) membranes. The membranes were then blocked with 5% non-fat milk and incubated with primary antibodies including CDK4, CDK6, cyclin D1, γH2AX, p-γH2AX, c-caspase-9, c-caspase-3, Bcl-2, Bax, Cyto c and β-actin with ratio at 1:1,000 at 4°C overnight. β-actin was used as the internal reference. Subsequently, the membranes were incubated with horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (IgG, Cat. No. ab6789; 1:2,000; Abcam) for 1 h. Protein bands were quantified using enhanced chemiluminescence (ECL; Keygentec, Nanjing, China) and observed by ChemiDoc™ XRS systems (Bio-Rad, Hercules, CA, USA).

DAPI Staining Assay

Approximately 1×10^5 cells/well were plated into 6-well plates and treated with indicated concentrations of kaempferol for 48 h. Then, the

nuclear morphology of cells was observed by 4',6-diamidino-2-phenylindole (DAPI) staining assay as previously described[23] under an inverted fluorescence microscope (Olympus, Japan).

Xenograft In Vivo Animal Experiment

All animal experiments were performed according to the National Institutes of Health Animal Use Guidelines and the current Chinese Regulations and Standards for the Use of Laboratory Animals. This study was approved by the First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology. BALB/c mice (4-week-old female) were subcutaneously injected with 100 µL GBC-SD cells (approximately 2×10^6 cells) *via* a sterile microsyringe. Mice were randomly divided into two groups: the model group and kaempferol group when tumor volume reached about 40 mm^3. Subsequently, the kaempferol group were intragastrically administered with 200 mg/kg kaempferol, and the model group were treated with equal volume of saline as control. Tumor volumes were measured using the following formula V = length × $width^2/2$ every week for 5 weeks. At the end of experiments, mice were sacrificed by cervical dislocation, then the wight and size of tumor were measured.

Ki-67 Staining Assay

The Xenograft tumors were removed from mice, fixed in 4% formaldehyde (Life Technologies, Grand Island, NY, USA), and embedded in paraffin. The paraffin sections were mounted on slides and immunostaining was performed using an anti-Ki67 antibody (Santa Cruz Biotechnology) as previously described[24].

Statistical Analysis

All experiments were repeated at least three times and data were presented as the mean ± standard deviation (SD). SPSS software (version 18.0) was used for statistical analyses. The differences between two groups were analyzed by Student's *t*-test. $p < 0.05$ was considered as statistically significant difference.

Results

Kaempferol Inhibited the Growth of GBC-SD and SGC996 Cells

The structural formula of kaempferol is shown **Supplementary Figure 1**. To investigate the effect of kaempferol in GBC, the cells of the GBC cell line GBC-SD and SGC996 were treated with 100 and 200 µg/mL for different time periods. The CCK-8 results indicated that kaempferol significantly suppressed the cell viability of both GBC-SD ($p < 0.01$) (Figure 1A) and SGC996 cells ($p < 0.01$) (Figure 1B) in a dose dependent manner. Cells were treated with the indicated concentrations of kaempferol for two weeks and the numbers of colony formation were evaluated. Similarly, kaempferol also significantly inhibited the colony formation of both GBC-SD ($p < 0.01$) (Figure 1C) and SGC996 cells ($p < 0.01$) (Figure 1D) in a dose dependent manner. These results indicated that kaempferol could significantly inhibit the cell growth of GBC cell lines *in vitro*.

Kaempferol Suppressed Invasion and Migration of GBC-SD and SGC996 Cells

The effects of kaempferol in the invasion and migration of GBC cell lines were then evaluated. Cells were treated with different concentrations of kaempferol for 48 h. The results showed that kaempferol significantly inhibited the migration ($p < 0.01$) (Figure 2A) and invasion ($p < 0.01$) (Figure 2B) ability of both GBC-SD and SGC996 cells in a dose dependent manner. These data indicated that kaempferol could significantly inhibit invasion and migration of GBC cell lines *in vitro*.

