J Huazhong Univ Sci Technol［Med Sci］
DOI 10.1007/s11596-015-1499-x
35(5):736-741,2015

Total Triterpenoids from *Ganoderma Lucidum* Suppresses Prostate Cancer Cell Growth by Inducing Growth Arrest and Apoptosis*

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Summary: In this study, one immortalized human normal prostatic epithelial cell line (BPH) and four human prostate cancer cell lines (LNCaP, 22Rv1, PC-3, and DU-145) were treated with *Ganoderma Lucidum* triterpenoids (GLT) at different doses and for different time periods. Cell viability, apoptosis, and cell cycle were analyzed using flow cytometry and chemical assays. Gene expression and binding to DNA were assessed using real-time PCR and Western blotting. It was found that GLT dose-dependently inhibited prostate cancer cell growth through induction of apoptosis and cell cycle arrest at G_1 phase. GLT-induced apoptosis was due to activation of Caspases-9 and -3 and turning on the downstream apoptotic events. GLT-induced cell cycle arrest (mainly G_1 arrest) was due to up-regulation of p21 expression at the early time and down-regulation of cyclin-dependent kinase 4 (CDK4) and E2F1 expression at the late time. These findings demonstrate that GLT suppresses prostate cancer cell growth by inducing growth arrest and apoptosis, which might suggest that GLT or *Ganoderma Lucidum* could be used as a potential therapeutic drug for prostate cancer.
Key words: *Ganoderma Lucidum* triterpenoids; prostate cancer; cell cycle arrest; apoptosis

Ganoderma (*G.*) *lucidum* is a Basidiomycetes fungus from the order Polyporales and popular medicinal mushroom used as a folk remedy in Asia since ancient times due to its diverse health-promoting properties[1, 2]. It has been shown that this fungus is useful in treating and preventing high blood pressure, hyperglycemia, hepatitis, chronic bronchitis, asthma, heart diseases, cancer and HIV[3–5], as well as its great effect on slowing down cell senescence and its antioxidant content[6–8].

G. lucidum's beneficial properties are related to a broad variety of bioactive compounds present in the fruiting body, mycelium and spores. Polysaccharides, triterpens, phenols, steroids, amino acids, nucleosides and nucleotides can be found amongst such compounds. Of special interest were the reported chemo-preventative properties of both the polysaccharides and triterpenoids isolated from *G. lucidum*[9–12]. There were about over 400 compounds isolated and identified from *G. lucidum*, of whom more than 150 compounds belonged to the kinds of triterpenoids, as ganoderic acid A–H[13, 14].

In this study, one immortalized human normal prostatic epithelial cell line (BPH) and four human prostate cancer cell lines (LNCaP, 22Rv1, PC-3, and DU-145) were treated with *G. Lucidum* triterpenoids (GLT) at different doses and for different time periods. Cell viability, apoptosis, and cell cycle were analyzed using flow cytometry and chemical assays. Gene expression and binding to DNA were assessed using real-time PCR and Western blotting.

1 MATERIALS AND METHODS

1.1 Preparation of Triterpenoids from *Ganoderma Lucidum*

The fresh fruit bodies of *G. lucidum* were collected from the Mushroom Garden of Guangdong Yuewei Edible Fungi Technology Co., Ltd. (China) and identified by Professor Xie Yizhen. A voucher specimen (No. GL20141105) has been deposited in the Herbarium of Microbiology Institute of Guangdong (China), dried at

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*This project was supported by Science and Technology Planning Project of Guangdong Province, China (No. 2011B080701075).

70°C and then ground into fine powders that could pass through a sieve with 30 pores per square inch. 500 g of powders were incubated with hot petroleum ether (1:10, w/v) in an incubator at 70°C for 1 h, and the process was repeated once. After cooling down to room temperature, the extracts were filtered, the filter residue was kept incubating with hot 90% of aqueous ethanol solution in an incubator at 100°C for 2 h, and the process was repeated once. Following immediate filtration, the filtrate was evaporated under vacuum to dryness using a rotary evaporator. The partially dried samples were re-dissolved in methanol, and then added into a chromatographic column with absorbing material C18, gradient elution with water and methanol. The eluant of 40%–75% aqueous methanol solution was collected. Then the eluant was evaporated under vacuum to dryness using a rotary evaporator and then the extracts of GLT were got, and stored at 4°C environment until used.

