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Full Length Research Paper

Qianliening capsule treats benign prostatic hyperplasia (BPH) by down-regulating the expression of PCNA, CyclinD1 and CDK4

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Benign prostatic hyperplasia (BPH) is characterized by abnormal proliferation of epithelial and stromal cells in prostatic tissue, which is closely correlated with increased expression of PCNA, CyclinD1 and CDK4. Therefore, inhibition of cell proliferation by suppressing the expression of the above genes is a promising strategy in the development of novel anti-BPH therapies. The aim of this study was to investigate the effect of Qianliening capsule (QC), a traditional Chinese formulation that has been shown to be clinically effective in the treatment of BPH, on the expression of PCNA, CyclinD1 and CDK4 in prostatic tissues of BPH rats. Male Sprage-Dawley (SD) rats were castrated and subcutaneously injected with testosterone propionate to generate BPH model. Meanwhile, BPH rats were orally treated with QC, or with finasteride that was used as a positive control drug. Treatment with QC or finasteride significantly reduced the PI (Prostate Index, PI = prostate wet weight / body weight × 100%) in BPH rats (*P*<0.05). In addition, QC or finasteride treatment significantly inhibited model construction-induced upregulation of expression of PCNA, CyclinD1 and CDK4 in prostatic tissues of BPH rats (*P*<0.05). Our findings for the first time demonstrated that QC can obviously reduce the PI, the expression of PCNA, CyclinD1 and CDK4 in the prostatic tissues of BPH rats, which may in part explain its anti-BPH activity.

Key words: Qianliening capsule, benign prostatic hyperplasia, cell proliferation, PCNA, CyclinD1, CDK4.

INTRODUCTION

Benign prostatic hyperplasia (BPH) is a pathological overgrowth of the human prostate that develops in a majority of aging men older than 50 years. BPH causes increased resistance to urine flow through the urethra and sometimes kidney damage, bladder stones and urinary tract infections, and thereby affects the quality of life (Djavan, 2003). BPH is a proliferative process of both the stromal and epithelial elements of the prostate arising in the periurethral and transition zones of the gland, and is hormonally dependent on testosterone and dihydrotestosterone production (Berry et al., 1984). Despite the prevalence of BPH, its pathogenesis remains controversial. Multiple partially overlapping and complementary theories have been proposed, such as embryonic reawakening, stem cell defects, chronic inflammation, imbalance between androgen/estrogen signaling and increased TGF-β signaling, all of which seem to partly reveal the abnormal growth in BPH.

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Abbreviations: QC, Qianliening capsule; **BPH,** benign prostatic hyperplasia; **PI,** prostatic index; **DHT,** 5-dihihydrotestosterone; **LUTS,** lower urinary tract symptoms

However, recently, it was widely accepted that increase in the total number of stromal and epithelial cells, which resulted from excessive cell proliferation and/or reduction of cell apoptosis, plays a critical role in the development of BPH (Zhang et al., 2009; Kyprianou et al., 1996; Claus et al., 1997; Roehrborn, 2008).

The cell cycle takes place in prostatic stromal and epithelial cells leading to their division and duplication. G1/S transition is one of the two main checkpoints used by cells to regulate the cell cycle progress and thus the cell proliferation. G1/S progression is highly regulated by Cyclin D1 and Cyclin-dependent kinase 4 (CDK4) (Chen et al., 1996; Grana and Redy, 1995). PCNA is an acidic nuclear protein that has been recognized as a histological marker for the G1/S phase in the cell cycle (Zhong et al., 2008). Therefore, the expression of PCNA, CDK4 and CyclinD1 can reflect the proliferation state of BPH cells to some extent.

To date, there is no completely effective treatment for BPH. The mainstay of pharmacotherapy is the combination of 5α-reductase inhibitors, such as finasteride and dutasteride which regulate the levels of 5-dihhichydrotestosterone (DHT), and alpha adrenergic-blockers, including terazosin, doxazosin and tamsulosin which inhibit α-adrenergic receptors, relaxing smooth muscle in the prostate and the bladder neck, thus decreasing the blockage of urine flow. Moreover, in some patients, surgery, transurethral resection of the prostate, is the only effective intervention (Tiwari et al., 2005). However, all these therapies may have troubling side effects such as orthostatic hypotension, decreased libido and ejaculation or erectile dysfunction. Because of these adverse effects, natural products that appear to have limited adverse events are becoming more and more important in treatments of BPH, such as *Saw palmetto*, *Pygeum africanum* and *Hypoxis rooperi* (Boyle et al., 2000; Wilt et al., 2000, 2002) which have long been used to treat BPH successfully.

