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

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A B S T R A C T
Tangeretin (TANG), present in peel of citrus fruits, has been shown to possess 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 antitumor 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 G0/G1 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 knockeddown 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.

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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].
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, arrests cells at G2/M phase, induces 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% CO2 at 37°C. Tangeretin (TANG) was obtained from Indofine Chemical Company, USA and dissolved in DMSO. All other chemicals 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′-GGCCGTATGATATTATATA-3′, respectively. Scrambled control (siCtl) was 5′-AATTCTCGAAGCTGTCAGT-3′. Transfection of siRNAs were carried out using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. In brief, about 3 × 105 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, the 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 pelletted 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.
overnight at 4°C with primary antibodies specific for PTEN, cyclin-D, cdc-2 and GAPDH. The corresponding secondary IgG antibodies with alkaline phosphatase markers were used and incubated for 2 h at room temperature. The membranes were developed using ECL reagent.

2.8. Statistical analysis

All data are expressed as mean ± standard deviation (SD). Significant differences were assessed using Student’s t-test and p value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Effect of TANG on glioblastoma cell growth

We first performed extensive dose findings of different concentrations of TANG (0, 15, 30, 45 and 60 μM) using two different human glioblastoma cell lines, U-87MG and LN-18. U-87MG and LN-18 cells were treated with different concentrations of TANG for 48 h and measured cell growth by MTT assay. As shown in Fig. 1A, TANG significantly inhibited the cell growth in both cells in a dose dependent manner and 50% of inhibition was observed at the concentration of 45 μM of TANG. Further, a subsequent time course study (Fig. 1B, C) showed that 45 μM of TANG inhibited 50% of cell growth at 48 h in both cell lines.

3.2. Effect of TANG on cell-cycle progression

To evaluate the inhibitory effect of TANG on cell proliferation, we measured cell-cycle progression by using flow cytometry. U-87MG and LN-18 cells were treated with 45 μM of TANG for 48 h and measured cell-cycle as outlined in materials and methods. As shown in Fig. 2A-B, TANG treated U-87MG and LN-18 cells resulted in an increase of G2/M phase from 21% to 46% and 50%, respectively (21%), which was accompanied by decrease of G1 phase as compared to vehicle treated cells (Control).

3.3. Effect of TANG on cell death

Next we examined the effect of TANG on cell death by measuring apoptotic cells. U-87MG and LN-18 cells were treated with 45 μM of TANG for 48 h and measured apoptotic cells as outlined in materials and methods. The TANG treated cells showed a significant increase (p < 0.0001) of apoptotic cells in both U-87MG and LN-18 cells as compared to vehicle treated cells (Fig. 3A and B).

3.4. Effect of TANG on PTEN and cell-cycle regulated mRNA and protein expressions

To demonstrate the molecular mechanism of TANG on cell growth and observed cell-cycle distribution, we measured the mRNA and protein levels of PTEN, cyclin-D and cdc-2 in U-87MG cells. U-87MG cells were treated with 45 μM of TANG for 24 h and measured mRNA and protein expressions as described in materials and methods. The mRNA expression of PTEN was significantly increased, whereas the mRNA expressions of cyclin-D and cdc-2 were markedly decreased in TANG treated cells as compared to control cells (Fig. 4A). Further, the PTEN protein level was also increased and concomitant decrease of cyclin-D and cdc-2 protein levels in TANG treated cells (Fig. 4B), indicating that reduction of cyclin-D and cdc-2 due to TANG treatment may be responsible for TANG-induced G2/M phase cell-cycle arrest.
3.5. Effect of TANG on cell growth and apoptosis in PTEN knockdown cells

To investigate whether TANG-induced cell growth suppression and apoptosis are mediated through upregulation of PTEN, we knockdown PTEN in U-87MG cells and the knockdown efficiency was confirmed by western blot (Fig. 5A). Further, PTEN knockdown cells were treated with TANG for 48 h and measured cell growth and apoptosis. As shown in Fig. 5B, TANG did not suppress the cell growth in PTEN knockdown cells (siPTEN + TANG) as compared to siCtrl cells treated with TANG (siCtrl + TANG). Similarly, TANG did not induce cell death in PTEN knockdown cells (siPTEN + TANG) as compared to siCtrl cells treated with TANG (siCtrl + TANG), suggesting that PTEN is a mediator for TANG induced cell death and growth suppression.

4. Discussion

Glioblastoma remains the major causes of death worldwide and the treatment strategy is chemotherapy with cytotoxic agents, which increases patient survival but also has side effects that severely limit its clinical effectiveness such as acquisition of drug resistance [19,20]. Existing therapies, including chemotherapy and radiotherapy has been the backbone for cancer treatment; however, these therapies is somewhat limited with severe side effects. Therefore, novel therapeutic methods with natural compounds may increase anti-cancer activities by decreasing the side effects of chemotherapy. Recently, the use of natural dietary supplements have increased significant consideration as chemopreventive agents, which can prevent the process of carcinogenesis [21]. Despite TANG has been shown to possess chemopreventive effect in many cancer models, the exact mechanism of anticancer activity is not well understood, particularly in glioblastoma. Thus, the aim of this study was to examine the effect of TANG on glioblastoma cells with regulation of cell-cycle, apoptosis and PTEN expression that required for the induction of cancer cell death.

In the current study, we demonstrated that cells treated with TANG exhibited decreased cell growth in a dose and -time dependent manner, increase cells at G2/M phase and induce apoptosis. Further, TANG treatment increased PTEN expression and decreased cell-cycle regulatory genes including cyclin-D and cdc-2 at transcription and translational levels. The ability of TANG to decrease cell growth and to induce cell death was compromised when PTEN was knockdown by siRNA.

Cell-cycle checkpoints are play major role in protecting genomic DNA against environmental agents that may cause DNA damage and chromosome segregation [22,23]. Environmental agents-induced these errors transform signals to proteins that affect apoptosis, DNA repair, cell-cycle arrest at different checkpoints [24]. In the present study, TANG-increased cell-cycle arrest at G2/M phase may allow cells to complete DNA replication through repair damaged DNA and thereby suppressing the tumor growth process. The M-phase-promoting factor is an important regulator of the G2 to M transition that consists of regulatory
cell-cycle and apoptosis. Thus, our findings suggest that TANG might use as a chemopreventive agent for glioblastoma treatment. However, further studies are required to determine whether TANG can be implemented as a chemopreventive for the treatment of glioblastoma.

References


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