1.2 Cell Culture

The sources and cell culture conditions of one immortalized human normal prostatic epithelial cell line (BPH) and four human prostate cancer cell lines (LNCaP, 22Rv1, PC-3, and DU-145) were described previously[15, 16]. Cells were cultured in a 5% CO_2 humidified incubator at 37°C.

1.3 Cell Viability Assay

Cell viability in the presence of GLT was measured by quantitative colorimetric assay with cell counting kit-8 (CCK-8; Dojindo Laboratories, Japan)[17]. Briefly, the cells were seeded onto 96-well culture plate at a density of 2×10^4 cells/well. After drug treatment, 10 μL/well of CCK8 solution was added, and cells were incubated at 37°C for 1 h. The absorbance (*A*) of each well was determined at 450 nm using a microplate reader (RT-2100C, USA). Cell viability was expressed as the ratio of *A* value of the treated cells to that of the non-treated control. The data were presented as the mean and standard error of the mean (SEM) of three independent experiments.

1.4 Detection of Apoptotic Nucleosomes

Cells were seeded onto 24-well plates with 1×10^5 cells/well in triplicate per group in the complete culture medium with FBS. After incubation overnight, cells were treated with 50 or 100 μg/mL GLT for 48 h, and a control group was treated with PBS. Apoptotic nucleosomes were detected using Cell Death Detection ELISA kit (Roche Diagnostics Corporation, USA) according to the manufacturer's instructions. *A* value was measured at 405 nm (A_{405}) with a reference wavelength at 490 nm (A_{490}) using a microplate reader (RT-2100C, USA). The amount of apoptotic nucleosomes was represented by A_{405}–A_{490}.

1.5 Cell Cycle Analysis

Cells were treated without or with 10, 50 or 100 μg/mL GLT for 48 h. The percentage of cells at G_1/G_0, S and G_2/M phases was determined by flow cytometry as described previously[18].

1.6 Reverse Transcription (RT) and Quantitative (q) PCR

Cells were treated with 50 μg/mL GLT for 0, 12, 24, and 48 h. Total RNAs were extracted for RT and qPCR analysis. Results were normalized against GAPDH levels using the formula: ΔCt (Cycle threshold)=Ct of target gene–Ct of GAPDH. The mRNA expression in the control group was used as the baseline. Therefore, ΔΔCt was calculated using the formula: ΔΔCt=ΔCt of target gene–ΔCt of the baseline. The fold change of mRNA level was calculated as fold=$2^{-\Delta\Delta Ct}$. PCR primers used are shown in table 1.

Table 1 PCR primers

Primers	Sequences (5′-3′)
p21	Forward: TGCAACTACTACAGAAACTGCTG
	Reverse: CAAAGTGGTCGGTAGCCACA
CDK4	Forward: GGGGACCTAGAGCAACTTACT
	Reverse: CAGCGCAGTCCTTCCAAAT
E2F1	Forward: ACGCTATGAGACCTCACTGAA
	Reverse: TCCTGGGTCAACCCCTCAAG
Cyclin D1	Forward: GCTGCGAAGTGGAAACCATC
	Reverse: CCTCCTTCTGCACACATTTGAA
GAPDH	Forward: TAAAAGCAGCCCTGGTGACC
	Reverse: CCACATCGCTCAGACACCAT

1.7 Western Blotting Analysis

Cells were treated without or with GLT at concentration of 50 μg/mL GLT for 0, 12, 24, 48, and 72 h. Proteins were extracted for Western blotting analysis. Mouse anti-p21 antibody was obtained from Santa Cruz Biotechnology (USA). Mouse anti-GAPDH and rabbit anti-cleaved poly (ADP-ribose) polymerase (PARP) antibodies were purchased from EMD Millipore Corp (USA). Mouse anti-CDK4, Mouse anti-E2F1, rabbit anti-cyclin D1 antibodies were bought from Abcam, USA.