Qianliening capsule (QC) is a traditional Chinese medicine formulation consisting of wine rhubarb, leech, Milkvetch root, *Achyranthes aspera* and dodders. These components together confer QC properties of heat-clearing, detoxification, promotion of blood circulation, removal of blood stasis, tonifying the kidney and nourishing vitality (replenishing the kidney qi in Chinese). It has been shown that QC can obviously improve a series of lower urinary tract symptoms (LUTS) in BPH patients, such as frequency of urination, urinary urgency, thin urine flow, urinary endless and some other voiding disorders. In addition, QC can ameliorate the urodynamic evaluation indexes of BPH patients such as maximum free urinary flow rate and average urinary flow rate. Our preliminary study on BPH model rats showed that QC could significantly decrease the prostatic volume and weight, and inhibit enlargement of prostate (Lin et al.,2010; Zhou et al., 2008, 2010a, b, 2012), further confirming that QC has a good therapeutic effect on BPH.

However, the mechanism of its anti-BPH activity still remains largely unknown. Therefore, using a rat model of BPH which was generated by castration and sub-cutaneous injection with testosterone propionate, in this study, we evaluated the therapeutic effect of QC on BPH, and investigated the underlying molecular mechanism.

MATERIALS AND METHODS

Thirty-two SPF grade male adult Sprague-Dawley (SD) rats (200 to 220 g) were purchased from Shanghai Si-Lai-Ke Experimental Animal Ltd. (Shanghai, China). The rats were housed in clean pathogen-free rooms in an environment with controlled temperature (22°C), humidity and a 12 h light/dark cycle with free access to water and standard laboratory food. All animal treatments were strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals, and the experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

Except for the eight control group (Cont) rats, orchiectomy was carried out for the other 24 rats under napental anesthesia through the scrotal route in an asepsis condition. Seven days after castration, they were randomly assigned to three experimental groups with eight animals in each: the model group (Model), the finasteride group (Finast) and the QC group (QC). Testosterone propionate (Shanghai GM Pharmaceutical Co., Ltd. China) were given to the castrated rats by daily intraperitoneal injections at a does of 5 mg/kg for 4 weeks to generate BPH model. Following BPH induction, the finasteride group received finasteride (Hangzhou Merck. China) at a does of 0.5 mg/kg every day and QC group received the Qianliening capsules (QC, Fujian, China, FDA approval No.: Z09104065) at a dose of 4.5 mg/kg (equivalent to 6 times the dose for a human adult) for 4 weeks. After 4 weeks of treatment, the prostates from the rats in all groups were removed, weighed and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) assays and immunohistochemistry examination.

Prostatic index (PI)

An analytical balance was used to measure the prostate weight (PW) and the prostatic index (PI) was calculated as: PW / BW × 100%.

RNA extraction and RT-PCR analysis

Total RNA was isolated from fresh prostate tissues with TriZol Reagent (Invitrogen, Carlsbad, CA, USA). Oligo(dT)-primed RNA (2 µg) was reverse-transcribed with SuperScript II reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of PCNA, cyclinD1 and CDK4 by PCR with Taq DNA polymerase (Fermentas). β-Actin was used as an internal control. The sequences of the primers used for amplification of PCNA, cyclinD1 and CDK4 are as follows: PCNA forward 5′- GA CAC ATA CCG CTG CGA TCG -3′ and reverse 5′- TCA CCA CAG CAT CTC CAA TAT -3′ (Tm = 59°C, 307 bp); Cyclin D1 forward 5′-GGA GCA GAA GTG CGA AGA-3′ and reverse 5′- GGG TGG GTT GGA AAT GAA-3′ (Tm = 57°C,394 bp); CDK 4 forward 5′-CTT CCC GTC AGC ACA GTT C-3′ and reverse 5′- GGT CAG CAT TTC CAG TAG C′ (Tm = 55°C, 687 bp); β-actin forward 5′-ACT GGC ATT GTG ATG GAC TC-3′ and reverse 5′-CAG CAC TGT GTT GGC

Table 1. Comparison of the prostate wet weight and PI among different groups ($\bar{x} \pm s$).

