Bcl-2/BCL2L12 mediated apoptosis and cell cycle arrest induced by Kaempferol through the suppression of PI3K/AKT signaling pathway in Hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is a highly aggressive and third leading cancer-related death. PI3k/AKT and p53 signaling pathways play a vital role and regulate cellular proliferation, migration, cell cycle, apoptosis, and autophagy during cancer conditions. BCL2L12 is a bcl-2 family protein and encoded by the BCL2L12 gene, which is involved in the execution phase of apoptosis. However, the biological function of PI3K/AKT/Bcl-2 mediated BCL2L12, and its molecular mechanism in HCC is largely unknown. Herein, we investigated the effect of Kaempferol, on PI3K/AKT/Bcl-2/BCL2L12 expressions in HCC cells. The expression level of PI3K/AKT/Bcl-2/BCL2L12, and its target genes in polyphenolic flavonoid Kaempferol treated HCC cells analyzed by Quantitative Real-Time PCR and Western blotting. Subsequently, the effect of Kaempferol in HCC chemosensitivity, cell proliferation, migration, cell cycle, and apoptosis were analyzed using AO/EtBr staining, ROS staining, mitochondrial membrane potential assay, wound healing assay, Transwell migration assay, MTT assay, and PI Staining. We found that the downregulation of PI3-K, AKT, LC3A/B, MMP9, Bcl-2, and BCL2L12 and upregulation of PTEN, p53, p21, and caspase 3 in Kaempferol treated HCC cells. Subsequently, we observed that Kaempferol effectively inhibits cellular proliferation, migration and enhances apoptosis, cell cycle arrest, autophagy, and 5-Fluorouracil mediated chemosensitivity in HCC cells. Furthermore, Kaempferol has dominant role against HCC and inhibits 50-60% of cells after 24h treatment with 30μM in HepG2 and 40μM in Huh7 cells. Therefore, Kaempferol plays a promising role in cancer therapeutics by inhibiting PI3K/AKT/Bcl-2 mediated BCL2L12 expression in HCC.

Keywords: BCL2L12; Kaempferol; HCC; Apoptosis; Chemosensitivity; PI3K/AKT signaling

Introduction

Hepatocellular carcinoma (HCC) is a highly aggressive tumor and the third leading cause of cancer-related death [1]. Hepatitis B virus (HBV) is the major risk factor for human HCC, approximately 50-80 % cases, and the hepatitis C virus (HCV) is 10 to 25 % [2].HCC usually diagnosed at the advanced stages, and Chemotherapy is the most frequent treatment used for HCC [3].Previous studies revealed that signaling pathways having an important role and regulate cell proliferation, cell cycle, apoptosis, and autophagy during carcinogenesis especially, Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway
and p53 signaling pathway [4]. The p53 is a tumor suppressor (TS) protein [5] encoded by the p53 gene, and it is a crucial gene in the prevention of cancer. PI3K/AKT signal transduction serves an important role and associated with certain gynaecological tumors [6-8]. Molecular targets for the PI3K/AKT is the possible way to treat cancer cells.

B-cell lymphoma 2 (Bcl-2) family consists of pro-apoptotic (BAX, BAK) and anti-apoptotic (Bcl-2, Bcl-xL, and myeloid cell leukemia-1) proteins that control apoptosis by the mitochondrial pathway [9]. Bcl2-like 12 (BCL2L12) is a member of the Bcl-2 family and encoded by the BCL2L12 gene and which is involved in several cancers [10]. The BCL2L12 role was first discovered in the glioma cancer pathogenesis. BCL2L12 inhibit p53-dependent DNA damage-induced apoptosis in glioma [11], and latterly found their role in breast cancer [12], Leukemia [13], and Gastric adenocarcinoma (AGS) [14]. The pro-apoptotic gene caspase-7 directly and caspase-3 indirectly regulating the BCL2L12 expressions [15] and their enzymes play an important role in the execution phase of apoptosis. Bcl-2 regulated by PI3K/AKT in human cancers such as prostate cancer [16] and Breast cancer [17].