Kaempferol Significantly Induced Apoptosis of GBC Cells by Regulating Cell Cycle Distribution

Next, flow cytometry was performed to explore the effect of kaempferol in cell cycle phase distribution and apoptosis of GBC cells. The results indicated that both GBC-SD and SGC996 cells were significantly arrested in G0/G1-phase and exhibited a significant decrease in S and G2/M phase in a dose dependent manner with the increase in the concentration of kaempferol ($p < 0.01$) (Figure 3A). Meanwhile, kaempferol significantly induced cell apoptosis of both GBC-SD and SGC996 cells in a dose dependent manner ($p < 0.01$) (Figure 3B). These results indicated that kaempferol could remarkedly promote apoptosis of GBC cell lines partly through arresting GBC cells at G0/G1-phase.

Kaempferol Induced DNA Damage in GBC-SD and SGC996 Cells

The DAPI staining assay showed that kaempferol could evidently promote DNA condensation in a dose dependent manner (Figure 4A). It has

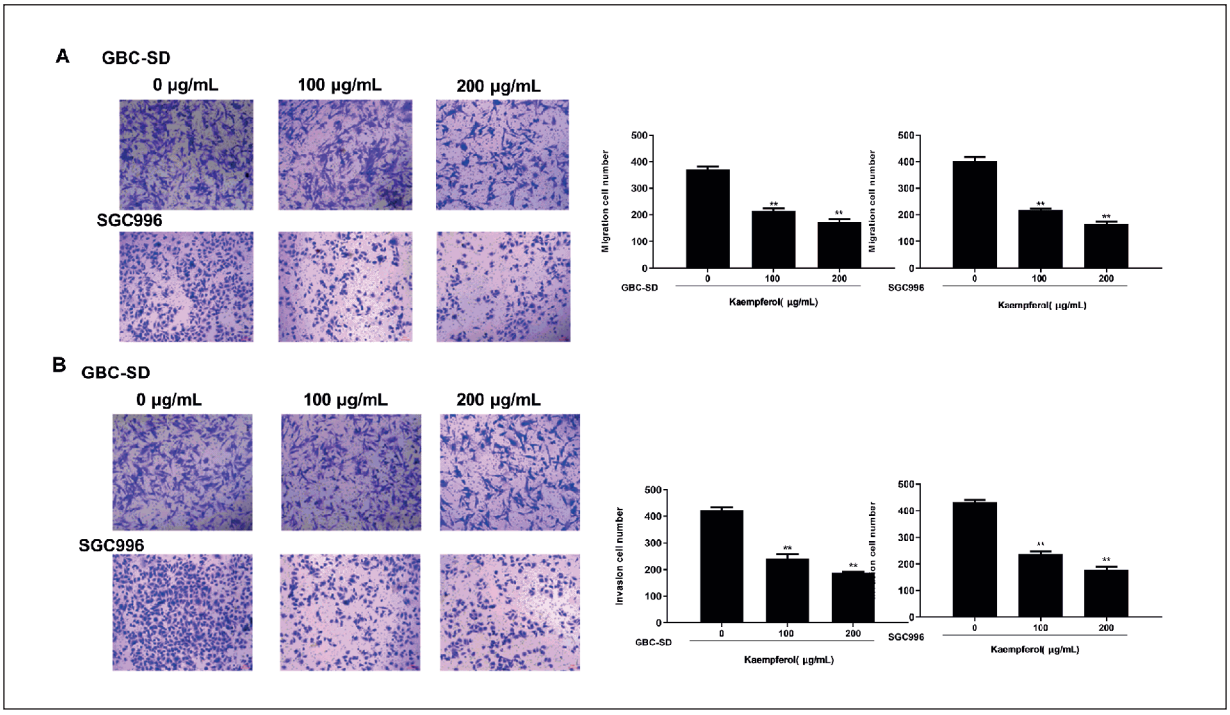

Figure 2. Effect of kaempferol on the invasion and migration of GBC-SD and SGC996 cells. The migration (**A**) and invasion (**B**) of GBC-SD and SGC996 cells was evaluated by transwell assay (magnification: 20×). N = 3, * $p < 0.05$ and ** $p < 0.01$.

