Ganoderma lucidum polysaccharide inhibits prostate cancer cell migration via the protein arginine methyltransferase 6 signaling pathway

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Abstract. Prostate cancer is one of the most common types of malignant tumor of men worldwide and the incidence and mortality rate is gradually increasing. At present, the molecular mechanisms of growth and migration in human prostate cancer have not been completely elucidated. Studies have demonstrated that Ganoderma lucidum polysaccharides (GLP) can inhibit cancer. Therefore the present study investigated the effect and molecular mechanism of GLP on cell growth and migration of LNCaP human prostate cancer cells. LNCaP cells were transfected with either a protein arginine methyltransferase 6 (PRMT6) overexpression plasmid or PRMT6 small interfering (si)RNA. The cell growth and migration, and the expression of PRMT6 signaling-associated proteins, were investigated following treatment with 5 and 20 µg/ml GLP. The results demonstrated that GLP inhibited cell growth, induced cell cycle arrest, decreased PRMT6, cyclin-dependent kinase 2 (CDK2), focal adhesion kinase (FAK) and steroid receptor coactivator, (SRC) expression, and increased p21 expression in LNCaP cells, as determined by using a Coulter counter, flow cytometry, and reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. Furthermore, GLP significantly inhibited cell migration, as determined by Transwell migration and scratch assays, and altered CDK2, FAK, SRC and p21 expression in LNCaP cells transfected with the PRMT6 overexpression plasmid. By contrast, PRMT6 knockdown by siRNA reduced the effect of GLP on cell migration. These results indicate that GLP was effective in inhibiting cell growth, the cell cycle and cell migration, and the suppressive effect of GLP on cell migration may occur via the PRMT6 signaling pathway. Therefore, it is suggested that GLP may act as a tumor suppressor with applications in the treatment of prostate cancer. The results of the present study provide both the preliminary theoretical and experimental basis for the investigation of GLP as a therapeutic agent.

Introduction

Prostate cancer is one of the most common types of malignant tumor in Europe and the United States (1,2). Prostate cancer is the second most common non-skin cancer in men and is the fifth leading cause of cancer-associated mortality in men worldwide. A total of ~14% (1,22,000) of men diagnosed with prostate cancer worldwide in 2008 were in the Asia Pacific region, with 32% in Japan, 28% in China and 15% in Australia (3). It was recently reported that the incidence and mortality of prostate cancer in the majority of Asian countries gradually increased between 2012 and 2016 (4). It is established that prostate cancer is associated with genetic factors, diet, infection and hormonal factors. At present, the molecular mechanisms of growth and migration in human prostate cancer have not been completely elucidated.

Protein arginine methyltransferase 6 (PRMT6) is a type I arginine methyltransferase that is primarily expressed in the nucleus and has functions in the regulation of transcription and the cell cycle, and DNA repair (5). PRMT6 has also been demonstrated to act as a coactivator in estrogen, progesterone and glucocorticoid receptor transcription. Furthermore, El-Andaloussi et al (6) reported that PRMT6 had a key role in DNA base excision repair regulation as it forms a complex with methylated DNA polymerase β. Several studies have indicated that the expression of PRMT6 was usually observed in various types of tumor cells, including non-small cell lung cancer (7), hepatocellular carcinoma (8), breast cancer (9) and prostate cancer (10). In addition, studies have reported that
PRMT6 knockdown inhibits cell growth and the cell cycle in lung cancer and U2OS human osteosarcoma cells (11,12). Phakle et al (13) reported that PRMT6 exhibited an oncogenic function by directly binding to and inhibiting the promoter of p21, which stimulated cell growth and protected the cell cycle from senescence in breast cancer cells. Although certain studies have reported that the expression of PRMT6 may be associated with the motility and invasion of tumor cells (10,14), the molecular mechanisms of PRMT6 in the regulation of cell growth and migration have not been completely elucidated.