Group	N	PI (%)
Control group	8	0.13 ± 0.0225
Model group	8	0.25 ± 0.01356[1)]*
Finasteride group	8	0.20 ± 0.01927[2)] **
QC group	8	0.21 ± 0.01885[2)]**

PI = PW (prostate wet weight) / BW (body weight) ×100%. *P < 0.05 for the difference between PI in normal group and model group. **P<0.05 versus model group.

ATA GA-3′ (Tm = 55°C, 453 bp). Samples were analyzed by gel electrophoresis (1.5% agarose). The DNA bands were examined using a Gel Documentation System (BioRad, Model Gel Doc 2000, USA).

Immunohistochemistry analysis

A 0.5 × 0.5 × 0.1 cm block of tissue was collected from the lateral lobe of the prostate gland of each rat. The tissue blocks were rinsed with phosphate buffer solution (PBS), fixed with 10% formaldehyde for 12 to 24 h, and subsequently embedded in paraffin, archived, and finally, sliced. The paraffin sections were processed for PCNA, cyclinD1 and CDK4 immunohistochemistry using streptavidin-perosidase (SP) method: endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide for 10 min, followed by incubation with 10% serum for 30 min at room temperature. Sections were incubated with primary antibodies at room temperature for 2 h. Primary antiserum was detected after incubation with a biotinylated secondary antibody. The primary antibodies employed were polyclonal rabbit anti-rat PCNA, cyclinD1 and CDK4 (Bohai Biotechnology Development Co., Ltd. Hebei, China). Secondary antibody, SP was purchased from Bohai Biotechnology Development Co., Ltd. (Hebei, China). PBS was used to replace the primary antibody as a negative control. Color was developed using DAB chromogen (Bohai Biotechnology Development Co., Ltd. Hebei, China). After staining, five high-power fields (400x) were randomly selected in each slide and the immunohistochemistry slides were examined with the method of immunohistochemical score (IHS), which was calculated by combining an estimate of the percentage of immunoreactive cells (quantity score) with an estimate of the staining intensity (staining intensity score), as follows: no staining is scored as 0, 1 to 10% of cells stained scored as 1, 11 to 50% as 2, 51 to 80% as 3 and 81 to 100% as 4. Staining intensity is rated on a scale of 0 to 3, with 0 = negative; 1 = weak; 2 = moderate and 3 = strong. The raw data were converted to the IHS by multiplying the quantity and staining intensity scores: 0 as “-”, 1 to 4 as “+”, 5 to 8 as “++” and 9 to 12 as “+++” (Table 3).

Statistical analysis

Data were expressed as means ± standard deviation (s.d). The comparisons between the four groups were performed using the one-way ANOVA (analysis of variance) with a post hoc test. For categorical variables, data were presented by number and percentage. The associations between categorical variables were tested using Fisher’s exact test. All statistical hypothesis tests were set with a significance level of 0.05. Statistical analyses were performed using SPSS 15.0 statistics software (SPSS Inc, Chicago, IL, USA).

RESULTS

QC treatment reduced prostatic index (PI) in BPH rats

To evaluate the efficacy of QC in treatment of BPH, we first examined the effect of QC on prostatic index (PI) in BPH rats by calculating the ratio of prostatic weight to body weight. As shown in Table 1, the mean PI in the model group was significantly elevated when compared with that in the control group (P<0.05). However, administration with either QC or finasteride significantly reduced PI in BPH rats ($p < 0.05$).

QC treatment suppressed the mRNA expressions of PCNA, CyclinD1 and CDK4 in the prostatic tissue of BPH rats

The mRNA expression was determined by RT-PCR assay. As shown in Figure 1 and Table 2, model construction obviously enhanced the mRNA expression of PCNA, CyclinD1 and CDK4 in rat prostatic tissues which however, was significantly neutralized by both QC and finasteride treatment.

QC treatment inhibited the protein expressions of PCNA, CyclinD1 and CDK4 in the prostatic tissue of BPH rats and quantitative analysis

Data from immunohistochemistry analysis (IHC) showed that the protein expression level of PCNA, CyclinD1 and CDK4 in prostatic tissues of model group was significantly higher than that of the control group (P<0.05) (Figures 2 to 4 and Table 3), whereas treatment with QC or finasteride profoundly inhibited the protein expression of PCNA, CyclinD1 and CDK4 in the prostatic tissues of BPH rats (P<0.05; Figures 2 to 4 and Table 3).