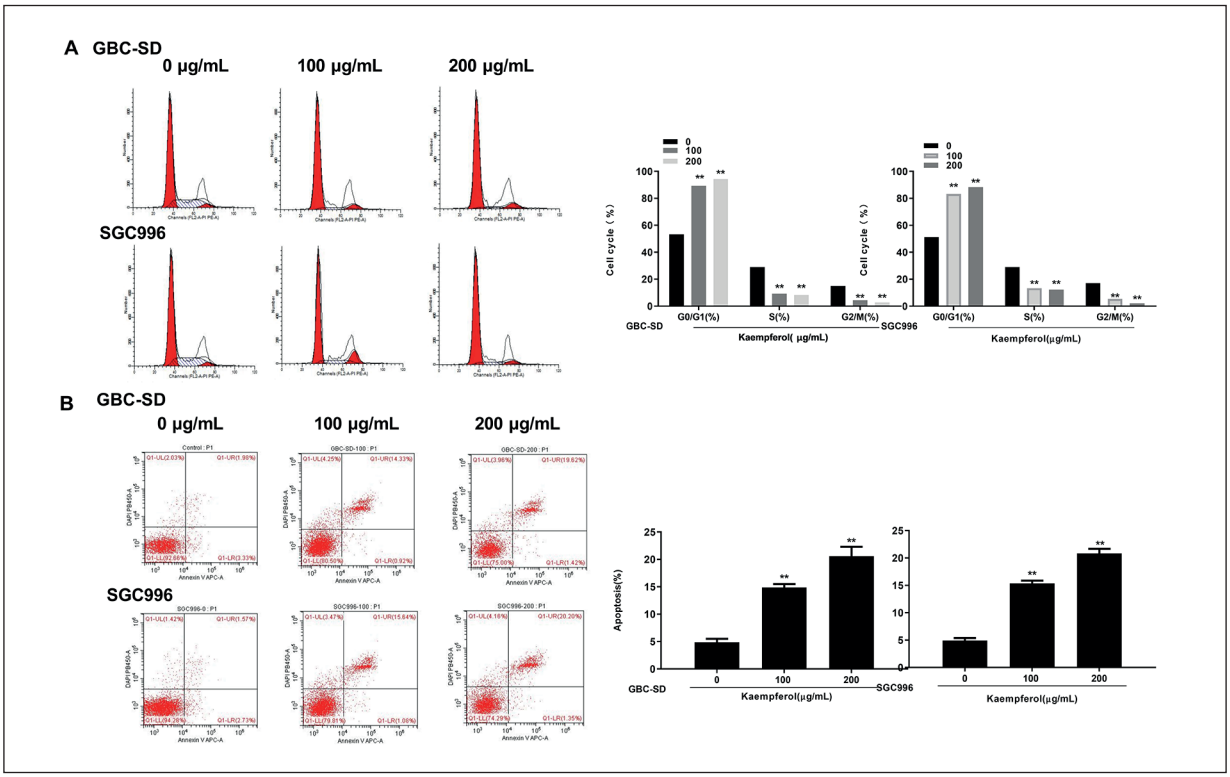

Figure 3. Effect of kaempferol on cell cycle and apoptosis of SGC996 and GBC-SD cells. Cell cycle (**A**) and apoptosis (**B**) of SGC996 and GBC-SD cells was evaluated by flow cytometry. N = 3, * $p < 0.05$ and ** $p < 0.01$.