It has been reported that *Ganoderma lucidum* (*G. lucidum*) exhibited preventive and therapeutic effects in cancer (15), chronic bronchitis (16), bronchial asthma (17) and hepatitis (18). Polysaccharides, which consist of glucose, mannose, galactose, xylose, fucose and arabinose, are one of the most important active components of *G. lucidum* (19). Several *in vitro* and *in vivo* studies have demonstrated that polysaccharides extracted from *G. lucidum* (GLP) exhibited significant effects on tumorigenesis, oxidative stress, inflammation and immunoregulation (20,21). Xu et al (22) reported that GLP affected the function of T lymphocytes, B lymphocytes, macrophages and natural killer cells. Although certain studies have reported that GLP exhibited potential antiproliferative, pro-apoptotic and inhibitory effects on migration in several cancer cell lines, including colon cancer (23,24), hepatocellular carcinoma (25), acute myeloid leukemia (26,27) and breast cancer (28,29), it is not established whether GLP is effective in regulating the growth and migration of prostate cancer cells. Therefore, the present study aimed to investigate the effect of GLP on the growth and migration of human prostate cancer cells, and to investigate the underlying molecular mechanism.

**Materials and methods**

**Isolation and analysis of GLP.** *G. lucidum* was provided by the College of Food Science of Shenyang Agricultural University (Shenyang, China). GLP was extracted from *G. lucidum* as described previously (30). To obtain a crude polysaccharide sample, fermentation broth of *G. lucidum* was concentrated and precipitated by 90% alcohol. Identification and quantification of the monosaccharides was performed using a gas chromatography (GC) analyzer (Beckman Coulter, Inc., Brea, CA, USA). According to the method described by He et al (31), 5 mg dry GLP was hydrolyzed in 2 M trifluoroacetic acid at 120°C for 5 h. The hydrolysate was reduced by NaBH₄ and was acetylated using acetic anhydride. The acetylated monosaccharides of GLP were added into a GC analyzer and GLP was analyzed by gas chromatography, which determined that GLP was composed of arabinose, galactose, glucose and xylose in an approximate molar ratio of 4:2:10:1.

**Cell culture and GLP treatment.** LNCaP prostate cancer cells were purchased from ScienCell Research Laboratories, Inc., (San Diego, California, USA), and were cultured according to supplier instructions. Cells in culture bottle were washed three times with PBS and were cultured in RPMI-1640 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), which was supplemented with 10% fetal calf serum (Thermo Fisher Scientific, Inc.), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. A total of 1.2x10⁵–1.8x10⁷ cells/well were subsequently treated with 5 and 20 µg/ml GLP and the control cells were treated with 0.01 M PBS (1XPBS) 1 at 37°C for 72 h. The concentrations of GLP employed were selected based on a previous study (32). All cells were cultured at 37°C, 5% CO₂ and 100% humidity.

**Plasmids and small interfering (si)RNA transfection.** The PRMT6 expression plasmid pVAX1 (4 µg) acted as a control plasmid purchased from Thermo Fisher Scientific, Inc., PRMT6 siRNA (50 mM) and siR-Ribo™ (50 mM) acted as negative control were purchased from ScienCell Research Laboratories, Inc., which were transfected into LNCaP cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol at 37°C for 4 h. The following siRNA sequences were employed: PRMT6 siRNA, 5'-GAGCAAGACGCGACGUUU-3' (GenBank, accession number BC073866). Cells following 4 h transfection were subsequently treated with 5 and 20 µg/ml GLP and the control cells were treated with 0.01 M PBS (1XPBS) at 37°C for 72 h.