DISCUSSION

BPH, also known as benign prostatic hypertrophy, is a non-cancerous enlargement of the prostate that involves hyperplasia of prostatic stromal and epithelial cells. As

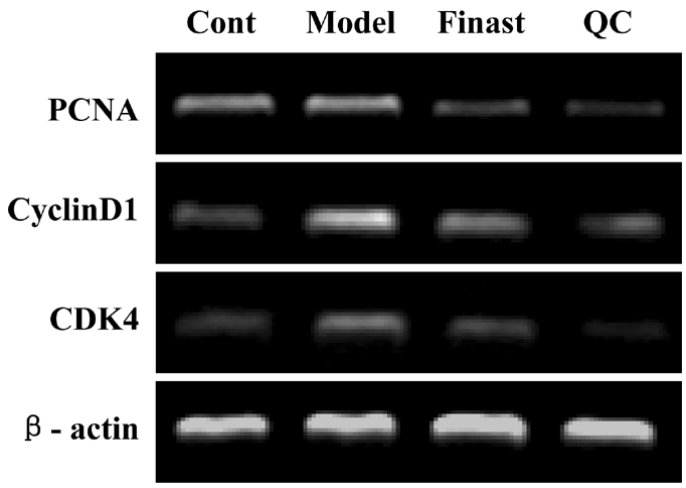

Figure 1. Effects of QC on the mRNA expressions of PCNA, Cyclind1 and CDK4 in the prostate cells. QC treatment was able to downregulate the expression of PCNA, CyclinD1 and CDK4.

Table 2. Quantitative analysis of PCNA, CyclinD1, CDK4 mRNA expression in each group ($\bar{x}$ ±s).

Group	N	PCNA/β- actin	CyclinD1/β- actin	CDK4/β- actin
Control group	8	0.65±0.22	0.29±0.18	0.25±0.19
Model group	8	0.93±0.13*	0.61±0.11*	0.44±0.08*
Finasteride group	8	0.56±0.15**	0.42±0.15**	0.25±0.18**
QC group	8	0.45±0.26**	0.28±0.21**	0.10±0.16**

* *P*<0.05 versus control group; ** *P*<0.05 versus model group.

Table 3. Expression of PCNA, CyclinD1, CDK4 in the prostate tissues of different groups of rats.

Group	N	PCNA				Cyclin D1				CK4			
		-	+	++	+++	-	+	++	+++	-	+	++	+++
Control group	8	0	6	2	0	2	6	0	0	3	5	0	0
Model group	8	0	0	5	3 *	0	3	3	2 *	0	3	3	2 *
Finasteride group	8	1	5	2	0 **	2	4	1	1 **	2	5	1	0 **
QC group	8	2	4	2	0 **	1	3	4	0 **	1	5	2	0 **

* *P*<0.05 versus control group; ** *P*<0.05 versus model group.

the volume of the prostate sufficiently enlarges, the nodules compress the urethral canal to cause partial or sometimes virtually complete obstruction of the urethra, which interferes with the normal flow of urine. It leads to the symptoms of urinary hesitancy, frequent urination, dysuria (painful urination), increased risk of urinary tract infections and urinary retention. Treatment options for BPH include surgery and medications to reduce the amount of tissue and increase the flow of urine. Usually, medication treatments are the most common choice for BPH patients with slight or midrange symptoms. The two main medications for management of BPH are alpha blockers and 5α-reductase inhibitors. But both two of these medicines have their own side effects. So people often seek herbal remedies for BPH since they usually generate less negative effects and display therapeutic efficacy (Lieber, 1998). As a traditional Chinese herbal formulation which has been used for a long time in clinical practice, QC has been shown to be effective in the treatment of BPH (Lin et al., 2010; Zhou et al., 2008, 2010a, b). In this study, we calculated the PI of each group to observe the general changes of prostate tissues. The result shows that both QC and finasteride significantly reduced PI in BPH rats, which validate the clinical effect of QC. However, the mechanism of QC action is still largely unknown.

