Int J Clin Exp Med 2016;9(11):21415-21423
www.ijcem.com /ISSN:1940-5901/IJCEM0033356

Original Article

Kaempferol targets estrogen-related receptor α and inhibits cell proliferation and invasion in retinoblastoma via Wnt/β-catenin signaling pathway

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Received January 22, 2016; Accepted September 20, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Retinoblastoma is a pediatric retinal tumor caused by mutational inactivation of the tumor suppressor pRb, and the present study aims to examine the role of kaempferol in retinoblastoma and explore its underlying molecular mechanisms. The mRNA and protein expression levels were measured by quantitative real time PCR (qRT-PCR) and western blot, respectively; cell growth and invasion was determined by CCK-8 assay, colony formation assay, and cell invasion assay; cell cycle was determined by flow cytometry, and cell apoptosis was examined by flow cytometry and TUNEL assay. The target of kaempferol was confirmed by luciferase reporter assay. Kaempferol inhibited cell growth and invasion in a concentration-dependent manner in retinoblastoma SO-RB50 cells. Clinical sample analysis showed that estrogen-related receptor α (ERRα) is up-regulated in retinoblastoma tissues and SO-RB50 cells. Luciferase reporter assay further showed that kaempferol exerted its effect via targeting ERRα. Mechanistic study also showed that kaempferol caused G_2/M arrest as well as apoptosis in SO-RB50 cells and also suppressed Wnt/β-catenin signaling via targeting ERRα. In conclusion, our studies indicate that kaempferol targets ERRα and inhibits cell proliferation and invasion in retinoblastoma via Wnt/β-catenin signaling pathway.

Keywords: Retinoblastoma, kaempferol, SO-RB50 cells, ERRα, apoptosis, Wnt/β-catenin

Introduction

Retinoblastoma is the most primary intraocular tumor represented by a mutation of the retinoblastoma gene on both alleles in childe worldwide, and the prevalence of this disease ranges from 1:15,000 to 1:20,000 in children under the age of 5 years old [1, 2]. Chemotherapy, focal therapy such as laser or cryotherapy, and enucleation are the commonly used treatments for retinoblastoma [3-5]. However, there is an increased risk of developing other retinoblastoma-related monocular cancers later in life, as tumor cells frequently invade the optic nerve, orbit and choroid [6]. Therefore, therapeutics to minimize the requirement of enucleation, decrease the risk tumor metastasis and the development of secondary cancers, preserve vision and prevent systemic toxicity are of great importance.

Kaempferol (3,4,5,7-tetrahydoxyflavone), one the most commonly found dietary flavonoids, has been isolated from grapefruit, tea, broccoli, and other plant sources [7]. Kaempferol has been shown to inhibit cell growth and induce apoptosis in various types of cancers including colon, lung, and prostate cancers [8-10], and kaempferol has been proposed to exert its anti-cancer functions via inhibiting DNA synthesis, including nuclear DNA degradation, and inhibiting kinase activities [11, 12]. However, other molecular mechanisms involving the anti-cancer effect of kaempferol have also been proposed. For example, kaempferol induces apoptosis in renal cell carcinoma via EGFR/p38 signaling [13]. Kaempferol can also induce apoptosis of HT-29 cells via activation of cell surface death receptors and the mitochondrial pathway [12]. In addition, kaempferol induces apoptosis in lung cancer cells via activation of MEK-MAPK signaling [14]. Recent studies demonstrated that kaempferol is an estrogen-related receptor α (ERRα) inverse agonist, and inhibits cell growth in different cancer cell lines via

antagonizing ERRα activities [15, 16]. ERRα belongs to the nuclear receptor superfamily identified on the basis of their high levels of sequence identity to estrogen receptors [17]. The primary role of ERRα is in energy metabolism, and additional studies suggested that ERRα may be a prognostic marker for colon, prostate, ovarian, and breast cancer [17-20], and antagonizing of ERRα inhibits cancer cell growth, while overexpression of ERRα promotes cancer cell growth, which suggest that ERRα may be a novel target for cancer treatment.