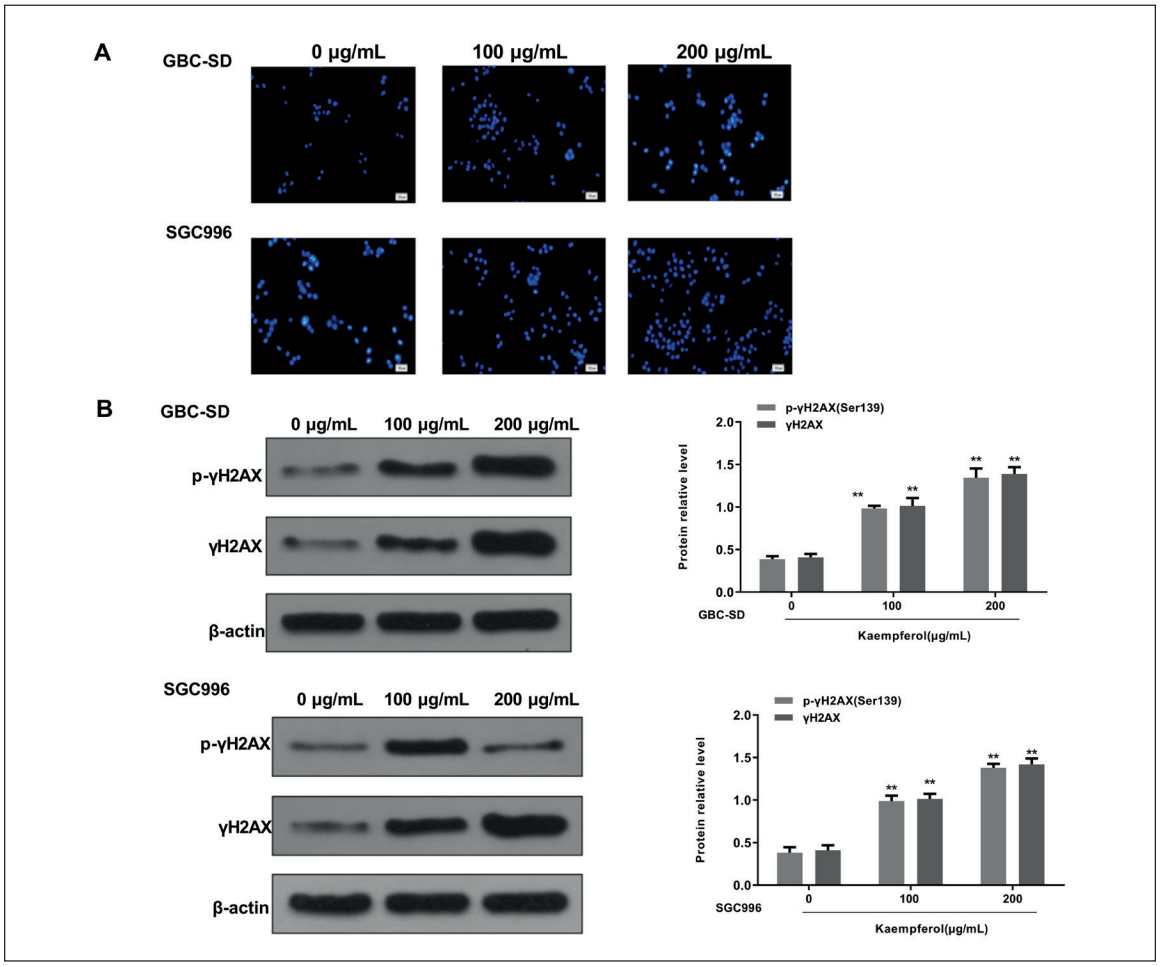

Figure 4. Effect of kaempferol on DNA damage of SGC996 and GBC-SD cells. **A,** The DNA damage of SGC996 and GBC-SD cells was evaluated by DAPI staining assay (magnification: 400×). **B,** The protein level of p-H2AX and total H2AX was detected by Western blot. N = 3, * $p < 0.05$ and ** $p < 0.01$.

been reported that double-strand breaks (DSBs) of DNA are always accompanied by histone H2AX phosphorylation[25], suggesting that phosphorylated level of H2AX can be used to detect DNA damage. Therefore, the effect of kaempferol in the H2AX phosphorylation was detected by Western blot. The results indicated that kaempferol significantly enhanced H2AX phosphorylation as well as the total H2AX level compared with the negative control in both GBC-SD and SGC996 cells in a dose dependent manner ($p < 0.01$) (Figure 4B). These results demonstrated that kaempferol could induce DNA damage in GBC cell lines *in vitro*.

