

# Anticancer effect of triterpenes from *Ganoderma lucidum* in human prostate cancer cells

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Received February 29, 2016; Accepted July 14, 2017

DOI: 10.3892/ol.2017.7153

**Abstract.** *Ganoderma lucidum*, within the Polyporaceae family of Basidiomycota, is a popular traditional remedy medicine used in Asia to promote health and longevity. Compounds extracted from *G. lucidum* have revealed anticancer, antioxidant and liver protective effects. *G. lucidum* has been associated with prostate cancer cells. *G. lucidum* extracts contain numerous bioactive components; however, the exact functional monomer is unknown and the role of triterpenes from *G. lucidum* (GLT) in prostate cancer remain obscure. The present study investigated the effects of GLT on cell viability, migration, invasion and apoptosis in DU-145 human prostate cancer cells. The results demonstrated that a high dose (2 mg/ml) of GLT inhibits cell viability in a dose- and time-dependent manner by the regulation of matrix metalloproteases. Furthermore, GLT induced apoptosis of DU-145 cells. In general, GLT exerts its effect on cancer cells via numerous mechanisms and may have potential therapeutic use for the prevention and treatment of cancer.

## Introduction

Prostate cancer is the most common type of malignancy and the second leading cause of cancer-associated mortalities in the USA (1). Prostate cancer cells first respond to androgen ablation therapy, but long-term anti-androgen treatment results in loss of responsiveness. Prostate cancer types may eventually develop to be androgen-independent with markedly metastatic behavior. Prostate cancer cells metastasize to other parts of the human body; therefore, prostate cancer has deleterious effects on the survival time and quality of life of patients. Until 2007, ~80% of patients who were diagnosed with prostate cancer developed bone metastasis (2,3). Thus, understanding the

molecular mechanisms underlying the metastasis of prostate cancer is critical for the prevention and therapy of prostate cancer metastasis.

Pharmaceutical compounds extracted from mushrooms have demonstrated benefits for the treatment of a variety of diseases, including various types of cancer, immunological disorders and neurodegenerative diseases (4-6). *Ganoderma lucidum*, a basidiomycetous fungus, is a type of mushroom that has widely been used as a traditional medicine for thousands of years, particularly in Asia (7). A number of bioactive chemical substances, including polysaccharides, triterpenoids and proteins are extracted from the fruiting bodies, cultured mycelia and spores of *G. lucidum* (8). Previous studies have indicated that the active compounds isolated from its fruiting body 'Lingzhi' participate in a variety of biological processes, including anti-inflammatory, antioxidant, antitumor and immunomodulatory activities (9-13). Triterpenes isolated from *G. lucidum* (GLT) also exhibit cytotoxic activity against mouse sarcoma and mouse lung carcinoma cells (14).

*G. lucidum* has been reported to inhibit the metastasis of human prostate cancer cells (15,16); however, because extracts of *G. lucidum* contain numerous bioactive compounds, the exact functional compound remains to be clarified. The present study was performed to elucidate the role of GLT on prostate cancer cells. The present study demonstrated that GLT significantly inhibited cell viability and induced apoptosis in highly invasive DU-145 prostate cancer cells. Additionally, GLT suppressed the migration and invasion via inhibition of matrix metalloproteinase (MMP) expression.

## Materials and methods

**Cell culture and reagents.** DU-145 human prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). DU-145 cells were maintained in F-12 medium containing penicillin (50 U/ml), streptomycin (50 U/ml) and 10% fetal bovine serum (FBS; all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. GLT was purchased from Nanjing Zhongke Pharmaceutical Co. Ltd. (Nanjing, China). The principal component of GLT is ganoderic acid H, with a purity of ~99%. GLT was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a concentration of 40 mg/ml and

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**Key words:** *Ganoderma lucidum*, triterpenes, prostate cancer, metastasis, apoptosis

stored at 4°C (the final concentration of DMSO in the controls and GLT was <0.1% to exclude its toxicity). DMSO was used as the control.

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was isolated from cells using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse-transcribed using a SuperScript First Strand cDNA system (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The following primers were used for amplification of MMP-2: Sense primer 5'-GTCCAC TGTGGTGGGAACT-3' (sense) and 5'-CTCCTGAATGCC CTTGATGT-3' (antisense); Thermocycling conditions were: 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 68°C for 1 min, then 68°C for 10 min.