**Cell growth curve, cell clones and cycle analysis.** For cell growth curve analysis, 5x10⁴ cells were plated in each well of 12 well plates in triplicate. Cells were maintained in culture with 10% fetal calf serum at 37°C, 5% CO₂ and 100% humidity for 12 h, and then cells were transfected with PRMT6 overexpression plasmid or PRMT6 siRNA for 4 h. Following transfection, 5x10⁴ cells were treated with 5 or 20 µg/ml GLP and control cells were treated with 0.01 M PBS (1XPBS) at 37°C for 0, 24, 48, 72, 96 and 120 h and were immediately counted by a Coulter counter (Beckman Coulter, Inc.) through an optical microscope (Olympus Corporation, Tokyo, Japan) following staining with 0.4% trypan blue solution for 10 min at room temperature. A total of 5x10⁴ cells/well were plated in 6-well plates in triplicate. Cells were maintained in culture with 10% fetal calf serum at 37°C, 5% CO₂ and 100% humidity for 12 h, and then cells were transfected with the PRMT6 overexpression plasmid or PRMT6 siRNA for 4 h. Following transfection cells were treated with 5 or 20 µg/ml GLP and with 0.01 M PBS (1XPBS) as control at 37°C for 120 h, and then were fixed using 4% paraformaldehyde for 15 min at room temperature and stained with 0.4% trypan blue solution for 10 min at room temperature. Images were taken with an optical microscope (Olympus Corporation) magnification, x100 and gray value analysis was using by imagepro version 6.0 (Media Cybernetics, Inc.) of five fields of view. For cell cycle analysis, 2x10⁵ cells were fixed 70% pre-cooled ethanol, stored at 4°C overnight and then stained with PBS containing 5 mg/ml RNase, 0.1% Triton X-100 and 20 mg/ml propidium iodide in the dark at room temperature for 30 min and analyzed using a flow cytometer (Attune NxT; Thermo Fisher Scientific, Inc.). The amount of DNA in G1, S and G2/M phases was analyzed using ModFit 161 LT version 3.0 software (Verity Software House, Inc., Topsham, ME, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and RT was performed using ABScript II cDNA First-Strand Synthesis kit...
(ABclonal Biotech Co., Ltd., Wuhan, China) according to the manufacturer’s protocol. cDNA was quantified by qPCR using SYBR Premix Ex Taq II (Takara Bio, Inc., Otsu, Japan) on a Mx3000P qPCR system (Agilent, Santa Clara, CA, USA). The reaction was as follows: cDNA 2 µl, ddH2O 6.4 µl, upstream and downstream primers 0.8 µl, SYBR Premix Ex Taq™ II 10 and 20 µl total reaction system. Amplification conditions: annealing 56°C, 30 sec; extending 72°C, 30 sec; 45 cycles. The experiment was repeated 3 times. Gene expression levels were calculated using Stratagene Mx3000P software (version Mx3005P; Shanghai PuDi Biotechnology Co., Ltd., Shanghai, China). The relative amount of PCR products generated from each primer set was determined on the basis of the threshold cycle (Ct) number using the 2^ΔΔCt method (33). GAPDH was used as control to normalize the amount of cDNA used.

Figure 1. GLP inhibited cell growth of LNCaP cells. LNCaP cells were cultured in RPMI-1640 and treated with 5 or 20 µg/ml GLP. (A) The growth ratio of cells was determined using growth curve analyses. Cell growth was also detected by (B) morphological observation and (C) quantified. Data are presented as the mean ± standard deviation. *P<0.05 vs. Ctrl. GLP, Ganoderma lucidum polysaccharide; Ctrl, control.
Western blot analysis. Western blot analysis was performed as described previously (34). The cell protein was extracted by radioimmunoprecipitation lysate kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The protein concentration was determined by bicinchoninic assay protein concentration kit (Vazyme, Piscataway, NJ, USA). Briefly, 20 µg protein/lane was loaded and resolved by 10% SDS-PAGE. Protein was subsequently transferred to polyvinylidene difluoride membranes. Following blocking with 5% bovine serum albumin (Thermo Fisher Scientific, Inc.) for 1 h at room temperature, primary antibodies against PRMT6 (1:500; cat. no. sc-271744; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), p21 (1:500; cat. no. sc-136020; Santa Cruz Biotechnology, Inc.), CDK2 (1:1,000; cat. no. sc-70829; Santa Cruz Biotechnology, Inc.), FAK (1:200; cat. no. sc-271195; Santa Cruz Biotechnology, Inc.), SRC (1:1,000; cat. no. sc-32789; Santa Cruz Biotechnology, Inc.) and GAPDH (1:5,000; cat. no. sc-66163; Santa Cruz Biotechnology, Inc.) were incubated with the membranes at 4˚C. After 12 h incubation, the blot was washed and incubated with goat anti-mouse immunoglobulin (Ig) G-horse radish peroxidase (HRP; 1:2,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) and goat anti-rabbit IgG-HRP (1:2,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 2.5 h at room temperature. Proteins were visualized using a Clarity Western enhanced chemiluminescence Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a Tanon 5200 Full automatic chemiluminescence image analysis system (Tanon Science and Technology Co., Ltd., Shanghai, China).