Kaempferol is a polyphenolic flavonoid present in many plants. Several pre-clinical studies have shown that Kaempferol has anticancer and anti-inflammatory effect [18] and inhibit invasion and migration in various human cancer cells, such as non-small cell lung cancer [29], breast cancer [20], colon cancer [21], and leukaemia [22] and induces apoptosis in HepG2 cells [23], and autophagic cell death in SK-HEP-1 human hepatic cancer cells [24]. Kaempferol induces apoptosis and cell cycle arrest through the inhibition of PI3K/AKT signaling in gastric cancer [25]. Since, the correlation between Kaempferol and PI3K/AKT/Bcl-2/BCL2L12 signaling in HCC chemosensitivity, and apoptosis has not elucidated. In the present study, we focused to validate the effect of kaempferol on PI3K/AKT/Bcl-2-mediated BCL2L12 proteins in cell cycle, apoptosis, and autophagy regulation of HCC cells.

Materials and methods

Reagents

Kaempferol (C15H10O6), 5-FU, and DMSO purchased from Sigma Aldrich Co., St. Louis, MO, USA. 2’,7’-Dichlorofluorescin diacetate (DCFH-DA) purchased from Thermo Fisher Scientific, Waltham, MA, USA. Dulbecco’s Modified Eagle’s Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin Streptomycin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) purchased from HiMedia Laboratories Pvt. Ltd., L.B.S.

**Cell Line, Cell Culture**

Hepatocellular Carcinoma cell line HepG2 and Huh7 purchased from National Centre for Cell Science (NCCS), Pune, India. The cells maintained in DMEM supplemented with 10% FBS and 1% antibiotics. Normal liver epithelial cell line THLE-2 (THLE-2; ATCC-CRL-2706) purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Bronchial Epithelial Cell Growth Medium (BMEM) supplemented with 0.08% phosphoethanolamine (Sigma Aldrich Co, St. Louis, Missouri, United States), 0.06% human recombinant EGF (Corning, NY, USA) and 10% FBS. All the cells incubated at 37°C under a humidified atmosphere with 5% CO₂.

**MTT assay**

HCC cells cultured in 96-well plates, at a concentration of 1x10⁴ cells/well after overnight growth, Kaempferol with concentration ranging from 0-60μM dissolved in DMSO further incubated for 24h. At the end of treatment 200μl, culture medium containing 20μL MTT (5 mg/mL) added to each well and further incubated at 37°C for 4h. The supernatant removed, crystals formations dissolved in 200μl DMSO, and the absorbance measured at 570 nm using a Microplate reader (Bio Rad, Hercules, CA, USA). For chemosensitivity, following treatment of 24 h, cells harvested and seeded into 96-well plate (5X10⁴). Cells treated with the IC₅₀ concentration of 5-fluorouracil for different time intervals (0, 12 and 24 h). The IC₅₀ values taken from the previous studies for HepG2 cell line (323.2 μM) [26]; from Genomics of Drug Sensitivity in Cancer Project for Huh7 cell line (346.49μM). After the incubation, the chemosensitivity assay performed by MTT assay, as described in the cell proliferation procedure.

**Transwell Migration Assay**

HCC cells (2x10⁵ cells/well) plated in 12-well plates and treated with Kaempferol for 24 h. Then cells harvested and seeded onto Transwell chamber inserts (with a membrane pore size of 8 μM), without Matrigel and DMEM medium placed in the lower chamber for overnight. After 16 h incubation, the medium removed and washed with phosphate-buffered saline (PBS) twice. Cells fixed with 500 μl of formaldehyde in both well at room temperature
(RT) for 2 minutes. After the fixation, inserts washed with PBS twice, and permeabilize cells by 100% methanol in inserts and lower well at RT for 20 minutes. After the incubation, methanol removed and washed with PBS twice and cells stained with 0.2% crystal violet for 15 minutes and washed with PBS twice. Non-migrated cells removed from the upper chamber by using a cotton swab, and the migrated cells in underside of the filter examined and counted using a light microscope at 20X magnification. Stained cells collected by washing with 10% acetic acid and quantified by measuring absorbance at 595 nm using a Microplate reader.