MMP-9, 5'-GACAAGAAGTGGGGCTTCTG-3' (sense) and 5'-TCAAAGACCGAGTCCAGCTT-3' (antisense); Thermocycling conditions were: 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, then 72°C for 10 min.

$\beta$ -actin (internal control) 5'-CGAAACTACCTTCAA CTCCATCA-3' (sense) and 5'-CGGACTCGTCATACTCCT GCT-3' (antisense). Thermocycling conditions were 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 68°C for 1 min, then 68°C for 10 min. The PCR products were analyzed by 2% agarose gel electrophoresis and confirmed by their appropriate size. The image was taken with the gel imaging analysis system and the gray scale values were analyzed using ImageJ software (V2.1.4.7; National Institutes of Health, Bethesda, MD, USA). Experiments were repeated three times.

**Cell viability assay.** Cell viability was determined using an MTT assay, according to the manufacturer's protocol (Promega Corporation, Madison, WI, USA). Briefly, DU-145 cells cultured in a 96-well plate at a density of  $1 \times 10^4/\text{cm}^2$  were treated with 0.1, 0.5, 1 and 5 mg/ml GLT for 24 h and with 2 mg/ml GLT for 1, 2 and 3 days. At the end of the incubation period, absorption was determined using a microplate reader at 570 nm.

**Wound-healing assay.** DU-145 cells were cultured in 6-cm plates until confluent and subsequently the monolayer was scratched using a fine sterile pipette tip to produce a narrow wound. The medium and debris were aspirated away and replaced with fresh medium in the presence of 0.1 and 2 mg/ml GLT. Images were captured prior to and 6, 12 and 24 h after wounding using a Nikon TMS-F phase-contrast microscope (Nikon Instruments, Florence, Italy).

**Transwell migration assay.** Migration assays with DU-145 cells were performed using a Transwell kit from BD Biosciences (Franklin Lakes, NJ, USA). Cells were removed from culture plates with trypsin and washed twice with PBS. Cells ( $4 \times 10^4$ ) in 250  $\mu\text{l}$  Dulbecco's modified Eagle's medium (DMEM) with or without 0.1 and 2 mg/ml GLT were placed in the upper invasion chamber and the lower compartment was loaded with DMEM supplemented with 10% FBS. The cell migration chamber was inserted into the lower compartment and incubated for 24 h at 37°C. Cells on the upper side

of the filter were removed with a cotton swab. Cells attached to the filter were fixed with 100% methanol for 10 min at room temperature. Cells attached to the filter were stained with Giemsa stain (5%) for 1 h at room temperature. Filters were destained by washing with water and the number of cells attached to the filter was then quantified by enumerating cells in images captured using a light microscope of the stained filters.

**Transwell invasion assay.** An invasion assay with DU-145 cells was performed using BD BioCoat Matrigel invasion chambers (BD Biosciences), according to the manufacturer's protocol. DU-145 cells were seeded at  $4 \times 10^4$  cells per well in serum-free DMEM in the upper compartment, with or without 0.1 and 2 mg/ml GLT, and DMEM supplemented with 10% FBS was placed in the lower compartment of the chamber as a chemoattractant. Following a 24-h incubation, the non-invading cells on the upper side of the chamber were removed and the membranes were fixed with 100% methanol for 10 min at room temperature, washed with PBS and stained with Giemsa stain (5%) for 1 h at room temperature. Invasiveness was evaluated by counting the invading cells under a light microscope.

**Flow cytometric assay.** Cells were washed with ice-cold PBS, resuspended in 100  $\mu\text{l}$  binding buffer (1% bovine serum albumin (Thermo Fisher Scientific, Inc.) in PBS) and stained with fluorescein isothiocyanate (FITC)-annexin V (BD Biosciences). The cells were incubated for 15 min at 37°C in the dark. Subsequently, 200  $\mu\text{l}$  binding buffer supplemented with propidium iodide (PI; 20  $\mu\text{g}/\text{ml}$ ) was added immediately prior to flow cytometry. The cells were analyzed using FACSCanto cytometer and FACSDiva software (V.5.0.2; BD Biosciences). Early and late apoptosis was evaluated. Cells at early and late apoptosis were counted.