1.8 Apoptosis Associated Genes Caspase-3 and Caspase-9 Activities Assay

At the end of drug treatment, the cells were washed with D-Hanks solution, then scraped from the plates into 1 mL ice-cold PBS (0.1 mol/L, containing 0.05 mmol/L EDTA), and homogenized. The homogenate was centrifuged at 4000 g for 10 min at 4°C. The resulting supernatants were stored at –80°C until the following analyses. The activities of caspase-3 and caspase-9 were measured using the Detection kits purchased from Nanjing Jiancheng Bioengineering Institute (China) according to the manufacture's instruction. Levels were normalized to the protein concentration of each sample and expressed as the ratio of level of the treated cells to that of the non-treated control.

1.9 Statistical Analysis

Results from this study were presented as $x\pm s_{\bar{x}}$. Statistical analysis was performed using two-tailed Student's *t* test. A *P*-value <0.05 was considered statistically significant.

2 RESULTS

2.1 Cell Viability Assay

To study the effects of GLT on prostate cancer cell growth, we treated BHP, LNCaP, 22Rv1, PC-3, and DU-145 cells with 10, 50, and 100 μg/mL of GLT. As shown in fig. 1, we found that GLT inhibited cell growth in all of the five cell lines in a dose-dependent fashion (fig. 1A–1E). Interestingly, four prostate cancer cell lines (LNCaP, 22Rv1, PC-3, and DU-145) were more sensitive to GLT than BHP, as the number of viable cells was decreased by approximately 55% to 45% in the four prostate cancer cell lines (fig. 1B–1E), whereas it was only reduced by 12% in BHP cells treated with 50 μg/mL GLT (fig. 1A).

2.2 GLT-induced Apoptosis of Prostate Cancer Cells

To test if GLT induces apoptosis of prostate cancer cells, we measured apoptotic nucleosomes in untreated and GLT-treated cells. We found that 50 μg/mL of GLT treatment for 48 h significantly increased the amount of apoptotic nucleosomes in LNCaP, 22Rv1, PC-3, and DU-145 cells, compared to the untreated control groups (fig. 2A–2D, P<0.05 or 0.01). Consistently, PARP cleavage in all four prostate cancer cell lines was induced by GLT in a dose- and time-dependent manner (fig. 3A–3H). Since PARP cleavage has been widely used as an indicator of apoptosis[24, 25], these results indicate that GLT induces apoptosis of four prostate cancer cell lines.

