Puerarin modulation of CENPA affects downstream PLK1 and CCNB1 expression to inhibit bladder cancer cell proliferation

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Abstract

Background: The treatment alternatives for bladder cancer (BLCA), the 10th most prevalent cancer in the world, need to be further investigated, and many active substances like Puerarin in herbal medicine were found to be effective in treating BLCA. The purpose of this study was to investigate the potential treating mechanisms of Puerarin on BLCA. Methods: The cell counting kit 8 assay and flow cytometry were performed to confirm Puerarin’s ability to suppress BLCA. The differentially expressed proteins (DEPs) were obtained by Tandem Mass Tags technology and functional enrichment analysis performed by R studio. The most enriched pathways were selected for study and the DEPs were screened out. Protein-protein interaction network maps were created using String and Cytoscape and key proteins, which will be analyzed for survival, expression, and upstream transcription factor prediction, were screened out using the cytoHubba plugin. CHEA3 was used to obtain upstream transcription factor validated by molecular docking and western blotting experiments. Results: Cell counting kit 8 showed that Puerarin inhibited BLCA cells, with 50% inhibitory concentration of 218 μmol/L T24 and 198 μmol/L in 5637. Flow cytometry reveals that Puerarin blocks T24 and 5637 cells in G1 phase. 1,385 DEPs were obtained and the enrichment analysis revealed that cell cycle and DNA replication were the two main areas in which DEPs were enriched. Cyclin-B-cyclin dependent kinase 1 (CDK1), cyclin B1 (CCNB1), and polo-like kinase 1 (PLK1) were identified as key proteins, and their upstream transcription factor was predicted to be centromere protein A (CENPA). Puerarin’s binding energy to CENPA was determined by molecular docking to be −6.3 kcal/mol, indicating a strong binding interaction. Western blot showed that Puerarin significantly reduced the expression of CENPA. Conclusion: We hypothesize that Puerarin may inhibit the proliferation of bladder cancer cells by inhibiting CENPA expression to regulate PLK1 and CCNB1 expression, thereby affecting cell cycle.

Keywords: Puerarin; BLCA; Tandem Mass Tags; cell cycle

Author contributions

Pang K and Xu H conducted article conceptualization, writing and experimental implementation. Gao W, Pan D, and Ma YY conducted the article writing, experimental implementation. Zhang RR, Cao YL, Zhou YC, and Xu MY conducted data compilation and article revision. Pang K and Zhang PY conducted article revisions, technical support, financial support.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

BLCA, bladder cancer; CENPA, centromere protein A; BP, biological pathways; CC, cellular components; CCK-8, the cell counting kit 8; DEP, differentially expressed proteins; CCNB1, cyclin B1; CDK1, cyclin-B-cyclin dependent kinase 1; GO, Gene Ontology; IC50, 50% inhibitory concentration; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction; MIBIC, muscle-invasive bladder cancer; MF, molecular functions; NMIBC, non-muscle invasive bladder cancer; PLK1, polo-like kinase 1; TMT, Tandem Mass Tags; CENPAs, centromere proteins.

Citation


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**Highlights**

Screen the differentially expressed proteins using the method of Tandem Mass Tags. Use bioinformatic analysis to explore the mechanism of Pueraria's action in bladder cancer. Perform molecular docking and cellular experiments to validate the analysis results.

**Medical history of objective**

Pueraria was first published in China in the Eastern Han Dynasty in the "Shennong Materia Medica", and subsequently recorded in the "Treatise on Medicinal Properties", "Tang Materia Medica", "Gleamings of Materia Medica", it has the effect of relieves thirst, vomiting, and headaches, and helps with appetizers, detoxification, and relief of breathlessness and so on. Modern pharmacological research shows that PuerariaMirifica has the effect of improving cardiovascular and cerebrovascular circulation, lowering sugar, lowering fat, protecting the liver, and detoxifying alcohol.

**Background**

With 573,000 new cases and 213,000 fatalities per year, bladder cancer (BLCA) is the tenth most prevalent malignancy worldwide and the second leading cancer in the male genitourinary system [1, 2]. BLCA is classified as either muscle-invasive bladder cancer (MIBC) or non-muscle invasive bladder cancer (NMIBC), depending on the extent of bladder wall invasion; NMIBC accounts for 80% of newly diagnosed BLCA [3, 4]. The first-line treatment for NMIBC is still transurethral resection of the bladder tumor plus postoperative cisplatin-based chemotherapy, but the postoperative recurrence rate can reach 50%–70%, and there is still a 10%–15% possibility of progression to MIBC [5, 6]. Moreover, the risk of surgery, the intolerance of chemotherapy drugs, and the high cost of treatment increase the burden on patients' lives, so it is urgent to find new therapeutic drugs or adjuvant agents for BLCA patients. Exploring the potential of novel drugs and combination therapies, especially when many patients are resistant to chemotherapeutic agents, is a key area of research today [7].