**Statistical analysis.** Results are presented graphically as the mean  $\pm$  standard error of the mean of at least three independent experiments. The statistical significance of the differences was analyzed using Student's t-test between two groups and one-way analysis of variance with Newman-Keuls post-hoc tests for comparisons among more than two groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**GLT extract inhibits the viability of prostate cancer cells in a dose- and time-dependent manner.** To examine the effect of GLT extract on cell viability, cultured DU-145 cells were treated with increasing concentrations of GLT (0.1, 0.5, 1 and 5 mg/ml), and the viability of cells was determined using an MTT assay. As presented in Fig. 1A, a low concentration of GLT (0.1 mg/ml) had less effect on the viability of DU-145 cells compared with the control ( $P > 0.05$ ). Once the concentration reached  $\geq 0.5$  mg/ml, the viability of DU-145 cells was significantly inhibited compared with the control ( $P < 0.05$ ). Furthermore, as presented in Fig. 1B, 2 mg/ml GLT markedly inhibited the proliferation of DU-145 cells at 24 h after the treatment. Subsequently, 2 mg/ml GLT was selected as the treatment concentration and 0.1 mg/ml GLT as an additional control for further experiments.

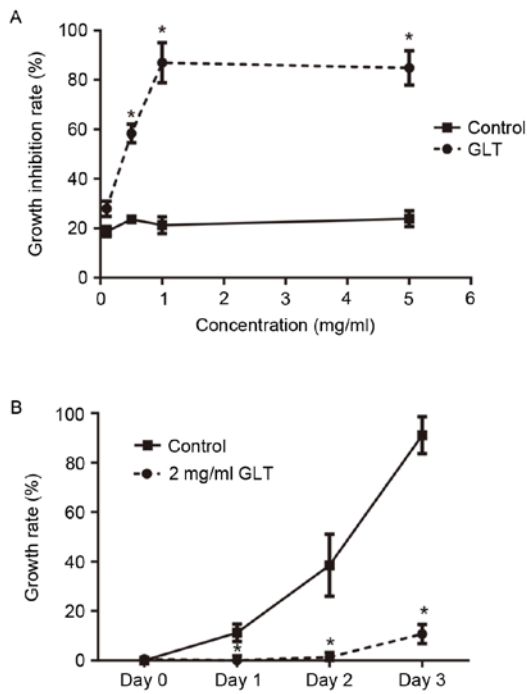


Figure 1. GLT inhibits the viability and proliferation of DU-145 prostate cancer cells. (A) DU-145 cells were treated with 0.1, 0.5, 1 and 5 mg/ml GLT for 24 h. Cell viability was determined using an MTT assay. (B) Cultured DU-145 cells were treated with 2 mg/ml GLT for 1, 2 and 3 days. The proliferation rates were determined using an MTT assay. \*P<0.05 vs. control. GLT, *Ganoderma lucidum* triterpene.