In the present study, we examined the role of kaempferol in retinoblastoma and explored its underlying molecular mechanisms. The anti-proliferative and anti-invasive properties of kaempferol were examined in retinoblastoma SO-RB50 cells. Clinical sample analysis as well as luciferase reporter assay showed that kaempferol exerted its effect via targeting ERRα. Mechanistic study also showed that kaempferol caused G_2/M arrest as well as apoptosis in retinoblastoma cells and suppressed Wnt/β-catenin signaling via targeting ERRα.

Materials and methods

Cell culture

Human retinoblastoma SO-RB50 cells were obtained from the Department of Pathology of Zhongshan Ophthalmic Center, Sun Yat-sen University (Guangzhou, China), and cells were cultured in RPMI 1640 medium (Sigma, St.Louis, USA) supplemented with fetal bovine serum (Hyclone) to a final concentration of 10% and antibiotics, and cells were incubated at 37°C with 5% CO_2.

Tissue samples

This study was approved by the Ethics Review Committees of Shenzhen Eye Hospital, and informed consent was obtained from all patients. A total of 25 human tissue samples were used in this study, which included five normal 11 normal pediatric retinas and 14 retinoblastomas. All the dissected samples were snap-frozen in liquid nitrogen for further quantitative real-time PCR (qRT-PCR) analysis.

Chemicals, plasmids and transfection

Kaempferol and XTC-790 were purchased from Sigma. pCMX-ERRα and pcDNA-PGC-1α plasmids and the respective plasmids (pCMX and pCDNA) used for negative control were purchased from Generay Biotechnology (Shanghai, China). All the transfection was done by using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

RNA extraction and qRT-PCR

Total RNA fractions were isolated from tissue samples and SO-RB50 cell lines using TRIzol reagent (Invitrogen). RNA was reverse transcribed into cDNA using PrimeScript RT reagent kit (TaKaRA, Dalian, China), and qRT-PCR was performed with SYBR Premix Ex Taq (TaKaRa). GAPDH was used as an internal control. Primers for ERRα were obtained from RiboBio (Guangzhou, China), the primers for *ERR*α are: forward, 5'-TTCHHCGACTGCAAGCTC-3' and reverse, 5'-CACAGCCTCAGCATCTTCAATG-3'. The primers for *GAPDH* were: forward, 5'-GGATATTGTTGGCATCAATCACC-3' and reverse, 5'-AGCCTACTCCATGGTGGTGAAGT-3'.

CCK-8 assay

SO-RB50 cells were seeded in 96-well plates (5×10^3 cells/well) and incubated for 24 h, and then treated with kaempferol (10-30 μM) or its vehicle control for 24 h. In the transfection studies, 24 h after seeding the cells, cells were transfected with pCMX-ERRα and pcDNA-PGC-1α or their respective control plasmids, 24 h after transfection, cells were then treated with kaempferol (30 μM) or its vehicle control for 24 h. CCK-8 kit (Beyotime) was used to detect cell proliferation index according to manufacturer's instructions.

Colony formation assay

SO-RB50 cells were seeded in 6-well plates (5×10^5 cells/well) and incubated for 24 h; and then treated with kaempferol (10-30 μM) or its vehicle control for every 2 days. In the transfection studies, 24 h after seeding the cells, cells were transfected with pCMX-ERRα and pcDNA-PGC-1α or their respective control plasmids, 24 h after transfection, cells were then treated with kaempferol (30 μM) or its vehicle control for every 2 days. Two weeks later, cells were fixed in 4% formaldehyde and stained with 0.1% crystal violet. The number of colonies was determined by using a microscope.

Invasion assay

SO-RB50 cells were seeded in 24-well transwell plates (5 × 10^4 cells/well) and incubated for 24 h, and then treated with kaempferol (10-30 μM) or its vehicle control for 24 h. In the transfection studies, 24 h after seeding the cells, cells were transfected with pCMX-ERRα and pcDNA-PGC-1α or their respective control plasmids, 24 h after transfection, cells were then treated with kaempferol (30 μM) or its vehicle control for 24 h. Cells were re-suspended in serum-free DMEM and grown in the upper chambers with Matrigel-coated membrane (BD Bioscience, USA), and 500 μl of DMEM containing 10% FBS was added into the bottom of the chambers. Cells were allowed to migrated through the 8 μm polyethylene terephthalate membrane for 24 h, Cells passed through the membrane were fixed in 4% formaldehyde and stained with 0.1% crystal violet.