Transwell assay. Briefly, 5x10^5 cells were treated with 5 or 20 µg/ml GLP, or control treatment, for 24 h at 37˚C and were seeded into the RPMI-1640 medium in the upper well of a Boyden chamber at a concentration of 5x10^4 cells per well. The lower compartment was filled with 1 ml RPMI-1640 (Thermo Fisher Scientific, Inc.), which was supplemented with 20% fetal calf serum (Thermo Fisher Scientific, Inc.). Following incubation at 37˚C for 48 h, migratory cells on the lower surface of the filter were fixed with 95% ethanol for 10 min at room temperature, stained with 0.4% trypan blue solution for 10 min at room temperature and imaged using an optical microscope (Olympus Corporation) and an AxioCam HRc CCD camera (Olympus Corporation; magnification, x400 times).

Scratch assay. Scratch assay was performed as described in a previous study (35). Briefly, 1x10^6 cells were plated into 6 well plates in triplicate at subconfluence and cultured for 24 h at 37˚C. Confluent cells were treated either 5 or 20 µg/ml GLP, or PBS for the control treatment for 24 h at 37˚C prior to cell scraping using 1 ml pipette tips. Cells were then washed with growth medium and continually cultured in RPMI-1640 (Thermo Fisher Scientific, Inc.), which was supplemented with 10% fetal calf serum (Thermo Fisher Scientific, Inc.), 2 mM glutamine, 100 U/ml penicillin and
100 mg/ml streptomycin for 48 h at 37˚C, and during this time, cells were also treated either 5 or 20 µg/ml GLP, or PBS for the control treatment. Cell migration was photographed in ten regions at 0 and 48 h.

Statistical analysis. Data are presented as the mean ± standard deviation, and were analyzed by analysis of variance followed by Student-Newman-Keuls post-hoc test, and Chi-squared test using SPSS software (version 20.0; IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

GLP inhibits cell growth in LNCaP cells. The effects of different GLP concentrations on cell growth were determined using growth curve analyses and morphological observation. The results demonstrated that the growth ratio of cells was significantly decreased in either 5 or 20 µg/ml GLP groups compared with the control group following incubation for 72 h (Fig. 1A). Furthermore, morphological results also revealed that GLP (5 and 20 µg/ml) significantly inhibited cell growth compared with the control group (Fig. 1B-C).
GLP induces cell cycle arrest in LNCaP cells. Flow cytometry was performed to investigate the effect of GLP on the cell cycle. The results demonstrated that 5 and 20 µg/ml GLP significantly induced cell cycle arrest, with an increased number of cells in the G1 phase, of LNCaP cells in a dose-dependent manner, compared with control cells (Fig. 2).

GLP inhibits cell migration in LNCaP cells. Transwell and scratch assays were performed to investigate the effect of GLP on the migration of LNCaP cells. The results demonstrated that 5 and 20 µg/ml GLP significantly inhibited cell migration compared with the control group (Fig. 3).

Effect of GLP on the PRMT6 signaling pathway and migration-associated proteins in LNCaP cells. To determine the effect of GLP on the PRMT6 signaling pathway and migration-associated proteins in LNCaP cells, the expression of PRMT6, p21, CDK2, FAK and SRC were determined by RT-qPCR and western blot analysis. The results demonstrated that 5 and 20 µg/ml GLP decreased the protein expression of...
PRMT6 and CDK2, and increased p21 expression (Fig. 4A). In addition, the protein expression of migration-associated proteins FAK and FRC were decreased in GLP groups compared with the control group (Fig. 4A). Results for mRNA expression demonstrated that PRMT6, CDK2, FAK and FRC levels were significantly reduced at 5 and 20 µg/ml GLP, while