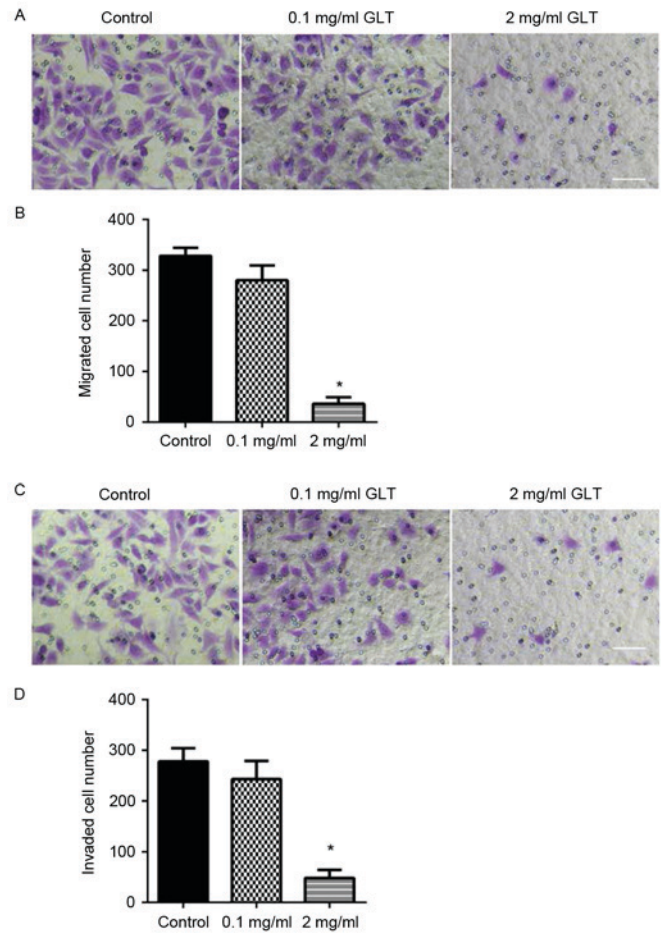


Figure 3. Transwell migration assay and invasion assay. DU-145 cells were placed in the upper invasion chamber and the lower compartment was loaded with DMEM containing 10% FBS. The cell migration chamber was inserted into the lower compartment and incubated at 37°C for 24 h, with or without the administration of GLT at the indicated concentrations. (A) Representative images. (B) Migrated cells were counted. (C) DU-145 cells were seeded at  $4 \times 10^4$  cells per well in serum-free DMEM in the upper compartment, with or without various concentrations of GLT and 24 h after the cells were harvested. Representative images. (D) Migrated cells were counted. \*P<0.05 vs. control and 0.1 mg/ml groups. Scale bar, 20  $\mu$ m. GLT, *Ganoderma lucidum* triterpene; DMEM, Dulbecco's modified Eagle's medium.

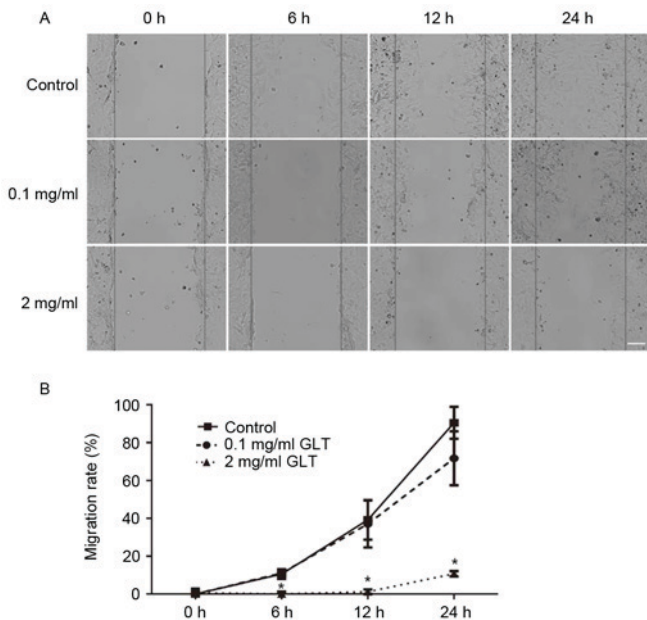


Figure 2. Wound healing assay. (A) DU-145 cells were scratched using a fine sterile pipette tip to produce a narrow wound. The medium and debris were aspirated away and replaced with fresh medium in the presence of 0.1 and 2 mg/ml GLT or an equal volume of fresh medium for the control group. Images were captured prior to and 6, 12 and 24 h after the treatments. Scale bar, 20  $\mu$ m. (B) Quantification of results. \*P<0.05 vs. control. GLT, *Ganoderma lucidum* triterpene.

GLT suppresses the migration and invasion of prostate cancer cells. The present study investigated the effect of GLT on cell

migration and invasion. First, a wound-healing assay was performed. As presented in Fig. 2, the control group cells migrated across the wound, which was healed after 24 h; however, 2 mg/ml GLT significantly inhibited this process and 0.1 mg/ml GLT exhibited no effect. Furthermore, cell migration and invasion were assessed using a Transwell system. DU-145 cells treated with or without GLT were allowed to migrate through a Transwell membrane into complete medium. Equal quantities of cells were transferred to the membrane surface and cell migration was assessed within 24 h. Compared with the control or 0.1 mg/ml GLT-treated cells, 2 mg/ml GLT led to a decreased level of cell migration (Fig. 3A and B). To evaluate cell invasion, DU-145 cells were plated on the Matrigel surface. As presented in Fig. 3C and D, 2 mg/ml GLT treatment significantly decreased DU-145 cell invasion. In addition, migration/invasion-associated genes, MMPs, have previously been implicated in prostate cancer metastasis (17,18). Thus, the mRNA expression level of MMP-2 and MMP-9 were investigated. As presented in Fig. 4, the results of