Luciferase reporter assay

SO-RB50 cells were seeded in 96-well plate (1 × 10^4 cells/well) and incubated for 24 h, and then co-transfected with pGL3-ERRE-Luci and pMCX-ERRα with or without pcDNA-PGC-1α plasmids. Renilla luciferase plasmid was used as an internal control. Twenty-four hours after transfection, cells were then treated with kaempferol (10-30 μM) or XTC-790 (15 μM) for 24 h before harvesting for luciferase assay. Luciferase activity was measured following Dual-Luciferase Reporter Assay System (Promega, Madison, USA).

Flow cytometry analysis

Cells seeded on a 6-well plate and incubate for 24 h, and then treated with kaempferol (30 μM) for 24 h; cells were then fixed with 75% ethanol at 4°C overnight and washed with cold PBS and treated with RNase I, followed by a 30 min staining with propidium iodide in dark. Cell cycle distribution were analyzed by a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Cell apoptosis was detected using an AnnexinV-FITC/PI apoptosis detection kit and analyzed by flow cytometry as per manufacturer's instruction.

TUNEL staining

Cells were seeded onto poly-L-lysine-coated slides in a 6-well plate and incubate for 24 h,and then treated with kaempferol (30 μM) for 24 h. Apoptotic cells were assessed using In Situ Cell Death Detection Kit, TMR red (TUNEL) (Roche, Mannheim, Germany) according to its procedure. Cell nuclei were counterstained with ProLong® Gold Antifade Reagent with DAPI (Invitrogen) and visualized on an Olympus fluorescence microscope. Cell death was calculated as the number of TUNEL-positive cells per total number of cells (DAPI positive).

Western blot analysis

Twenty four hours after seeding the cells, cells were transfected with pCMX-ERRα and pcDNA-PGC-1α or their respective control plasmids; 24 h after transfection, cells were then treated with kaempferol (30 μM) or its vehicle control for 24 h before havesting for western blot analysis. Western blot analysis was performed using anti-β-catenin (1:1000, Cell Signaling Technology, Beverly, USA); anti-GSK3β (1:15000, Cell Signaling Technology); anti-c-myc (1:1000, Cell Signaling Technology); anti-cyclin D1 (1:2000, Cell Signaling Technology); anti-b-actin (1:3000, Cell Signaling Technology), and b-actin was used a loading control.

Statistical analysis

All statistical analysis was performed using SPSS 15.0. Data was shown as mean ± SEM; and the differences among treatment groups were compared by one-way ANOVA, followed by Dunnett's multiple comparison test or unpaired t-test as appropriate. Differences were considered to be significant when $P<0.05$.

Results

The effects of kaempferol (10-30 μM) on cell growth and invasion in SO-RB50 cells

We showed that kaempferol (10-30 μM) treatment significantly reduced the number of SO-RB50 cells comparing to control (**Figure 1A**); CCK-8 assay results showed that kaempferol treatment suppressed the cell viability in SO-RB50 cells comparing to control (**Figure 1B**); colony formation assay further showed that the number of colonies were significantly lower in kaempferol-treated SO-RB50 cells than that in control group (**Figure 1C**). We also examined the effects of kaempferol on the invasive ability in SO-RB50 cells, and the tran-