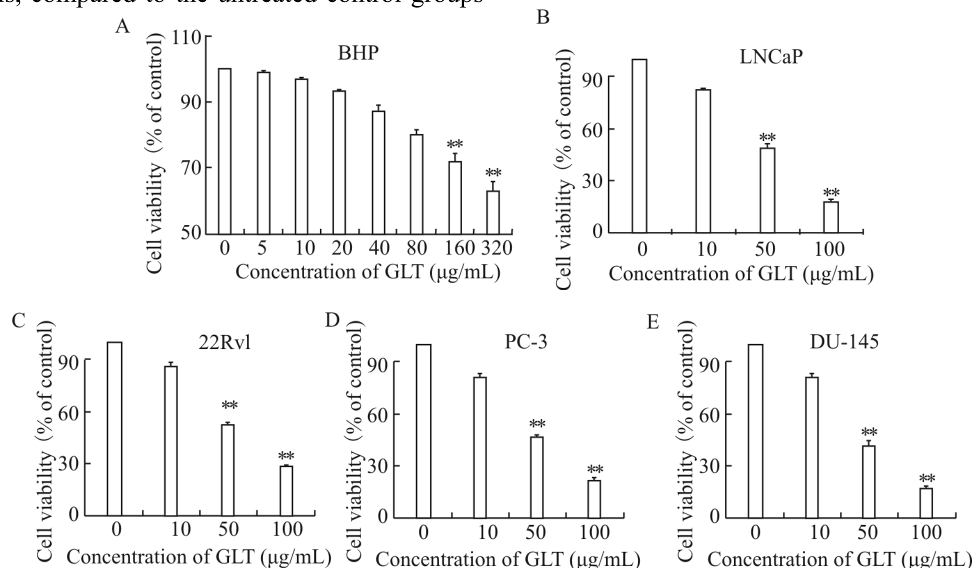

Fig. 1 Inhibitory effects of GLT on prostate cancer cell growth

A–E: BHP, LNCaP, 22Rv1, PC-3 and DU-145 cell lines, respectively. $^{**}P$<0.01 *vs*. control group

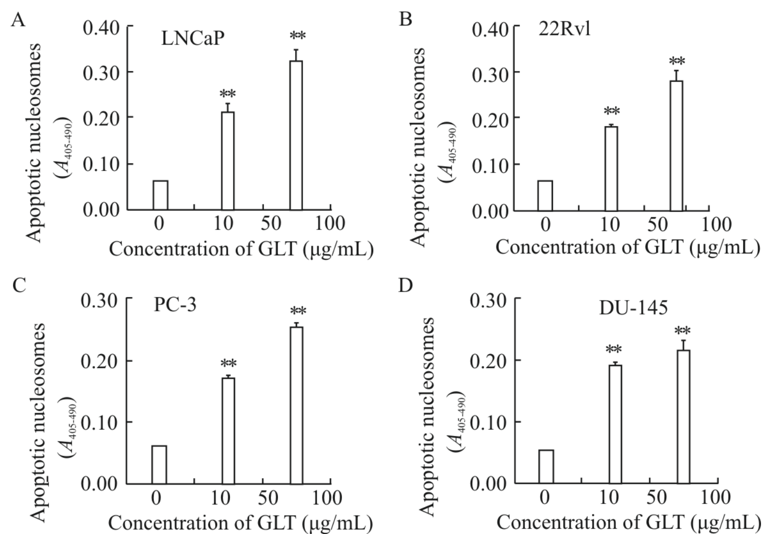

Fig. 2 GLT-induced apoptosis of prostate cancer cells

A, B, C and D: LNCap, 22Rv1, PC-3 and DU-145, respectively. $^{**}P$<0.01 *vs.* control group

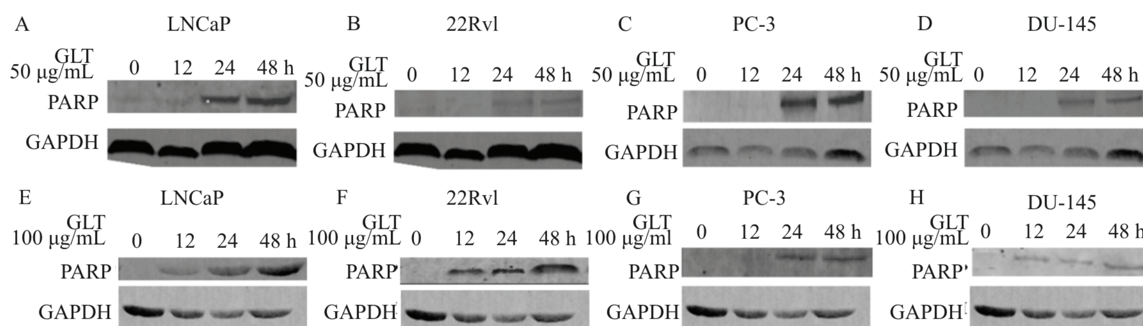

Fig. 3 GLT-induced apoptosis of prostate cancer cells

LNCap, 22Rv1, PC-3 and DU-145 cells were treated with 50 μg/mL (A, B, C and D respectively), or 100 μg/mL (E, F, G and H respectively) GLT.