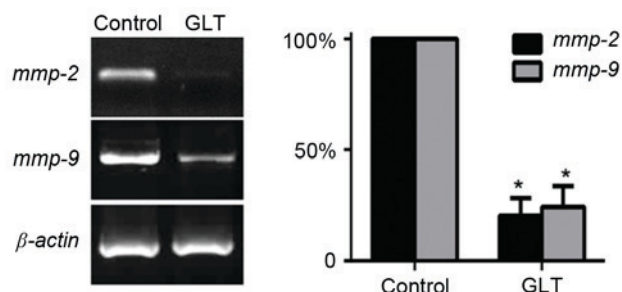


Figure 4. GLT administration suppresses the expression of MMPs. DU-145 cells were treated or not with 2 mg/ml GLT for 24 h, then cells were subjected to the reverse transcription-quantitative polymerase chain reaction. The expression levels of MMP-2 and MMP-9 are presented, with  $\beta$ -actin as the control (left panel). Normalized expression levels of MMPs are presented in the right panel. \* $P < 0.05$  vs. control. GLT, *Ganoderma lucidum* triterpene; MMP, matrix metalloproteinases.

RT-PCR indicated that 2 mg/ml GLT significantly suppressed the expression levels of MMPs. These results revealed that a high dose of GLT inhibited prostate cancer cell migration and invasion via the suppression of MMPs.

*GLT induces prostate cancer cell apoptosis.* It was investigated whether GLT was able to induce the apoptosis of prostate cancer cells. The apoptosis rate was determined by annexin V-FITC and PI double staining. Compared with the control group, 0.1 mg/ml GLT administration revealed no effect, whereas the apoptosis rate with 2 mg/ml GLT treatment was markedly increased (Fig. 5). These results suggested that GLT inhibited viability, migration and invasion, and also induces the apoptosis of prostate cancer cells *in vitro*.

## Discussion

The results of the present study demonstrated that GLT inhibits the growth of prostate cancer cells, suppresses the migration and invasion and induces apoptosis via the inhibition of MMP expression. The elucidation of the anticancer mechanism underlying GLT may contribute to the clinical usage of active compounds isolated from *G. lucidum*.

*G. lucidum* has been used as a preventive medicine in Asia for centuries (19). Various biologically active compounds have been isolated from *G. lucidum*, including polysaccharides, phenols, lipids and triterpenes. Polysaccharides possess antioxidant (20), immunomodulatory (21) and antitumor characteristics (22). Also, polysaccharides activate the immune response via the stimulation of production of inflammation mediators (5,23). Phenols have antioxidant properties (24) and lipids are able to inhibit the growth of hepatoma and sarcoma (25). Triterpenes demonstrate cytotoxicity towards hepatoma, cervical cancer and lung carcinoma cells (26-28). Besides the antitumor activity, GLT serve a role in a variety of other biological process, including anti-human immunodeficiency virus (HIV) and anti-HIV-1 protease activity (29,30), neurotrophic activity (31) and anti-obesity activity (32). The results of the present study identified that GLT inhibited the metastasis and induced the apoptosis of prostate cancer cells. These results are consistent with those of previous studies that identified the anti-prostate cancer effect of *G. lucidum* (15,16).