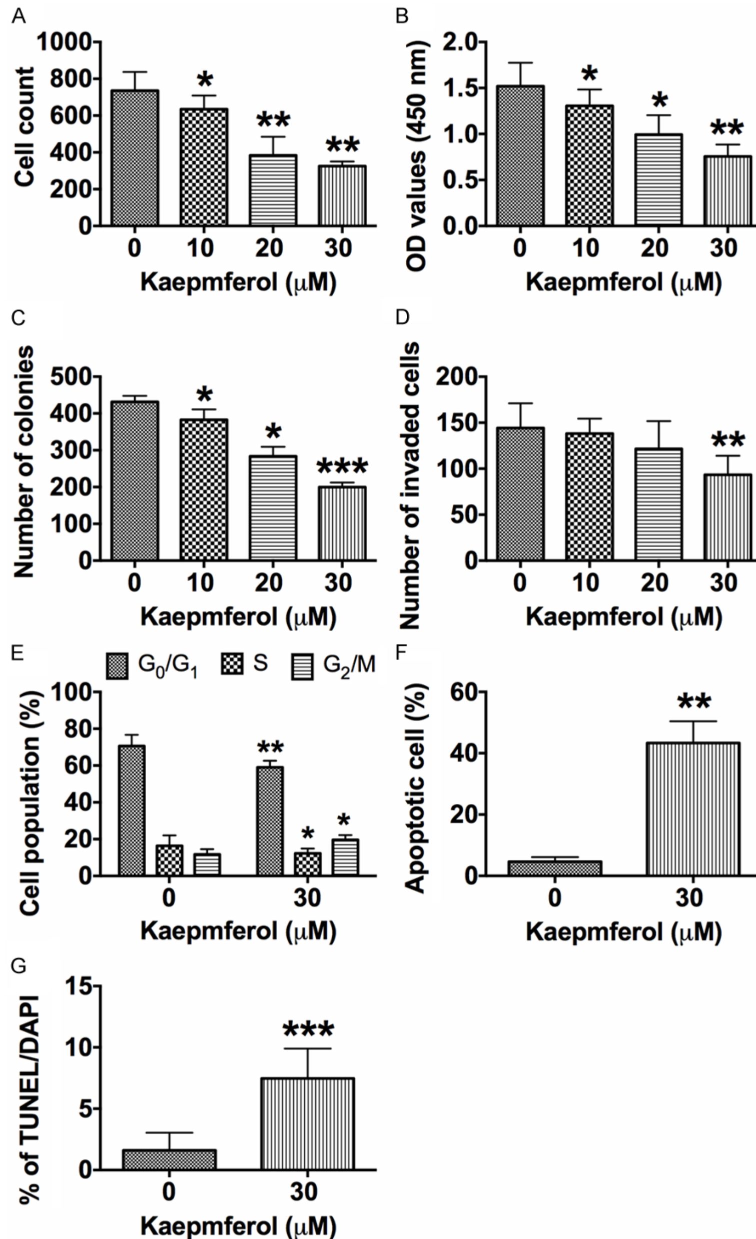

Figure 1. The effects of kaempferol (10-30 μM) on cell growth, invasion, cell cycle and apoptosis in SO-RB50 cells. A-C: Cell growth in kaempferol-treated SO-RB50 cells was measured by counting viable cell numbers, CCK-8 assay, or colony formation assay. D: The invasive ability in kaempferol-treated SO-RB50 cells was measured by transwell invasion assay. E: The effect of kaempferol on cell cycle was determined by FACS in SO-RB50. F: Cell apoptosis was measured by Annexin V/PI double staining in kaempferol-treated cells. G: The effect of kaempferol on cell apoptosis in SO-RB50 cells was measured by TUNEL assay, and the index of TUNEL-positive cells is expressed as the ratio of TUNEL stained cells to the total number of cells stained with DAPI. Data represents the mean ± SEM, n = 3. Significant differences relative to control were indicated as *P<0.05, **P<0.01, ***P<0.001 (One-way ANOVA followed by Dunnett's multiple comparison test).

swell invasion assay showed that kaempferol at 30 μM treatment significant decreased the invasive ability of SO-RB50 cells comparing to control group (**Figure 1D**). Kaempferol inhibited cell growth and invasion in a concentration-dependent manner (**Figure 1**).