With the research on cancer, gene-targeted therapy, novel drug-assisted therapy, and herbal therapy are gradually becoming an important part of the treatment of cancer [8]. Pueraria, also named 8-C-glucoside of daidzein, is a flavanone glycoside and is the major bioactive ingredient isolated from the root of the Pueraria lobata (Wild.) Ohwi [9, 10]. Pueraria has a very wide range of effects, according to the current research. Pueraria has been confirmed to be used in the treatment of cardiovascular diseases, diabetes, cancer, and other diseases [11, 12]. Other studies have proved that Pueraria has anti-inflammatory, anti-oxidant, anti-platelet, anti-apoptosis, and anti-arrhythmia properties [13]. Pueraria is widely used in clinical treatment because of its low cytotoxicity, strong pharmacological activity, and extensive pharmacological effects [14]. Verifying Pueraria's inhibitory impact on BLCA T24 cells and examining its mechanism were the goals of this investigation.

**Methods**

**Materials and reagents**

The Bio-Rad Laboratories (Shanghai) Co., Ltd. (Shanghai, China) was where the urea (161-0731) was acquired. Tris (T0826-500g), BSA (A0332), and SDS (SB0485-50g) were purchased from the Bioengineering (Shanghai) Co., Ltd. (Shanghai, China), NH4H2O (17837), C18 Empore MC Extraction Disks (66883-U), NH4HCO3 (A6141-25G), DL-dithiothreitol (43819-5G), HCOONH4 (17843), trifluoroacetic acid (TFA, T6508) were obtained from the Sigma Cable Company (Pte) Ltd. (Sigma High Tech Complex, Singapore), Beyotime Biotech Inc. (Shanghai, China) provided BCA Protein Assay kit (P0012) and SDS-PAGE Protein Sample Loading Buffer (P0015F). We bought formic acid (A117), 1 M TEAB (SE252676/90114), and a TMT 6plex Isobaric Mass Tag Labeling kit from Thermo Fisher Scientific Co., Ltd. (Waltham, MA, USA). Merck Chemicals (Shanghai) Co., Ltd (Hong Kong, China) provided Acetonitrile (1000304008). C18 Cartridge (WAT023590) was obtained from Waters Technology (Shanghai) Co., Ltd. (Shanghai, China). Shanghai Topland Biotechnology Co., Ltd. (Shanghai, China) provided Lysing Lysing Matrix A (6910-100-99219). Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China) provided HCl (10011018). Trypsin (V5117) was purchased from Promega (Beijing) Biotech Co., Ltd. (Beijing, China). Sartorius (Shanghai) Trading Co., Ltd. (Shanghai, China) provided 30KD Ultrafiltration centrifugal tube. Agilent Technologies (China) Co., Ltd. (Beijing, China) provided Multiple Affinity Removal LC Column – Human 1-4/Mouse 3. Human-derived T24, S637 cell line was obtained from Chinese Academy of Sciences (Beijing, China). CST Co., Ltd. (Danvers, MA, USA) provided CENPA antibody. Lamin B1 antibody, PVDF membrane, primary antibody dilution solution, secondary antibody dilution solution, protein blocking solution, secondary antibody were purchased from Servicebio Co., Ltd. (Wuhan, China). Protein extraction kit, Pueraria Traditional Chinese Medicine Monomer (98% drug purity) were purchased from Soleilbio Co., Ltd. (Beijing, China). Electrophoresis solution, trans-membrane kit, gradient gel were purchased from Kinsley Co., Ltd. (Zhengjiang, China).

**Cell culture**

Both BLCA T24 and S637 cells were grown in 1640 DMEM containing 10% FBS in an environment with a humidity and CO2 saturation of 5% at 37 °C. When the culture reached about 90% cells spread all over the bottom of the flask, after aspirating the original culture solution, add about 3 ml of D-Hanks buffer (Rangmei Biotechnology Co., Ltd., Xuzhou, China) to wash twice. Add 1 ml of Trypsin solution and gently shake the culture flask to fully digest the cells. When the cells could be seen to fall off in pieces under the microscope, immediately after, 3 ml of 1640 DMEM medium with 10% FBS was added to stop the digestion and blow up the cells. The cell suspension was centrifuged at 1,000 rpm and 4 °C for 5 min. The supernatant was discarded and 5 ml of complete medium was added, mixed thoroughly, and transferred to a new culture flask for further incubation in the incubator.