Kaempferol Significantly Inhibited the Activation of the CDK4/CDK6/Cyclin D1 Pathway

Kaempferol remarkedly decreased the expression levels of CDK4, CDK6 and cyclin D1 in both GBC-SD ($p < 0.01$) (Figure 5A) and SGC996 cells ($p < 0.01$) (Figure 5B) in a dose dependent manner, suggesting that kaempferol could inhibit the activation of the CDK4/CDK6/cyclin D1 signaling pathway. Meanwhile, we also detected the expression of apoptosis related makers including cytochrome C in the cytoplasm, c-caspase-3, c-caspase-9, Bax and Bcl-2. The results showed that kaempferol significantly upregulated cytochrome C in the cytoplasm, c-caspase-3, c-caspase-9 and Bax, while downregulated the expression of Bcl-2 in both GBC-SD ($p < 0.01$) (Figure 6A) and SGC996 cells ($p < 0.01$) (Figure 6B) in a dose dependent manner. These results indicated that kaempferol-induced apoptosis of GBC cell lines was due to the release of cytochrome C from the mitochondria to cytoplasm and the activation of c-caspase-9 and c-caspase-3.

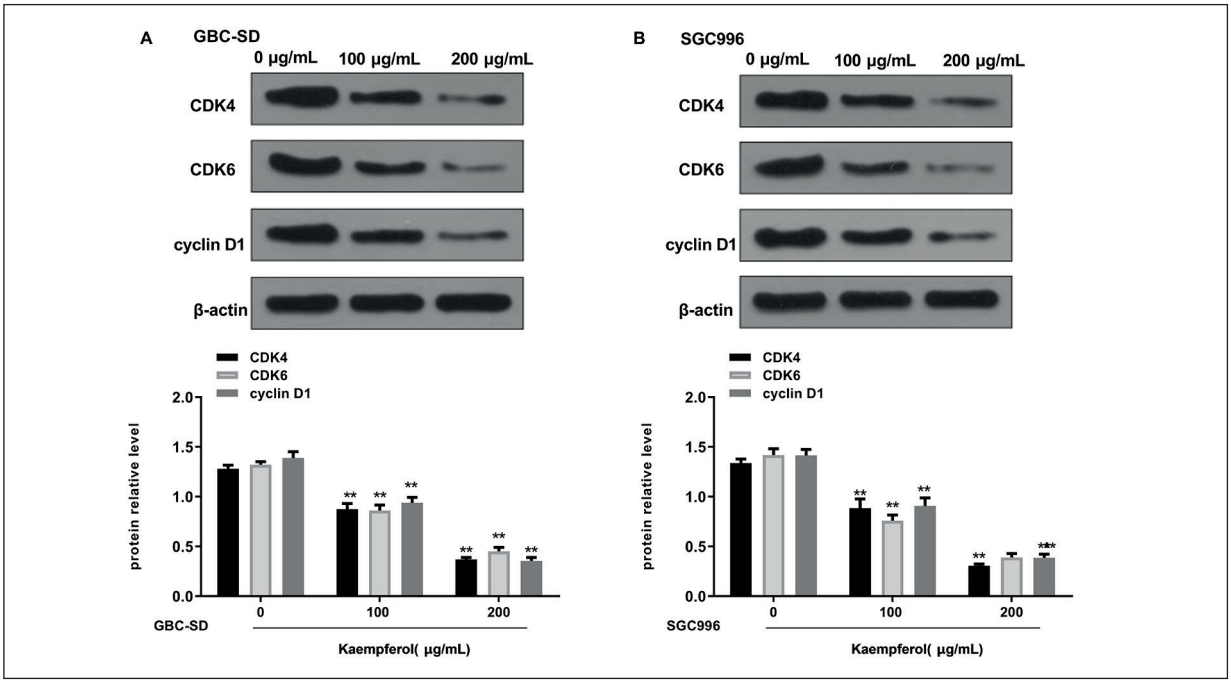

Figure 5. Effect of kaempferol on the CDK4/CDK6/cyclin D1 signaling pathway in GBC-SD and SGC996 cells. The protein expression of CDK4, CDK6 and cyclin D1 in GBC-SD (**A**) and SGC996 cells (**B**) was detected by western blot. N = 3, * $p < 0.05$ and ** $p < 0.01$.