**Wound Healing Assay**

HepG2 and Huh7 cells cultured in 6 well plates at a concentration of 1.5x10⁴ cells/well and allowed to grow overnight. The next day, cells wounded with help of a sterile 1 ml pipette tip by dragging it across the monolayer and cells treated with Kaempferol (40 μM) for 24h at 37°C. The wound healing process documented at 0h, 12h, and 24h, using a Floid cell imaging station and wound gap size or wound area calculated using ImageJ software (version 1.8.0).

**Detection of Intracellular ROS**

Measurement of intracellular ROS level in HCC cells made using DCFH-DA staining. The cells cultured in 6-well plates at a concentration of 1.5x10⁴ cells/well and treated with Kaempferol for 24h at 37°C. After treatment, cells incubated with 10μM DCFH-DA in Phosphate Buffer Saline (PBS) at 37°C for 30 mins. Later, the intracellular fluorescence signal visualized and recorded by Floid cell imaging station.

**Acridine Orange/Ethidium Bromide (AO/EB) staining**

HCC Cells seeded in 6 well culture plates and incubate with Kaempferol for 24 h. After treatment, cells stained with 10μl AO/EB (1mg/1ml for 5 minutes) and then visualized by Floid cell imaging station (Life Technologies, USA).

**Fluorescent Immunocytochemistry**

HCC cells seeded on a coverslip in six-well plates with a density of 1 x 10⁵ cells per ml. After 60% of confluency, cells treated with IC₅₀ (30µM and 40µM for HepG2 and Huh7 respectively) value of kaempferol. After 24 h incubation, cells fixed with 2% paraformaldehyde for 15 min. For permeabilization, cells incubated with 0.2% TritonX-100 in PBS for 5 min. 3% BSA in PBS used to block the cells. For the detection of BCL2L12,
cells incubated with an anti-BCL2L12 primary rabbit monoclonal antibody (EPR5201) (diluted 1:10,000, Abcam, Cambridge, MA, USA) at 4°C for overnight. Following the incubation, cells washed with PBS and stained with goat anti-rabbit Alexa Fluor 647-labeled secondary antibody (ab150079-Abcam) for 1 h at RT. For staining of filamentous actin, cells incubated with Alexa Fluor 488 phalloidin (Invitrogen) for 30 min at room temperature, and cells washed twice with PBS after each step of the staining. Coverslips mounted with ProLong Gold Antifade Mountant along with DAPI, and fluorescence images were taken using a fluorescence microscope (Carl Zeiss, Jena, Germany). The images captured and exported using ImageJ software (NIH).

**Propidium Iodide staining**

HCC cells treated with Kaempferol for 24 h. After the treatment, cells harvested by trypsinization and washed with 1x PBS once, and cells fixed with 70% ethanol and stored at 4°C for 2 h. After centrifugation, cells washed with PBS and incubated at dark with Propidium Iodide (PI) (1.5μg/mL) and RNase (100 U/mL) at 37°C for 30 minutes. The stained cells analyzed by Flow Cytometer. Cytometry data analyzed using FCSalyzer version 0.9.4.

**Quantitative real-time PCR**

Total RNA isolated using Trizol (Invitrogen, Carlsbad, CA, USA) from control and Kaempferol treated HepG2 and Huh7 cells. 2μg of total mRNA reverse transcribed with RevertAid First-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA,). Quantitative Real-time PCR performed on a Step One Plus RT - PCR (Applied Biosystem, Foster City, CA, USA) with sequence-specific primers and SYBER FAST qPCR master mix kit (Applied Biosystems) with following concentration: in 10 μl by the Step one plus RT-PCR (Applied Biosystem): 40 cycles at 95°C for 15 s, 60°C for 45 s and 72°C for 15 s. Primers used listed in Supplementary Table 1. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) used for normalization.