The effects of kaempferol (30 μM) on cell cycle and cell apoptosis in SO-RB50 cells

To further investigate the mechanisms involving the inhibitory effect of kaempferol on cell proliferation, we examined the effects of kaempferol on cell cycle and cell apoptosis in SO-RB50 cells. Kaempferol treatment increased cell population at S phase and G_2/M phase with an associated reduction of cell populations at G_0/G_1 phase comparing to control group (**Figure 1E**). Annexin V/PI staining further showed that kaempferol treatment caused an increase in the apoptotic rate of SO-RB50 cells comparing to control group (**Figure 1F**). Consistently, TUNEL assay results also revealed that the number of apoptotic cells was significantly higher in kaempferol treated cells comparing to control group (**Figure 1G**). Altogether, these results suggested kaempferol treatment induced apoptosis in SO-RB-50 cells.

The expression levels of ERRα in retinoblastoma tissues and cells, and the effect of kaempferol (10-30 μM) on the activities of ERRα

In the present study, we further examined if the anti-proliferative effects of kaempferol on retinoblastoma was via antagonizing the activities of EERα. The qRT-PCR results showed that the expression levels of ERRα mRNA in retinoblastoma tissues and cells were

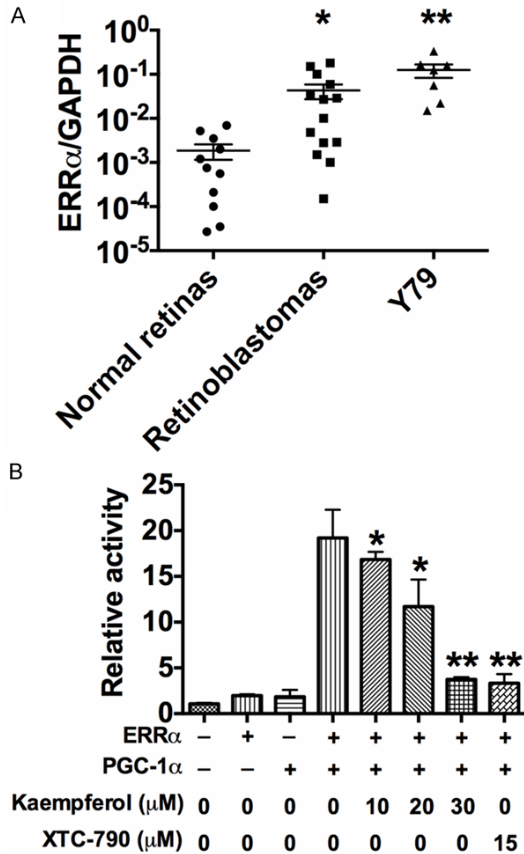

Figure 2. The expression levels of ERRα in retinoblastoma tissues and cells and the effect of kaempferol (10-30 μM) on the activities of ERRα. A: The expression levels of ERRα in normal retina tissues (n = 11), retinoblastoma tissues (n = 14), and SO-RB50 cells (n = 7) were determined by qRT-PCR. B: SO-RB50 cells were transfected with expression plasmids of pCMX-ERRα and/or pcDNA-PGC-1α together reporter pGL3-ERRE-Luc and control Renilla luciferase plasmid. Transfected cells were then treated with kaempferol or XTC-790 for 24 h before harvesting for luciferase assays. Data represents the mean ± SEM, n = 3. Significant differences between groups were indicated as *P<0.05, **P<0.01, P<0.001 (One-way ANOVA followed by Dunnett's multiple comparison test).

significantly higher than that in normal retina tissues (**Figure 2A**). SO-RB50 cells co-transfected with pCMX-ERRα and pcDNA-PGC-1α plasmids had a higher luciferase activity comparing to control groups (**Figure 2B**); kaempferol and XTC-790 treatment significantly reduced the luciferase activity in cells co-transfected pCMX-ERRα and pcDNA-PGC-1α plasmids comparing to their respective control groups, and kaempferol exhibited the inhibitory effect in a concentration-dependent manner.