2.3 GLT Blocked G_1/S Transition of Prostate Cancer Cell Cycle

To assess if GLT induces cell cycle arrest, we analyzed the percentages of cells in the G_1 (and G_0), S, and G_2 (and M) phases of the cell cycle using flow cytometry. We found that 10 μg/mL GLT treatment significantly increased the percentage of LNCaP and 22Rv1 cells at the G_1/G_0 phase, but significantly decreased the percentage of cells at the S phase (fig. 4A and 4B, $P<0.01$). However, although some effects were found in PC-3 and DU-145 cells, the differences were not statistically significant at the low dosage of GLT (fig. 4C and 4D, $P>0.05$). At a high dose such as 100 μg/mL, GLT treatment significantly increased the percentage of cells at the G_1/G_0 phase with the corresponding decrease of cells at the S phase in all four prostate cancer cell lines (fig. 4E–4H). These results imply that GLT treatment blocks the G_1/S transition, and thus inhibits cell proliferation.

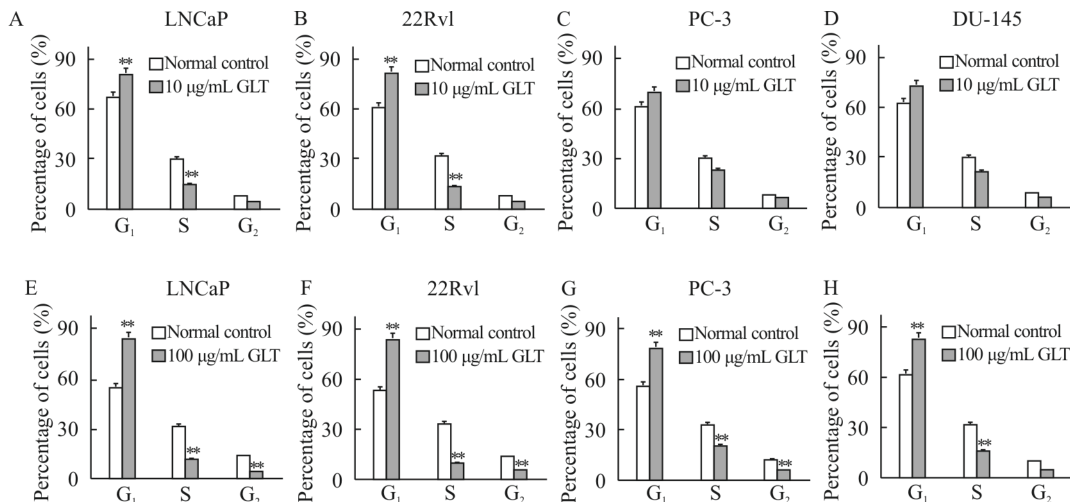

Fig. 4 GLT blocks G_1/S transition of prostate cancer cell cycle

A–H: Prostate cancer cells were plated in 60-mm dishes in triplicate per group and treated with 10 (A–D) or 100 μg/mL (E–H) GLT for 24 h. The normal control group was treated with PBS. The percentages of cells at G_1 (and G_0), S, and G_2 (and M) phases were determined by flow cytometry. $^{**}P<0.01$ *vs.* normal control group

2.4 GLT Regulated the Modulators of Prostate Cancer Cell Cycle

To assess how the GLT induces cell cycle arrest, we analyzed the modulators (p21, CDK4, E2F1 and Cyclin D1) of prostate cancer cell cycle. As shown in fig. 5, prostate cancer cells were treated with 50 μg/mL GLT for up to 48 h, and the levels of p21 were increased, and the levels of CDK4, E2F1 and Cyclin D1 were reduced in all four prostate cancer cell lines in a time-dependent manner. Fig. 5A–5D were used for Western blot analysis of the indicated proteins, for the loading control, the blots were probed for GAPDH, and E–H were RT and qPCR analysis results of the mRNA.