**Western blotting**

The cell lysates collected with RIPA lysis buffer (Himedia) (20 mM Tris HCl pH 8.150 mM NaCl, 0.5 % sodium deoxycholate, 5 mmol/L EDTA, 1 % Nonidet P-40, 0.1 % SDS) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich Co). The protein sample separated by SDS-PAGE transferred onto a 0.2 μm nitrocellulose membrane by transferred apparatus (BioRad Laboratories Incp., Hercules, CA, USA). The
membrane blocked using non-fat milk (5%) in TBS for 1 h at 37 °C; washed and proceed at primary antibodies (listed in above) overnight at 4°C. After washing, the membrane incubated at alkaline phosphatase (ALP) conjugated anti-rabbit (ab6722) or anti-mouse (ab97020) secondary antibody (1:1000; Abcam, Cambridge, MA, USA) and detected by BCIP/NBT solution (Merk, Millipore, Bedford, MD, USA). The amount of protein quantified by densitometric analysis by using ImageJ software.

Statistical Analysis

Statistical analyses performed using GraphPad Prism Software 7.0 (GraphPad Software, La Jolla, CA, USA), and the One-way ANOVA performed for comparison. P< 0.05 considered Significant. All the data presented at least three separate experiments.

3. Results

Kaempferol inhibits cellular proliferation and induces 5-Flurouracil mediated chemosensitivity in HCC cells

To understand the cytotoxic effect of Kaempferol, HCC (HepG2 and Huh7) cells treated with different concentrations of Kaempferol for 24 h (fig. 1.A) and then subjected to MTT assay to determine the percent viable cells in post treatment. The% viable cells decreased with the increase of Kaempferol concentration. For further studies, the Kaempferol dosage fixed at IC50 of 30μM for HepG2 and 40μM for the Huh7 cell line. Furthermore, Kaempferol enhances 5-fluorouracil sensitivity of HCC (HepG2 and Huh7) (Fig. 1.B) cells and confirmed by the MTT assay. The chemosensitivity assay showed that the IC50 values of 5-Fluorouracil decreased significantly due to Kaempferol treatment in HCC cells.
Fig.1. Kaempferol inhibits HCC cell viability and enhances Chemosensitivity
Effect of Kaempferol on HCC (HepG2 and Huh7) cells proliferation (A) and 5-Flurouracil mediated chemosensitivity (B) by MTT assay. HCC cells treated with Kaempferol and then treated with IC$_{50}$ Value of 5-Flurouracil (HepG2-323.2 μM; Huh7-346.4 μM) and incubated at different time intervals (6 h, 12 h, and 24 h). The scale bars represent 100μM. Error bars represent mean ± s.d. and P-values are represented as*P< 0.05, **P< 0.01, ***P< 0.001 compared to the corresponding controls.

Kaempferol regulates Post-wound migration

Cell mobility is essential for cancer cell proliferation. Therefore, we investigated the effect of Kaempferol on cell proliferation by wound-healing and Transwell migration assay in HCC cells. For the wound-healing assay, HepG2 and Huh7 cells treated with Kaempferol, at different time intervals (0 h, 12 h, and 24 h). The results showed that cell proliferation decreased (50-60%) after the 24 h treatment with Kaempferol in HCC cells when compared to control (Fig.2.A). Next, we assessed the effect of Kaempferol in HCC (HepG2 and Huh7) cell migration (Fig.2.B). The transwell migration assay showed that the HCC cell migration ability markedly reduced in Kaempferol treated group when compared to the control group. Therefore, our result suggests that Kaempferol effectively inhibits the cellular proliferation and migration of HCC cells in vitro.
**Fig.2. Kaempferol inhibits the cell proliferation and migration of HCC cells**

Kaempferol inhibits HCC cell migration *in vitro*. Scratch assay: HepG2 and Huh7 cell lines at 0, 12, and 24 h post-transfection with Kaempferol (A). Gap distance of cells was quantified by using Image J. Transwell migration assay (without Matrigel) was performed in Kaempferol treated HCC cells (B). The scale bars represent 100μm. Error bars represent mean ± s.d. and P-values are represented as ***P< 0.001 compared to the control.