Kaempferol Prevented Tumor Progression of GBC In Vivo

To further explore the protective role of kaempferol in GBC progression, the xenograft model was established. As expected, kaempferol could significantly prevent tumor progression compared with that in the control group *in vivo* (Figure 7A). Meanwhile, the tumor weight ($p < 0.01$) (Figure

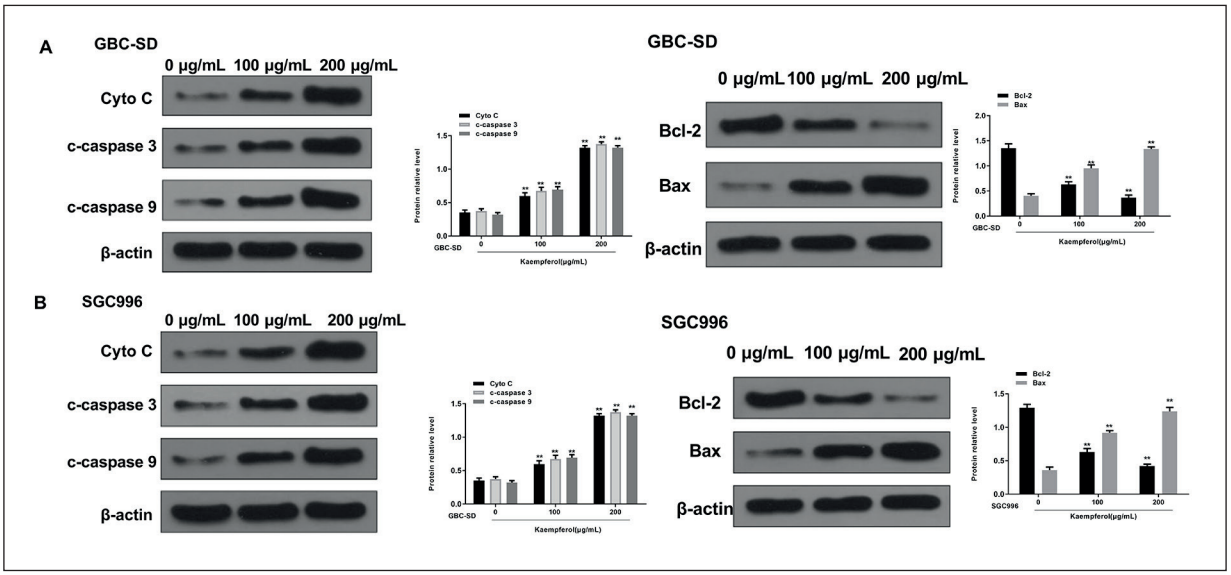

Figure 6. Effect of kaempferol on the expression of cytochrome C, caspases-3, caspase-9, Bcl-2 and Bax in GBC-SD and SGC996 cells. The protein level of cytochrome C, caspases-3, caspase-9, Bcl-2 and Bax in GBC-SD (**A**) and SGC996 cells (**B**) was detected by Western blot. N = 3, * $p < 0.05$ and ** $p < 0.01$.