The effects of kaempferol (30 μM) on cell growth and invasion in SO-RB50 cells transfected with pCMX-ERRα and pcDNA-PGC-1α plasmids

To further elucidate the anti-proliferative effects of kaempferol on retinoblastoma was via antagonizing the activities of EERα, we performed the *in vitro* functional studies. Co-transfection with pCMX-ERRα and pcDNA-PGC-1α plasmids in SO-RB50 cells significantly increased cell growth (**Figure 3A-C**) and cell invasion ability (**Figure 3D**); kaempferol treatment decreased the cell growth and cell invasion ability in SO-RB50 cells co-transfected with pCMX-ERRα and pcDNA-PGC-1α plasmids comparing to control (**Figure 3**).

Kaempferol (30 μM) inhibited Wnt/β-catenin signaling pathway in SO-RB50 cells transfected with pCMX-ERRα and pcDNA-PGC-1α plasmids

QRT-PCR and western blot showed that co-transfection with pCMX-ERRα and pcDNA-PGC-1α plasmids in SO-RB50 cells significantly increased the mRNA and protein expression levels of β-catenin, cyclin D1 and c-myc, while decreased the mRNA and protein expression levels of GSK3β (**Figure 4**); treatment with kaepmferol significantly suppressed the mRNA and protein expression levels of β-catenin, cyclin D1 and c-myc, and increased the mRNA and protein expression levels of GSK3β (**Figure 4**).

Discussion

In the present study, we for the first time demonstrated that kaempferol has anti-proliferative and anti-invasive property in retinoblastoma SO-RB50 cells. Our clinical sample analysis as well as luciferase reporter assay further showed that kaempferol exerted its effect via targeting ERRα. Mechanistic study also showed that kaempferol caused G_2/M arrest as well as apoptosis in retinoblastoma cells and also suppressed Wnt/β-catenin signaling via targeting ERRα.

Up to date, the anti-cancer property of kaempferol has been reported in various types of cancers, including lung, colon, prostate, and bladder cancers, these studies have demonstrated kaempferol treatment could inhibit cell prolif-