**The cell counting kit 8 (CCK-8) assay and flow cytometry CCK-8.** The vitality of T24 and S637 cells following Pueraria treatment was assessed using the CCK-8 test. Under the action of 1-methoxyPMS, dehydrogenase in the cell mitochondria reduces WST-8 to a yellow Formazan product that is very water-soluble. The quantity of live cells directly relates to the amount of formazan generated. Light absorption at a wavelength of 450 nm is detected by a microplate reader, and it can inadvertently indicate the quantity of living cells. It may indicate Pueraria’s capacity to suppress cancer cells when compared to the control group.

9 distinct Pueraria concentration groups and a control group were created using T24 cells and S637 cells. The concentrations were 0.001 µmol/L, 0.005 µmol/L, 0.025 µmol/L, 0.125 µmol/L, 0.625 µmol/L, 3.125 µmol/L, 15.625 µmol/L, 78.125 µmol/L, and 390.625 µmol/L, respectively.

The cells were digested with trypsin to make them detached from the wall, and a complete medium was added to resuspend the cells, 10 µl of Taipan blue stain and 40 µl of cell suspension were aspirated, and 10 µl of cell suspension was aspirated and placed on the cell counting plate after mixing thoroughly. The plate was placed in an automatic cell counter, and 5 fields of view were selected for counting and the average value was taken. Spread the 96-well plate at a cell density of 4,000 cells/well after sufficient blowing, try to make the same number of cells in each well, 100 µl cell suspension per well, 3–5 wells per group. Put into the thermostat and incubate for 24 h, wait for the cells to be completely attached to the wall, and observe the cell density of each experimental group under the microscope.
waste solution was discarded, and Puerarin was added according to the established concentration gradient. In the blank group, only complete medium was added without cells and Puerarin, in the control group, there were cells and complete medium, and in the experimental group, there were cells, complete medium, and different concentrations of Puerarin. After 24 h of incubation in the incubator, the waste liquid was discarded and 10 μL CKC-8 and 90 μL basal medium reagents were added to each well. Covered with tin foil and placed in the incubator at 37 °C, 5% CO2. The Optical Density value at the wavelength of 450 nm after 1 h and 2 h was detected by the enzyme marker.

Flow cytometry. T24 cells and 5637 cells were divided into two groups and cultured in six-well plates. When the cells were spread to the bottom of 80% of the bottles, 200 μmol/L Puerarin was added to the experimental group, and the control group was not given any special treatment. After 24 h of culture, the waste liquid was discarded, and the cells of both groups were added to the basal medium and cultured for 12 h to promote the synchronization of the cell cycle. The cells were fixed in 75% ethanol solution at 4 °C for 24 h. Then, the cells were stained with propidium iodide at 37 °C for 30 min and detected on flow cytometer.

Tandem Mass Tags (TMT) detection
Protein extraction-SDT lysis method. Cells were divided into an experimental group and a control group. Cells were added to an appropriate amount of SDT lysis solution (Servicebio, Wuhan, China), lysed by ultrasound, and placed in boiling water for 15 min. Centrifuge at 14,000 g for 15 min and save the supernatant. The BCA method was used for protein quantification.

SDS-PAGE electrophoresis. 20 g of protein from each group is taken, loaded with 6 × loading buffer, heated in a boiling water bath for 5 min, and electrophoresed at 12% SDS-PAGE (continuous pressure, 250 V, for 40 min), then Coomassie brilliant blue staining is applied.

FASP enzymatic hydrolysis. Take 200 g of protein solution for each group, mix with 100 mM DL-dithiothreitol, bring to a boil in a water bath for 5 min, and let cool to room temperature. 200 liters of UA buffer (Servicebio, Wuhan, China) should be added, mixed thoroughly, and then transferred to a tube for 30 kD ultrafiltration. The tube should then be centrifuged twice at 12,500 g for 25 min. Add 100 mL of IAA buffer (Servicebio, Wuhan, China), shake at 600 rpm for 1 min, allow the mixture to react for 30 min in the dark at room temperature, and then centrifuge it at 12,500 g for 25 min. Centrifuge at 12,500 g for 15 min while adding 100 L of UA buffer twice. Add 100 L of the 0.1 M TEAB solution, and centrifuge twice at 12,500 g for 15 min. Place at 37 °C for 16–18 h after adding 40 L of the Trypsin buffer and shaking for 1 min at 600 rpm. Replace the collection tube, centrifuge at 12,500 g for 15 min, then add 20 L of 0.1 M TEAB solution and collect the filtrate.