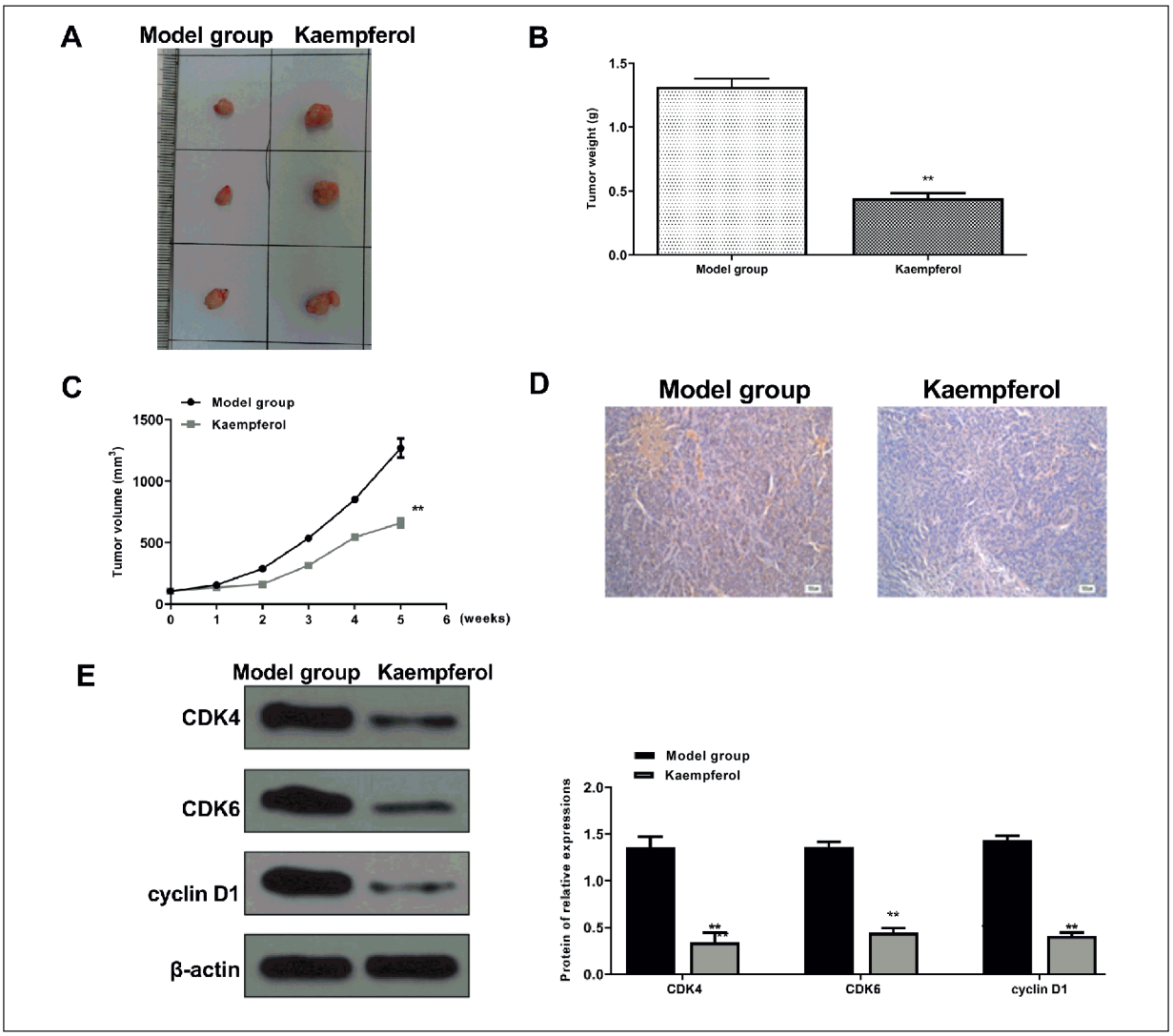

Figure 7. Kaempferol prevented GBC progression *in vivo*. Mice were randomly divided into two groups including the model group and kaempferol group. **A,** and **B,** Representative image (**A**) and weight (**B**) of subcutaneous tumors. **C,** Measurement of tumor volume weekly for six weeks. **D,** The positive cells in subcutaneous tumors was evaluated by Ki-67 staining assay (magnification: 10×). **E,** The protein level of CDK4, CDK6 and cyclin D1 was detected by Western blot. N = 3, * $p < 0.05$ and ** $p < 0.01$.

7B) and volume ($p < 0.01$) (Figure 7C) were significantly reduced by kaempferol compared with that in the control group. Ki-67 staining assay results showed that the numbers of positive cells in kaempferol treatment group were significantly decreased compared with that in the model group (Figure 7D). Similar to the inactivation of the CDK4/CDK6/cyclin D1 pathway induced by kaempferol *in vitro*, the expression levels of protein CDK4, CDK6 and cyclin D1 were also significantly decreased in kaempferol group ($p < 0.01$) (Figure 7E). These results suggested that kaempferol could efficiently prevent GBC progression in *vivo*.

Discussion

GBC is an aggressive malignancy and has become a global healthy challenge[26]. Due to the lack of efficient diagnostic approaches due to the asymptomatic phenotype at the early stage of GBC, most patients are diagnosed at advanced stages, which is unresectable and systemic therapy is the only option[27]. Therefore, it is necessary to develop effective drugs or treatment approaches to prevent GBC progression.