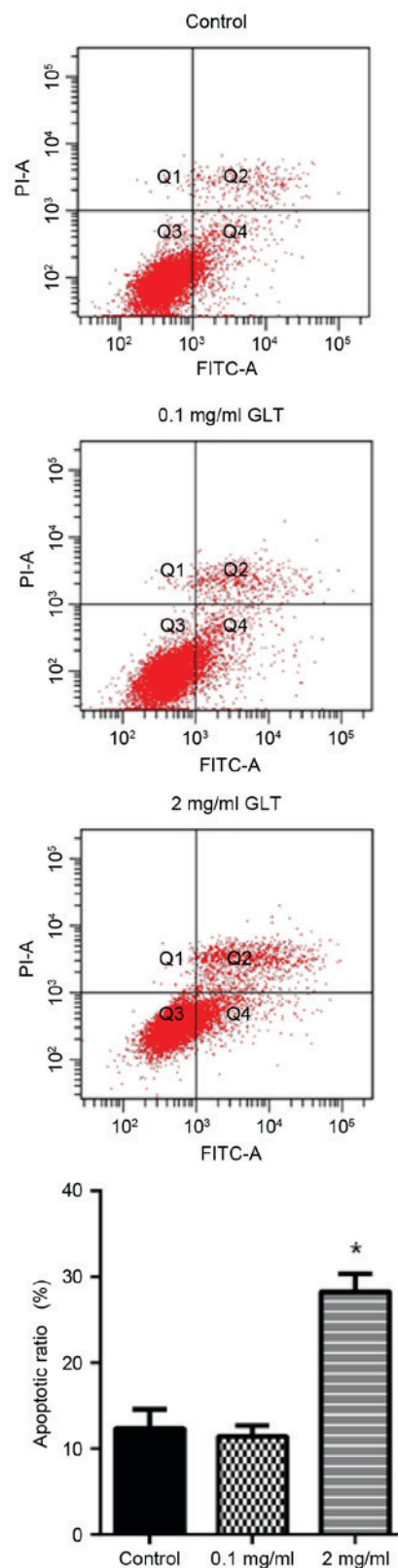


Figure 5. GLT induces the apoptosis of DU-145 cells. DU-145 cells were treated or not with 2 mg/ml GLT for 24 h, then cells were subjected to flow cytometry. The apoptotic rates were analyzed. \* $P < 0.05$  vs. control. GLT, *Ganoderma lucidum* triterpene; FITC, fluorescein isothiocyanate; PI, propidium iodide.

The identification of biologically active components of *G. lucidum* is important for the mechanistic characterization of their specific activity and the results of the present study

further revealed that triterpenes may be the active compounds responsible for the antitumor effect of *G. lucidum*. In addition, there is certain evidence that specific components in the natural herbal products interact with each other to function as the whole product (33). The present study did not identify other compounds extracted from *G. lucidum*, which requires further investigation.

The inhibition of the viability of prostate cancer cells by GLT may be caused by the induction of apoptosis. Apoptosis is a physiological process where cells are removed when they experience critical DNA damage (34,35). Inhibition of apoptosis, rather than enhanced cell proliferation, is particularly important for the development of cancer (36,37). The results of the present study revealed that GLT significantly induced apoptosis, confirmed by annexin V staining and flow cytometry. Generally, apoptosis can be divided into early and late stages. During the late stage of apoptosis, DNA fragmentation occurs following reactive oxygen species generation, caspase-3 activation and mitochondrial dysfunction (38). Additionally, apoptosis can be divided into the extrinsic and intrinsic pathways. The extrinsic apoptotic pathway can be initiated by death receptors (39) and the intrinsic pathway is also called the mitochondrion-dependent pathway (40). The majority of stimuli induce apoptosis via the mitochondrial pathway, which induces mitochondrial outer membrane permeabilization and these processes are regulated by members of the B cell lymphoma-2 (Bcl-2) protein family. This family may be subdivided into pro-apoptotic (Bcl-2-associated X protein and Bcl-2 homologous antagonist killer), pro-Bcl-2 homology 3-only proteins (including BH3-interacting domain death agonist, Bcl-2-like protein 11 and p53-upregulated modulator of apoptosis) and anti-apoptotic proteins [including Bcl-2 and B cell lymphoma extra-large (Bcl-xL)] (35). Decreased expression levels of anti-apoptotic proteins and increased expression levels or activation of pro-apoptotic proteins have been demonstrated to be critical for the induction of apoptosis (35,41). DU-145 prostate cancer cells have increased expression levels of Bcl-2 and Bcl-xL, protecting cells from apoptosis (42,43). The results of the present study revealed that GLT induced apoptosis; however, the specific underlying molecular mechanisms remain under investigation. The results of the present study suggested that GLT may decrease the expression of anti-apoptotic proteins and increase the expression levels of, or activate, pro-apoptotic proteins, thus inducing cell death.

The results of the present study revealed the antitumor effect of GLT. GLT administration inhibits the proliferation of human prostate cancer cells and induced apoptosis. Additional detailed signaling mechanisms and *in vivo* studies are required to establish GLT as a potential clinical agent for the prevention and/or treatment of prostate cancer.

### Acknowledgements

The present study was supported by the Fundamental Research Funds for the Central Universities (grant no. 21615498), Medical Scientific Research Foundation of Guangdong Province (grant no. A2015463) and Administration of Traditional Chinese Medicine of Guangdong Province, China (grant no. 20151186).

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