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Tangeretin induces cell cycle arrest and apoptosis through upregulation of PTEN expression in glioma cells

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ABSTRACT

Tangeretin (TANG), present in peel of citrus fruits, has been shown to various medicinal properties such as chemopreventive and neuroprotective. However, the chemopreventive effect of TANG on glioblastoma cells has not been examined. The present study was designed to explore the anticancer potential of TANG in glioblastoma cells and to investigate the related mechanism. Human glioblastoma U-87MG and LN-18 cells were treated with 45 μM concentration of TANG and cell growth was measured by MTT assay. The cell cycle distribution and cell death were measured by flow cytometry. The expression of cell cycle and apoptosis related genes were analyzed by quantitative RT-PCR and western blot. The cells treated with TANG were significantly increased cell growth suppression and cell death effects than vehicle treated cells. Further, TANG treatment increases G_2/M arrest and apoptosis by modulating PTEN and cell-cycle regulated genes such as cyclin-D and cdc-2 mRNA and protein expressions. Moreover, the ability of TANG to decrease cell growth and to induce cell death was compromised when PTEN was knockdown by siRNA. Taken together, the chemopreventive effect of TANG is associated with regulation of cell-cycle and apoptosis in glioblastoma, thereby attenuating glioblastoma cell growth. Hence, the present findings suggest that TANG may be a therapeutic agent for glioblastoma treatment.

1. Introduction

Glioma are the most predominant and common primary tumors of the central nervous system and lethal primary brain tumor [1]. Among all primary gliomas, glioblastoma is accounting for 55% of brain tumors [2]. Although the improvement of surgical operation with radiotherapy and chemotherapy for cancer treatment in the medical field, the prognosis for malignant glioblastoma remains poor, with a median survival of less than 1.5 years and 5-year survival rate among all cancers [3,4]. Long-term exposure of chemotherapeutic drugs leads to various physiological complications and some tumor cells are resistant to specific chemotherapy drugs [5]. Therefore, identifying newer chemopreventive drugs are necessary to develop novel treatment to improve the prognosis of glioma.

Several signaling pathways has been dysregulated and considered to be involved in the progression of cancer. Instance, phosphatase and tensin homolog (PTEN) is a potent tumor suppressor gene and its loss-of-function is encountered in human cancers. PTEN mutations are seen in 60% of glioblastoma and are among the most common genetic alterations in glioblastoma [6]. PTEN mutations in glioblastoma are associated with increased invasive behaviors and drug resistance [7,8].

Over the past few decades, research has been focused on number of dietary flavonoids and botanical natural compounds, which have chemopreventive properties that can reduce or prevent the tumorigenesis [9,10]. Flavonoids have also been shown to induce apoptosis in targeting cancer cells but not in normal cells [20]. For example, genistein, a soy isoflavone, has been shown to induce apoptosis in multiple cancer cells by down-regulating PI3-K/Akt pathway [11]. Tangeretin (TANG), a 4′,5,6,7,8-pentamethoxyflavone, rich in peel of citrus fruits and has been reported that TANG possess several biological activities such as suppression of cancer [12], metastasis [13], induction of apoptosis [14] and neuroprotective [15] properties. TANG has been shown to inhibit xenobiotic-induced genotoxicity in vitro [16]. The role and

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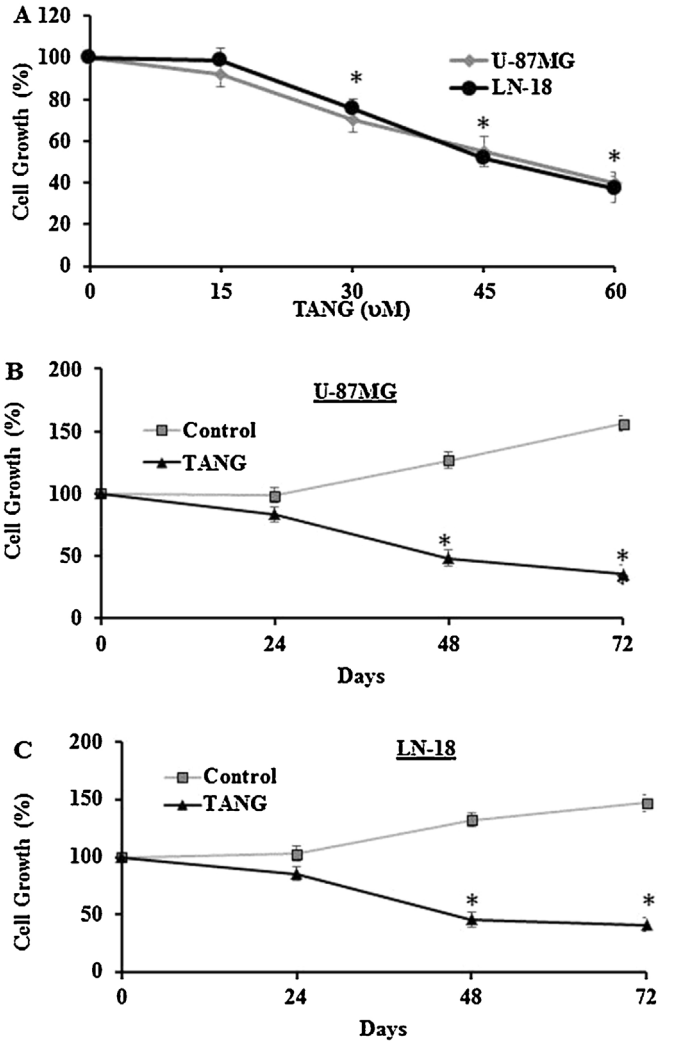