Figure 5. GLP inhibited the PRMT6 signaling pathway in LNCaP cells. LNCaP cells were transfected with either a PRMT6 overexpression plasmid or PRMT6 siRNA, and were subsequently treated with 5 or 20 µg/ml GLP. The protein expression of PRMT6, p21, CDK2, FAK and SRC was determined by western blot analysis in cells transfected with (A) PRMT6 overexpression plasmid and (B) PRMT6 siRNA. Reverse transcription-quantitative polymerase chain reaction was used to investigate mRNA expression. PRMT6 mRNA expression in cells transfected with (C) PRMT6 overexpression plasmid and (D) PRMT6 siRNA. p21 mRNA expression in cells transfected with (E) PRMT6 overexpression plasmid and (F) PRMT6 siRNA. CDK2 mRNA expression in cells transfected with (G) PRMT6 overexpression plasmid and (H) PRMT6 siRNA. FAK mRNA expression in cells transfected with (I) PRMT6 overexpression plasmid and (J) PRMT6 siRNA. SRC mRNA expression in cells transfected with (K) PRMT6 overexpression plasmid and (L) PRMT6 siRNA. Data are presented as the mean ± standard deviation. *P<0.05 vs. Ctrl. †P<0.05 vs. 0 µg/ml GLP + over-PRMT6/si PRMT6. GLP, Ganoderma lucidum polysaccharide; PRMT6, protein arginine methyltransferase 6; siRNA, small interfering RNA; CDK2, cyclin-dependent kinase 2; FAK, focal adhesion kinase; Ctrl, control; SRC, steroid receptor coactivator. 
p21 levels were significantly increased, compared with the control group (Fig. 4B-F).

GLP inhibits the migration of LNCaP cells via the PRMT6 signaling pathway. To determine whether GLP regulates the migration of LNCaP cells via the PRMT6 signaling pathway, cells were transfected with either a PRMT6 overexpression plasmid or PRMT6 siRNA, and were subsequently treated with 5 or 20 µg/ml GLP. The results demonstrated that overexpression of PRMT6 increased the expression of PRMT6, CDK2, FAK and FRC, and decreased p21 expression, compared with the vector-transfected control. By contrast, PRMT6 knockdown decreased PRMT6, CDK2, FAK and FRC expression, and increased p21 expression compared with the vector-transfected control. The results also demonstrated that GLP treatment inhibited PRMT6, CDK2, FAK and FRC expression, and promoted p21 expression in cells transfected with the PRMT6 overexpression plasmid, whereas PRMT6 knockdown by siRNA inhibited the effect of GLP on CDK2, p21, FAK and FRC expression (Fig. 5). In addition, Transwell results confirmed that GLP significantly inhibited the migration of cells transfected with the PRMT6 overexpression plasmid, and results for PRMT6 siRNA-transfected cells indicated that PRMT6 siRNA prevented the effects of GLP on cell migration (Fig. 6).

Discussion

In recent years, the morbidity and mortality associated with prostate cancer has been increasing in China. The present study demonstrated that GLP inhibited the cell growth, cell cycle and cell migration, decreased PRMT6, CDK2, FAK and FRC expression, and increased p21 expression in LNCaP cells. Furthermore, the results indicated that GLP significantly inhibited cell migration and altered CDK2, FAK, FRC and p21 expression in cells transfected with a PRMT6 overexpression plasmid. By contrast, PRMT6 knockdown by siRNA reduced the effect of GLP on cell migration and CDK2, FAK, FRC and p21 expression.

Similar results were reported in a study by Ghafar et al. (25), which demonstrated that GLP significantly inhibited the growth of hematoma cells and eliminated regulatory T cell suppression of T cell proliferation. Li et al. (36) also reported that GLP decreased the adhesion of PC-3 M human prostate carcinoma cells to umbilical cord vascular endothelial cells. Furthermore, Liang et al. (23) demonstrated that GLP exhibited potential antitumor activity by inhibiting migration and inducing apoptosis in human colon cancer cells. In addition, a previous study reported that Ganoderma atrum polysaccharide ameliorated the antitumor effect of cyclophosphamide,
which was mediated via the induction of apoptosis and immune system activation in sarcoma 180-bearing mice (37). Collectively, the results of the current study indicated that GLP exhibited antitumor activity, which may partially be mediated by inhibiting the growth and migration of LNCaP cells.