Recently, a series of natural extracts have been applied to the treatment of human cancers including GBC with wide anti-cancer activi-

ties and low toxicity. For instance, oxymatrine, extracted from *Sophora flavescens*, has been demonstrated to induce cell apoptosis and suppress cell viability, metastasis and invasion of GBC cells through the PTEN/PI3K/AKT signaling pathway[28]. Garcinol, a natural histone acetyltransferase inhibitor isolated from *Garcinia indica*, can inhibit cell proliferation and invasion of GBC-SD cells through inactivating the ATAT3 and AKT signaling pathway[29]. Shikonin, a natural extract isolated from the root of *Lithospermum erythrorhizon*, was reported to induce apoptosis and G0/G1 phase arrest of GBC cells through the JNK signaling pathway[30]. Piperlongumine (PL), a naturally occurring small molecule isolated from *Piper longum* L., has been identified to induce autophagy of GBC cells *via* reactive oxygen species (ROS)-activated ERK signaling pathway[31]. The anti-tumor activities of kaempferol have been demonstrated in various human cancers, but its role and the underlying mechanism in GBC has not been reported. Here, we explored the potential inhibitory effect of kaempferol on GBC progression. Cell viability and colony formation assay were employed to explore the effect of kaempferol on the growth of GBC cells. Our results indicated that kaempferol could dramatically inhibit the growth and colony formation of GBC cell lines SGC996 and GBC-SD cells *in vitro*.

Cell apoptosis is a programmed cell death and involves the regulation of various genes[32], making it a potential therapeutic target for GBC[33]. Tumor cells may use several molecular mechanisms to prevent apoptosis and acquire resistance to apoptotic agents, for example, through upregulation of antiapoptotic proteins such as Bcl-2, or downregulation or silencing of pro-apoptotic proteins such as BAX[34]. A previous study also indicated that Bax and Bcl-2 are co-regulators in cell apoptosis[32]. In the present study, our results indicated that kaempferol could significantly decrease the expression levels of Bcl-2 and increase the expression levels of Bax in both GBC cell lines SGC996 and GBC-SD cells. As we known, the releases of cytochrome C from the mitochondria to cytoplasm, as well as the activation of caspase-3 and caspase -9 play an essential role in the induction of the apoptotic cascade that subsequently results in programmed cell death[35]. Our results indicated that kaempferol could remarkably enhance the release of cytochrome C from the mitochondria to cytoplasm and also increase the expression levels of c-caspase-3 and c-caspase-9, which represents the activation of caspase-3 and caspase-9 in GBC cell lines *in vitro*. It has been reported that the dysregulation of the cyclin D1-CDK4/CDK6 axis is frequently occurred in almost all human cancers, which contributes to aberrant cell proliferation and consequent tumorigenesis[36]. Here, we found that kaempferol could significantly downregulate the expression of cyclin D1, CDK4 and CDK6 in both SGC996 and GBC-SD cells. Meanwhile, kaempferol remarkedly promoted apoptosis of GBC cell lines through arresting GBC cells at G0/G1-phase.

The cellular response to DNA damage is an important component of many cytotoxic therapeutics commonly used in cancer therapies[37]. When cells are exposed to DNA-damaging chemotherapeutic agents, double-stranded breaks (DSBs) occur and lead to the phosphorylation of histone H2AX at Ser 139[38]. Therefore, the phosphorylated level of H2AX is often used for the detection of DNA damage. As expected, kaempferol could remarkedly enhance the phosphorylation level of H2AX and total H2AX level in GBC cell lines, indicating that kaempferol could induce DNA damage of GBC cells. Moreover, the xenograft mice model was established to determine the anti-GBC effect of kaempferol. Our data further confirmed that kaempferol could efficiently decrease tumor size, weight and volume *in vivo*. All these results demonstrated that kaempferol might be a potential anti-GBC agent for clinical therapeutics. However, there are still some limitations in this study. First, the toxicity of kaempferol to gallbladder normal cells and the safe dose of kaempferol need to be elucidated in future. Second, further studies on cell line proliferation and EMT should be carried out to ensure a better understanding of the mechanism by which GBC occurs and develops.

Conclusions

In summary, our study highlighted the importance of kaempferol in efficiently preventing GBC progression by inhibiting the CDK4/CDK6/cyclin D1 axis. Our findings expand our knowledge of the functions of kaempferol in GBC. These results would provide valuable insights into the progression of new, effective therapeutic approaches for the treatment of GBC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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