**Kaempferol induces ROS mediated cell death**

To understand the morphological changes in HCC cells induced by Kaempferol treatment, Cells stained with AO/EtBr at 24 h post-treatment with 30 μM (HepG2) and 40 μM (Huh7) concentrations. About 50% of cells found to be apoptotic when compared with untreated cells. Apoptotic cells appeared orange in Kaempferol treated HCC cells, and there were no morphological changes observed in control cells (green) (Fig. 3.A). Next, we analyzed the ROS generation due to Kaempferol. After the treatment, DCFH-DA added and examined the level of ROS generation. Intracellular ROS generation was significantly higher in Kaempferol treated group when compared to the control group (Supplementary Fig. 3.B). It has shown that Kaempferol rapidly induces the production of intracellular ROS by mitochondria and promoting intrinsic apoptosis pathways in HCC cells.
Fig.3. Kaempferol induces ROS generation and morphological changes in HCC cells

HCC Cells were stained with AO/EB following the Kaempferol treatment and morphological changes were observed. Apoptotic cells appeared orange in Kaempferol treated HepG2 and Huh7 cells when compared to control cells (green) (indicated by arrows) (A). Intracellular ROS generation was analyzed by ROS staining in Kaempferol-treated HCC cells (B). Images were taken using Floid cell imaging station (×20).
Kaempferol induces cell cycle arrest, and inhibits BCL2L12 protein expression

Effect of Kaempferol on cell-cycle progression (Fig. 4.A) in HCC cells were studied by FACS. After 24 h of Kaempferol treatment, 34.87% of the HCC cells in Sub G0-G1 phase, 36.19% in G0-G1 phase, 18.2% in S phase and 6.8% in G2 phase when compared to the control cells consists of 5.62 % of the HCC cells in Sub G0-G1 phase, 59.35% in G0-G1 phase, 21.4% in S phase and 10.62% were in G2 phase. Thereby indicating that, Kaempferol significantly inhibits cell growth, and induces apoptosis in the Sub G0-G1 phase and cell cycle arrest in G0-G1 and G2-M phase in HCC cells. Next, we analyzed the BCL2L12 protein expression in Kaempferol treated HCC cells by Immunocytochemistry assay. BCL2L12 protein expression decreased in Kaempferol treated HCC (HepG2 and Huh7) cells when compared to control cells (Fig.4.B).
Fig.4. Kaempferol induces cell cycle arrest and inhibits BCL2L12 protein expression
HCC cells were treated with Kaempferol and 24 h later, the effect of Kaempferol on cell cycle (A) were determined by flow cytometry using Annexin V-FITC and PI staining. Effect of Kaempferol on BCL2L12 Protein expression was analyzed by Immunocytochemistry (B). Error bars presented as mean ± s.d. and P-values are represented as ***P< 0.001 compared to the control.

BCL2L12 Expression is significantly increased in HCC Cell Lines and Regulated by PI3K/AKT/Bcl-2 signaling
First, we analyzed the relative expression level of BCL2L12 in HepG2, Huh7, and THLE-2 cells by qRT-PCR (Fig.5.A) and western blot (Fig.5.B). We observed the overexpression of BCL2L12 in HCC (HepG2 and Huh7) cell lines when compared to the normal liver cell line (THLE-2). Next, we analyzed the effect of Kaempferol on regulatory genes of apoptosis (Bcl-2, Caspase3, and PTEN, BCL2L12), angiogenesis (MMP9) (Fig.5.C-D), cell cycle (p53 and p21), autophagy (LC3A), and PI3K/AKT signalling (Fig.5.E-F) in HCC cells. The results indicated that the BCL2L12, Bcl-2, PI3K, AKT, MMP9, and LC3A/B expression decreased, and p53, p21, PTEN, and caspase-3 expression increased in Kaempferol treated HCC cells when compared to control. GAPDH used as an internal control. This result showed that Kaempferol regulates apoptosis, cell cycle, autophagy, and angiogenesis through targeting Bcl-2/PI3K/AKT/BCL2L12 in HCC cells.
**Fig.5. Effect of Kaempferol on apoptosis and angiogenesis regulatory genes**