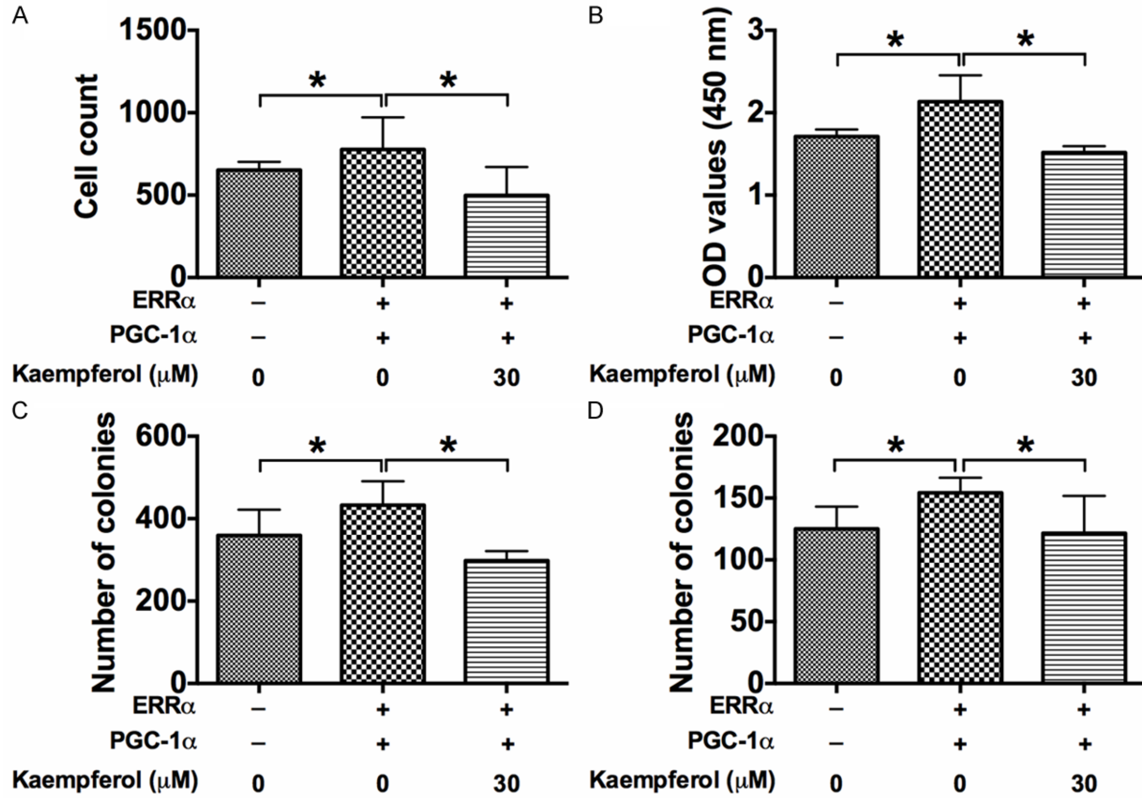

Figure 3. The effect of kaempferol (30 μM) on cell growth and invasion in SO-RB50 cells transfected with pCMX-ERRα and pcDNA-PGC-1α plasmids. A-C: Cell growth in the above transfected cells was measured by counting viable cell numbers, CCK-8 assay, or colony formation assay. D: The invasive ability in the above transfected cells was measured by transwell invasion assay. Data represents the mean ± SEM, n = 3. Significant differences relative to control were indicated as *P<0.05, **P<0.01, ***P<0.001 (One-way ANOVA followed by Dunnett's multiple comparison test).

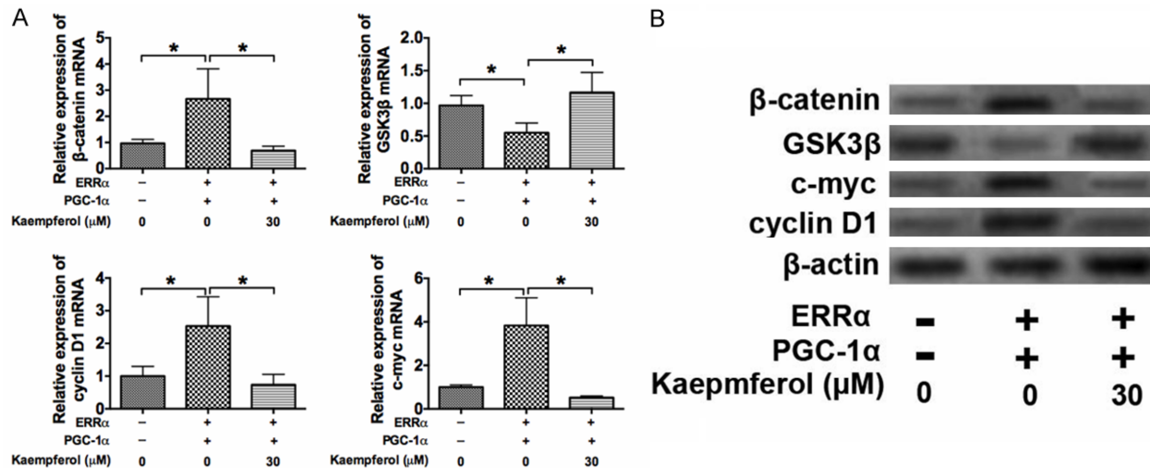

Figure 4. Kaempferol (30 μM) inhibited Wnt/β-catenin signaling pathway in SO-RB50 cells transfected with pCMX-ERRα and pcDNA-PGC-1α plasmids. A: The effects of kaempferol on the expression levels of β-catenin, GSK3β, cyclin D1, and c-myc mRNA in the above transfected cells were examined by qRT-PCR. B: The effects of kaempferol on the expression levels of β-catenin, GSK3β, cyclin D1, and c-myc protein in the above transfected cells were examined by western blot. Data represents the mean ± SEM, n = 3. Significant differences between groups were indicated as *P<0.05, **P<0.01, P<0.001 (One-way ANOVA followed by Dunnett's multiple comparison test).