BPH is considered to be a proliferative process of both the stromal and epithelial elements. Cell proliferation is

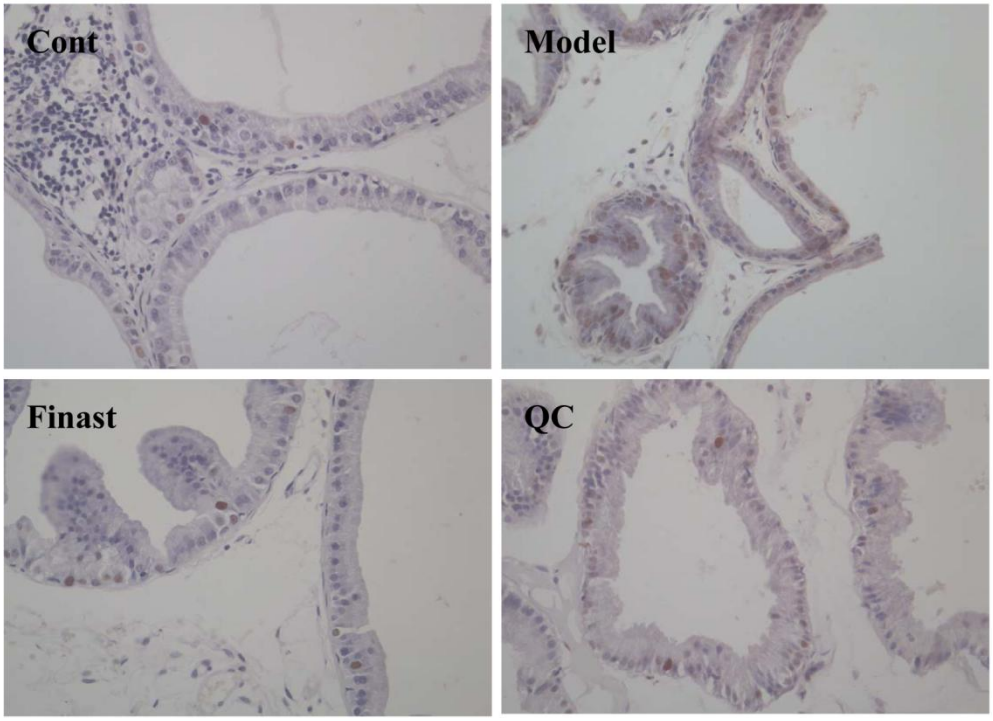

Figure 2. Immunohistochemical staining of PCNA in the prostate tissues of different groups of rats (immunohistochemistry 400×). There were more PCNA positive cells in model group than that in the control group prostates (P<0.05, Table 3). Few PCNA positive cells were found in the QC group and finasteride group prostates (P<0.05, Table 3).

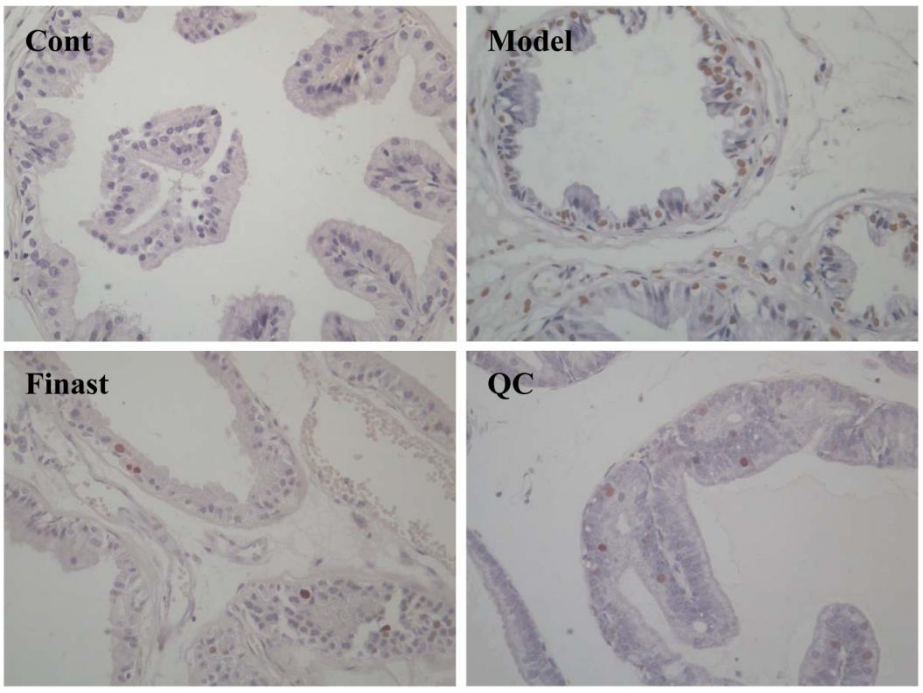

Figure 3. Immunohistochemical staining of in the prostate tissues of different groups of rats (immunohistochemistry 400×). There were more Cyclind1 positive cells in model group than that in the control group prostates (P<0.05, Table 3). Few CyclinD1 positive cells were found in the QC group and finasteride group prostates (P<0.05, Table 3).