Fig. 1. TANG inhibits glioblastoma cell growth in a dose and time dependent manner. (A) U-87MG and LN-18 cells were treated with different concentrations (0, 15, 30, 45 and 60 μM) of TANG for 48 h. The cell growth was measured as described in materials and methods and expressed as percentage of cell growth. (B) U-87MG and (C) LN-18 cells were treated with 45 μM of TANG for 24, 48 and 72 h and cell growth was measured. The data are expressed as mean ± standard deviation. Statistical analyses were performed with Student's *t*-test. $^{*}p < 0.01$ compared with vehicle treated cells (Control).

molecular mechanisms of TANG in therapy has been reviewed [17,18]. Though several earlier studies focused on TANG as anti-cancer agents in different cancer models, the role of TANG on glioblastoma and its mechanism has not yet been examined. Hence, in the present study, we demonstrated the anti-proliferative and apoptotic potential of TANG on two glioblastoma cell lines. Here we show that TANG inhibits cell growth, arrest cells at G_2/M phase, induce apoptosis by regulating PTEN and cell-cycle regulatory proteins.

2. Materials and methods

2.1. Cell lines and reagents

Human glioblastoma cells (U-87MG and LM_18A549 and H1299) were procured from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cells were maintained in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (FBS), 1X penicillin/streptomycin. All cells were incubated in a humidified atmosphere composed of 5% CO_2 at 37 °C. Tangeretin (TANG) was obtained from Indofine Chemical Company, USA and dissolved in DMSO. All other chemicals used were obtained from local commercial sources.

2.2. Cell proliferation assay

Cell growth was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Cells were plated in 96-well plates at a density of 10,000 cells per well. After 24 h, cells were treated with vehicle (DMSO) or different concentrations of TANG. Forty-eight hours after treatment, MTT reagents were added to each well and incubated for 3 h. DMSO was added to all wells and the plates were read at 595 nm.

2.3. Cell cycle analysis

Cell cycle analyses were performed by measuring cellular DNA content with propidium iodide as a dye. Cells were fixed with 75% ethanol at −20 °C. The ethanol fixed cells were re-suspended in PBS containing ribonuclease A (100 μg/ml) and incubated for 1 h at 37 °C. The ethanol fixed cells were stained with propidium iodide (50 μg/ml) and incubated for 30 min at room temperature in the dark. The data were acquired and analyzed using a FACSCalibur flow cytometer.

2.4. Transfection of siRNAs

PTEN-specific siRNA were synthesized from GenePharma (Shanghai, China). The sense and antisense oligos of PTEN siRNA sequences (siPTEN) were 5′-CAAGATGATGTTTGAAACTAT-3′ and 5′-GGCGCTATGTATATTATTATA-3′, respectively. Scrambled control (siCtl) was 5′- AATTCTCCGAACGTGTCACGT-3′. Transfection of siRNAs were carried out using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. In brief, about 3×10^5 cells/well were seeded in a six-well plate. One day after plating, the cells were transfected with either siCtl or siPTEN with Lipofectamine (Life Technologies, Grand Island, NY). After 48 h of transfection, cells were treated with vehicle or TANG and collected for subsequent analysis.

2.5. Apoptosis assay

Induction of apoptosis was determined using FITC Annexin V kit according to the manufacturer's instruction. Briefly, cells were trypsinized and pelleted by centrifugation at 1000 rpm for 5 min. After resuspending the pellets in binding buffer, add 5 μl of Annexin V-FITC and 5 μl of propidium iodide (50 μg/ml) and incubated at room temperature for 5 min in the dark. Flow cytometry was performed in a FACScan.

2.6. RT-PCR (qRT-PCR) analysis

Cells were harvested after 24 h of treatment with TANG and total RNAs were extracted using TRIzol reagent. cDNA were synthesized from 2 μg of total RNA using Oligo(dT) 12–18 primer and Superscript II reverse transcriptase. TaqMan probes were used to measure PTEN, cyclin-D and cdc-2 and GAPDH mRNA expressions. The PCR reactions were carried with 20 μl mixture containing 100 ng of cDNA, 10 μl of TaqMan 2X universal PCR master mix and 1 μl of specific probes. The PCR reactions were run on the ABI Prism 7900 Fast Real-time PCR system for each gene and each sample in triplicate as follows: 95 °C for 10 min, 45 cycles of a 15-s denaturing at 95 °C, and 1 min annealing at 60 °C. SDS 2.1 Software (ABI) was used to calculate mRNA expression levels, normalized to endogenous control gene (GAPDH), and relative to their corresponding controls.