eration, cell invasion, as well as cell migration [9-11, 14]. As far as we know, no study has been conducted to investigate the anti-cancer effect of kaempferol in retinoblastoma. In the present study, we reported that kaempferol treatment suppresses the proliferative and invasive ability of retinoblastoma cells, and our findings were consistent with previous studies in other types of cancers. In order to gain insight into the mechanisms involving the anti-cancer effect of kaempferol, flow cytometry was employed to examine the effect of kaempferol on cell cycle as well as apoptosis. The results showed that kaempferol treatment caused an increase in the cell population at S and G_2/M phase with an associated decrease in the population at G_0/G_1 phase. Consistently, previous studies in HT-29 human colon cancer cells also showed that 24 h treatment of kaempferol caused an in increase in the cell population at G_0/G_1 phase [9]. Similar findings were also reported in other types of cancers including gastric, liver, and bladder cancers [11, 21, 22]. Collectively, these studies suggest that kaempferol treatment caused G_2/M phase arrest in cancer cells. In our study, kaempferol treatment also induced apoptosis in retinoblastoma cells; however, the underlying molecular mechanism of kaempferol-induced apoptosis in retinoblastoma is unknown. In other types of cancers, kaempferol has been shown to modulate various apoptotic factors, which may in turn induce apoptosis. For example, kaempferol induces apoptosis in renal cell carcinoma via EGFR/p38 signaling [13]. Kaempferol can also induce apoptosis of HT-29 cells via activation of cell surface death receptors and the mitochondrial pathway [12]. In addition, kaempferol induces apoptosis in lung cancer cells via activation of MEK-MAPK signaling [14]. Therefore, the signaling pathway involving kaempferol-induced apoptosis may be cancer-type specific, and our data suggested that kaempferol induced apoptosis may be via G_2/M arrest in retinoblastoma cells.

Kaempferol has been shown to be an ERRα inverse agonist and inhibit cell growth by the ERRα activities [15, 16]. Whether kaempferol targets ERRα to exerts its anti-cancer function in retinoblastoma is unknown. Interestingly, the analysis of the clinical samples and retinoblastoma cell lines found that ERRα was up-regulated in retinoblastoma tissues and cells comparing to normal retina tissues, and luciferase reporter assay further demonstrated that kaempferol could antagonize the ERRα activities, which is consistent with previous findings in other types of cell lines [15]. In addition, the in vitro functional assay showed that overexpression of ERRα and its co-activator, PCG-1a, promotes cell proliferation and invasion in retinoblastoma cells. Indeed, the dysregulation of ERRα/PGC-1α has been demonstrated in various types of cancers. For instances, ERRα augments HIF-1 signaling by directly interacting with HIF-1α in normoxic and hypoxic prostate cancer cells [17]; ERRα metabolic nuclear receptor controls growth of colon cancer cells [19]. Kaempferol treatment suppressed the cell proliferation and invasion in ERRα and PCG-1α overexpressing retinoblastoma cells. Collectively, our results suggest that kaempferol target ERRα to exert its anti-proliferative and anti-invasive function in retinoblastoma cells.

The activity of Wnt/β-catenin signaling was up-regulated in ERRα/PCG-1α overexpressing SO-RB50 cells, and the treatment of kaempferol could prevent this up-regulation. Several lines of studies have demonstrated that the dysregulation of Wnt/β-catenin has been associated with cancer development [23-25]. A recent study also showed that inhibition of the Jagged/Notch pathway inhibits retinoblastoma cell proliferation via suppressing the Wnt/β-catenin signaling pathway [26]. In addition, ERRα has been demonstrated to regulate osteoblast differentiation via Wnt/β-catenin signaling [27]. Therefore, our studies may suggest that kaempferol suppress the activity of Wnt/β-catenin signaling via targeting ERRα.

In conclusion, these results indicate that kaempferol targets ERRα and inhibits cell proliferation and invasion in retinoblastoma via Wnt/β-catenin signaling pathway. Using kaempferol or the treatment of retinoblastoma in man may require more clinical investigation.

Acknowledgements

This work was supported by Shenzhen Innovative Research Program (NO. JCYJ20130401152829828).

Disclosure of conflict of interest

None.

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