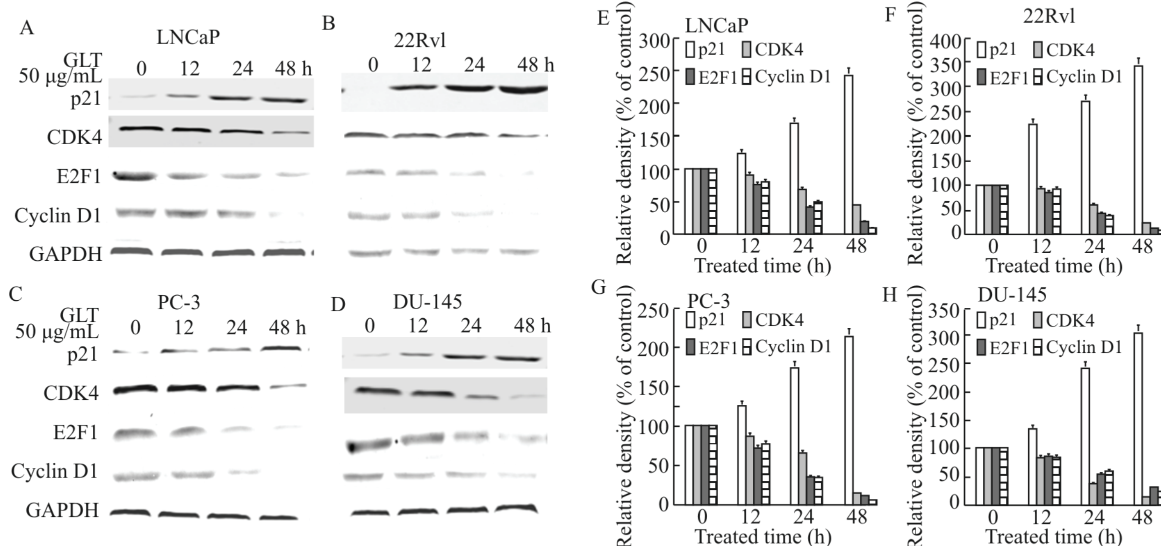

Fig. 5 Effects of GLT on p21, CDK4, E2F1 and Cyclin D1 levels

Prostate cancer cells were treated with 50 μg/mL GLT for up to 48 h. A–D: Protein extracts were used for Western blot analysis of the indicated proteins, and for the loading control, the blots were probed for GAPDH; E–H: RT and qPCR analysis results

2.5 Effect of GLT on Caspase-3 and Caspase-9 levels

To clarify the inhibitory role of GLT on prostate cancer cell growth by inducing apoptosis, the activities of caspase-3 and caspase-9 were detected using ELISA kit. As shown in fig. 6, the levels of caspase-3 were significantly reduced in all four cancer cell lines (fig. 6A–6D). After treatment with GLT at 50 or 100 μg/mL for 24 h, the levels of caspase-9 (fig. 6E–6H) showed the same trend.