PRMT6 expression is reported to be increased in various types of tumor cells, and it may participate in cell cycle regulation in tumor cells (38-41). p21 is an important downstream gene of PRMT6 that is involved in the development of numerous tumor types, including osteosarcoma (42,43), liver cancer (44) and prostate cancer (45-47). In addition, p21 functions in the promotion G1 cell cycle arrest (48,49). CDK2 has important roles in several tumor types by modulating the migration and motility of cancer cells (50,51). FAK and FRC are key markers of tumor cell migration, which are closely associated with the development of certain tumor types, including hepatocellular carcinoma (52), breast cancer (53) and U87-MG glioma (54). In the current study, the results demonstrated that overexpression of PRMT6 significantly increased expression of PRMT6, CDK2, FAK and FRC, and decreased p21 expression. By contrast, PRMT6 knockdown significantly decreased PRMT6, CDK2, FAK and FRC expression, and increased p21 expression in LNCaP cells. A similar report indicated that PRMT6 knockdown significantly increased the expression of p21 and induced cell cycle arrest in breast cancer cells (13). In addition, Wang et al (55) demonstrated that PRMT6 overexpression reduced cell cycle arrest at G1 phase and decreased the intensity of p16-CDK4 association in A549 human lung adenocarcinoma cells. A previous study demonstrated that PRMT6 overexpression significantly decreased the cell growth and colony forming ability of MCF7 breast cancer cells compared with controls (14). It has also been reported that PRMT6 promoted the proliferation of U2OS human osteosarcoma cells and inhibited cell senescence by suppressing p21 expression (40,56). In conclusion, these results indicate that PRMT6 knockdown may inhibit cell migration by upregulating the expression of p21 and downregulating CDK2 expression in LNCaP cells.

However, it has been reported that GLP significantly inhibited the proliferation of Si80 tumor-bearing mice by macrophage activation and improved immune system functions via the toll-like receptor 4-mediated nuclear factor-κB signaling pathway (57). The mitogen-activated protein kinase (MAPK) signaling pathway was also reported to be activated in GLP-induced RAW264.7 cells (58). In addition, a study by Liang et al (23) demonstrated that GLP inhibited migration and apoptosis by activating a Fas/caspase-dependent signaling pathway in human colon cancer cells. The results of the present study demonstrated that GLP significantly inhibited the migration of LNCaP cells transfected with a PRMT6 overexpression plasmid, whereas PRMT6 knockdown reduced the effect of GLP on cell migration, indicating that GLP may inhibit the migration of cells via the PRMT6 signaling pathway. A similar result was reported by Wu et al (59), which demonstrated that GLP inhibited the migration of MDA-MB-231 breast cancer cells, primarily by activating the FAK-Src signaling pathway. In addition, Yang et al (26) reported that GLP induced cell cycle arrest and apoptosis by blocking the extracellular signal-regulated kinase/MAPK pathway, and activating p38 and c-Jun N-terminal kinase MAPK pathways in HL-60 acute leukemia cells. WEES-G6, a triterpene-enriched extract from G. lucidum, was reported to inhibit the growth of Huh-7 human hepatoma cells, and activated JNK and p38 MAPK pathways in hepatocellular carcinoma (60).

In conclusion, the results of the present study indicated that GLP significantly inhibited the growth, cell cycle and migration of LNCaP cells. In addition, GLP inhibited the migration of cells transfected with a PRMT6 overexpression plasmid, whereas PRMT6 knockdown reduced the effect of GLP on cell migration, indicating that GLP may inhibit LNCaP cell migration via the PRMT6 signaling pathway. Therefore, it is suggested that GLP may act as a tumor suppressor have potential as a treatment for prostate cancer. The results of the present study provide both the preliminary theoretical and experimental basis for the investigation of GLP as a therapeutic agent.

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