The relative mRNA (A) and protein (B) expression level of BCL2L12 in HCC (HepG2 and Huh7) and THLE-2 cells. HCC cells were treated with Kaempferol for 24 h. The mRNA (C) and protein (D) expression level of BCL2L12, Bcl-2, Caspase 3, PTEN, and MMP9 evaluated by qRT-PCR and western blot. Kaempferol regulates p53, p21, PI3K, AKT, and LC3A/B, mRNA (E) and protein (F) levels in HCC cells. GAPDH and β-actin were used for normalization. Error bars presented as mean ± s.d and P value represented as **P < 0.01, ***P < 0.001, ****P < 0.0001 compared to control.

### 4. Discussion

Kaempferol is a flavonoid present in various foods such as apples, strawberries, beans, and citrus fruits, some of the major uses of Kaempferol are anti-oxidant and anti-inflammation, etc. The most important of all is that Kaempferol shows the significant anti-cancer activity demonstrated to be effective in inhibiting cell growth and proliferation in various cancers [27]. Kaempferol inhibits migration and invasion, reduce MMP2 expression and their enzyme activity and also inhibits the ERK1/2 phosphorylation in SCC4 (human tongue squamous cell carcinoma) cells [28]. Previous studies have shown that Kaempferol is a potential agent for HCC treatment due to its antitumor properties and also inhibit the Hep3B cell proliferation [29].

In this study, Kaempferol inhibits Bcl-2/PI3K/AKT mediated BCL2L12 expression and induces cellular apoptosis by the upregulation of p53 in HCC cells. p53 is a well-known tumor suppressor protein, and it works by activating p21. Kaempferol is known to modify several upstream regulators of p21 and enhance the phosphorylation of p53 [30]. p21 is a highly important checkpoint gene in the cell cycle and regulated by the transcription of p53 [31]. Rb phosphorylation is a more important target for regulation of G1/S progression by p53. The activation of cyclin kinase inhibitor p21 can, therefore, inhibit Rb phosphorylation and prevent E2F transcriptional activity leading to cell cycle arrest in untreated cells [32].

The Bcl-2 family members are essential regulators of apoptosis of this BCL2L12, a member of the Bcl-2 family, contains several evolutionarily conserved p53-binding sites and act as a potent inhibitor of caspase activation and pro-apoptotic activities [33]. BCL2L12 interacts with p53 and inhibit its binding capacity with their target genes. Thus, BCL2L12 suppresses the p53 transcription levels after genotoxic stress and inhibit DNA damage-induced apoptosis and cell cycle arrest activities by downregulation of p53. BCL2L12 neutralizes the transactivational activity of p53 by inhibiting the binding of p53 to particular target promoters and attenuated p53-mediated induction of selected target genes, including...
the cell cycle inhibitor p21 and the proapoptotic protein Bax. Besides, Bcl2L12 mediates neutralization of p53 activity and inhibits DNA damage-induced apoptosis in glioma cells [11]. Our result showed that Kaempferol effectively inhibits both G1 and G2 phase regulation, cellular proliferation, migration and induced chemosensitivity.

Subsequent data also showed that PI3K, Akt, Bcl-2, BCL2L12, MMP9, and LC3A/B protein expressions were downregulated and PTEN, p53, p21, and Caspase 3 expression significantly were upregulated in Kaempferol-treated HCC cells.

**Conclusion**

Kaempferol effectively enhances apoptosis, cell cycle arrest, autophagy, and chemosensitivity by targeting PI3K/AKT signaling pathway-dependent Bcl-2/BCL2L12 in HCC cells. The multi-functional BCL2L12, an important oncprotein regulated by PI3K/AKT/Bcl-2 and acting at the intersection of nuclear p53 and cytoplasmic caspase signaling and PI3K/AKT/Bcl-2/BCL2L12 complex could be a promising novel therapeutic target for the treatment of HCC. Thus, Kaempferol may act as a potential therapeutic agent for the treatment of metastatic HCC.

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**Disclosure of interest**

The authors declare that they have no conflicts of interest concerning this article.

**5. Reference**


**Supplementary Table 1.**

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