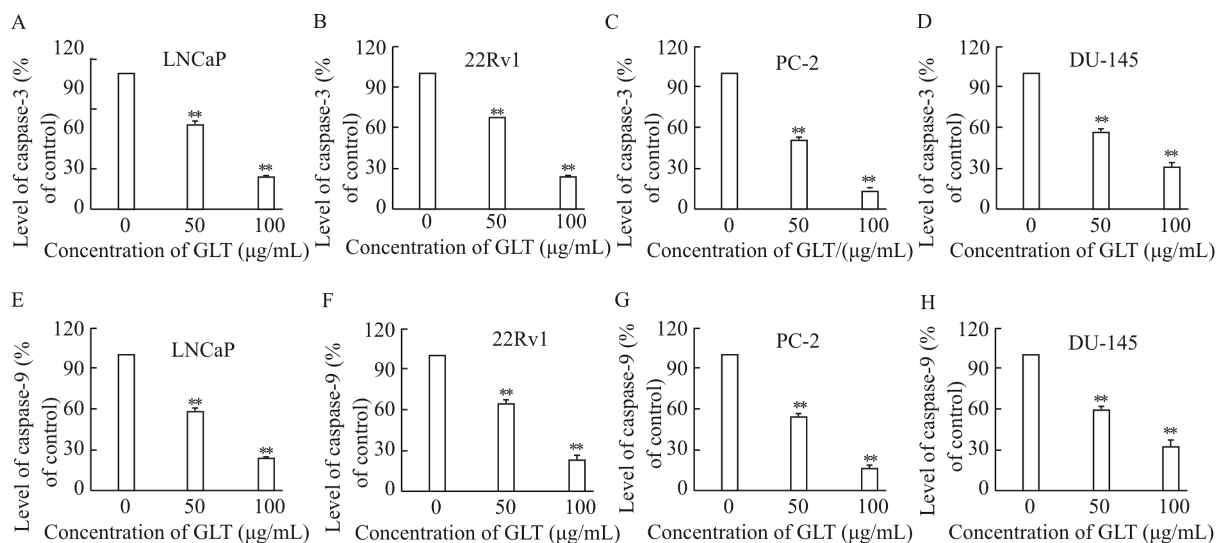

Fig. 6 Effects of GLT on caspase-3 and caspase-9 levels

Prostate cancer cells were treated with 50 and 100 μg/mL GLT for up to 24 h, and the control group was treated with PBS. The levels of caspase-3 (A–D) and -9 (E–H) were detected using ELISA kit. The data were presented as $\bar{x}\pm s_x$ of three independent experiments and % of the control group. $^{**}P<0.01$ *vs*. control group

3 DISCUSSION

Prostate cancer is the second most diagnosed cancer of men all over the world. Despite advanced therapy, prostate cancer patients still face poor prognosis, new drugs and strategies are needed to improve the clinical responses and outcomes. Here more and more medicinal mushrooms have been widely used as a miraculous herb for health promotion, especially by cancer patients[9, 11]. *G. lucidum* has been used traditionally for the prevention and treatment of cancers or tumors for a long time in traditional Chinese medicine. Its anti-tumor activity has attracted the attention of many researchers[19–21]. Phytochemical studies show it contains bioactive triterpenes, including ganoderic acid B, ganoderenic acid A, ganoderic acid A, lucideric acid A, etc.[22–25]. Accumulating evidence has shown that the GLT can inhibit the proliferation of hepatoma cells and HeLa cells[26, 27], as well as human colon cancer cells HT-29[28], lung cancer cells[29], breast cancer cells[30]. However, the potential anti-prostate cancer activity of GLT and its possible mechanism remain unclear.

P21 is a broad kinase inhibitory activity of the cell cycle inhibitor protein and negative regulator of CDK activation to control the period of conversion. CyclinD1/CDK4 is a key regulator for the regulation of G_1/S, once be activated, the cells will leave the G_0 phase and re-enter the cell cycle[31]. Results in the present study showed that the mRNA and protein of P21 expression increased, whereas the downstream components CDK4 expression decreased and its downstream transcription factor E2F1 mRNA and protein expression also decreased, which mediated cell apoptosis in prostate cancer. Results also showed GLT could decrease the protein and mRNA expression of CyclinD1, as well as decrease the activities of caspase-3 and caspase-9. The results suggest that GLT could modulate activation of P21, CDK4, E2F1 and CyclinD1 to induce apoptosis of prostate cancer cells.

In summary, the results as presented here demonstrate that GLT can inhibit prostate cancer cell growth through induction of apoptosis and cell cycle arrest. GLT-induced apoptosis is likely due to activation of caspases-3 and -9 and turning on the downstream apoptotic events. GLT-induced G_1 arrest is due to up-regulation of p21 expression at the early time and down-regulation of CDK4 and E2F1 expression at the late time after its treatment. However, it needs further pharmacological research and toxicology test *in vivo* to clarify whether GLT could be developed into a potential therapy for prostate cancer.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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(Received Apr. 15, 2015; revised Sep. 15, 2015)