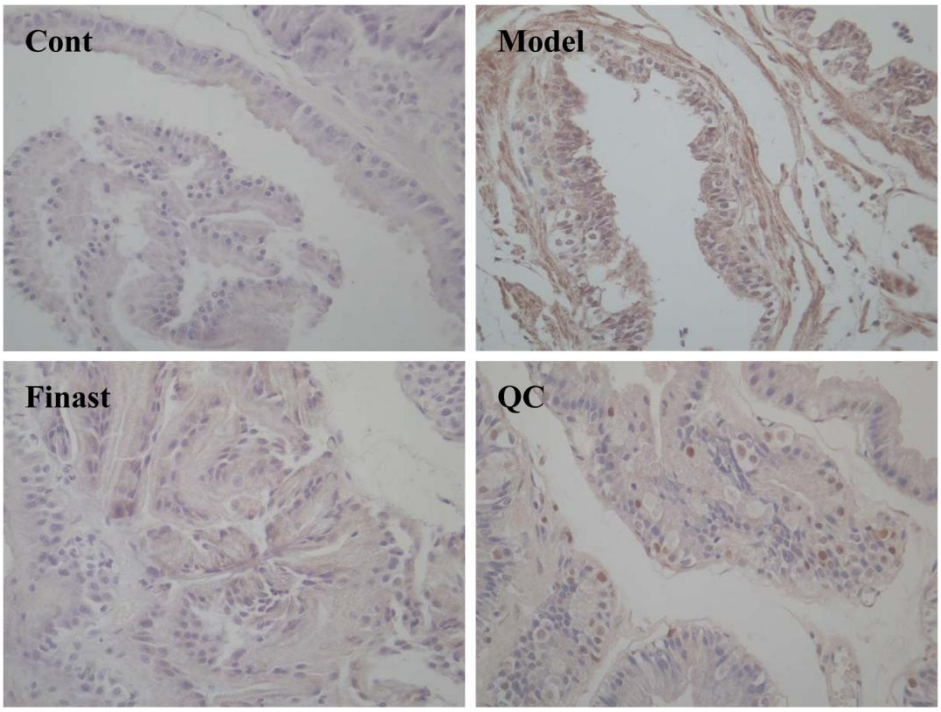

Figure 4. Immunohistochemical staining of CDK4 in the prostate tissues of different groups of rats (immunohistochemistry 400×). There were more CDK4 positive cells in model group than that in the control group prostates (P<0.05, Table 3). Few CDK4 positive cells were found in the QC group and finasteride group prostates (P<0.05, Table 3).

highly regulated by the cell cycle, which consists of four periods: S phase (DNA synthesis phase), M phase (mitosis), G1 and G2 phase. G1/S transition is one of the two main checkpoints of cell cycle (Nurse, 1994), which is responsible for initiation and completion of DNA replication. G1/S progression is precisely regulated by Cyclin D1 that exerts its function via forming an active complex with its CDK major catalytic partners (CDK4/6) (Morgan, 1995; Nurse, 2000). An unchecked or hyper-activated Cyclin D1/CDK4 complex may be responsible for enhanced cellular proliferation and the alteration of Cyclin D1/CDK4 complexes is becoming a possible target for the anti-proliferation therapies (Day et al., 2009; Sridhar et al., 2006; Dobashi, 2004; Nurse et al., 1998). PCNA is a 36 kD DNA polymerase delta auxiliary protein that involves proliferation and it is specifically expressed in proliferating cell nuclei. PCNA has been recognized as a histologic marker for the G1/S phase in the cell cycle (Bantis et al., 2004). Using immunohistochemistry and RT-PCR analyses, in this study, we found that the expression of PCNA, Cyclin D1 and CDK4 was significantly increased in the BPH model group which however, could be significantly inhibited by QC treatment.

In conclusion, here, we reported for the first time that Qianliening capsule can inhibit cell proliferation by down-regulating the expression of PCNA, Cyclin D1 and CDK4, which might be one of the mechanisms by which QC treats BPH.

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