TMT mark. Take 100 g of peptides from each sample and label them using the TMT labeling kit as directed.

High pH reversed-phase grade. Using an Agilent1260 infinityIIHLPLC system, each set of labeled peptides was combined and fractionated. Buffer solution A was 10 mmol/L HCOONH4, 5% ACN, pH 10.0, and B was 10 mmol/L HCOONH4, 85% ACN, pH 10.0. The samples were loaded onto the chromatographic column by an autosampler for separation at a flow rate of 1 mL/min after the chromatographic column had been equilibrated with solution A. The gradient of the liquid phase was as follows: 0% for solution B from 0 to 25 min; 0% to 7% for solution B from 25 to 30 min; 7% to 40% for solution B from 30 to 65 min; 40% to 100% for solution B from 65 to 70 min; and 100% for solution B from 70 to 85 min. During the elution process, monitoring the absorbance value at 214 nm, collecting elution components at an interval of 1 min, and about 40 samples of elution components were collected in total. The samples were lyophilized and reconstituted with 0.1% FA in 10 portions.

Mass spectrometry
Easy nLC chromatography. Using the Easy nLC nanoliter flow rate system, each sample was separated. Formic acid is present in buffers A and B in concentrations of 0.1% aqueous formic acid and 0.1% aqueous formic acid acetonitrile (acetonitrile is 80%). 100% solution A was used to equilibrate the column (Thermo Fisher Scientific, Waltham, MA, USA. Acclaim PepMap RSLC 50 μm × 15 cm, nano viper, P/N164943), and the samples were separated by loading onto an autosampler and running through an analytical column at a flow rate of 300 nL/min. Choose the corresponding gradient using the steps below. Gradient over an hour: 6% for solution B from 0 to 3 min; 6% to 28% for solution B from 3 to 45 min; 28% to 38% for solution B from 45 to 60 min; and 100% for solution B from 55 to 60 min.

Mass spec identification. Following chromatographic separation, samples were separated by mass spectrometry using a Q Exactive HF-X mass spectrometer. 60 or 90 min were allotted for the analysis (depending on the specific methodology). Positive ion detection was used. 350 to 1,600 m/z was the parent ion scanning range. Primary mass spectrometry has a resolution of 60,000. The primary Maximum IT was 50 ms, and the AGC target was 3e6. After each complete scan, ten fragmentation maps (MS2scan) were collected. The following were the secondary spectrum acquisition parameters: HCD was the MS2 Activation Type. The 2 m/z isolation window. The resolution of the secondary mass spectrometry was 15,000. AGC was aiming for 2e5. The AGC target was 2e3 at the least. 45 ms was the secondary Maximum IT. The acquisition charge was between 2 and 6. Ion dynamic exclusion time was 30 s, and Normalized Collision Energy was 30 eV.

Principal component analysis
The principal component analysis is the process of regrouping the signal values on a protein sample linearly to form a new set of composite variables. Depending on the problem being analyzed, several (usually 2–3) composite variables are selected from them so that they reflect as much information as possible about the original variables, thus achieving dimensionality reduction. At the same time, principal component analysis can also reflect the intra- and inter-group variability in a general way.

Volcano plot
The volcano plot was created by the R-package plot in R studio (2022.02.0). The horizontal coordinate is represented by Log(FoldChange), and the vertical coordinate is represented by −Log10(P-value), which shows the differentially expressed proteins (DEPs) that are significant. DEPs are defined as proteins with FoldChange > 1.2 and P-value (t-test) < 0.05. The results are visualized in the volcano plot.

Cluster analysis
To perform cluster analysis, the quantitative information of the target protein set is normalized, and then classify the two dimensions of sample and protein expression to generate a hierarchical clustering heat map.

Data analysis
Thermo Fisher Scientific’s Proteome Discoverer 2.2 software was utilized in this project to convert raw map files from Q Exactive HF-X (.Raw file) to .Mgf files, which were then sent to the Mascot 2.6 server for database retrieval through a built-in tool. Proteome Discoverer 2.2 returned the library file (.Dat files) created on the Mascot server to the program, where the data were screened using the false discovery rate 0.01 standard to produce highly reliable qualitative results. This project’s database is Uniprot_HomoSapiens_2039420210127. The final result will be a list of DEPs.

Basic functional analysis
Proteome subcellular localization analysis. We referred to Metascape (http://metascape.org) to obtain the subcellular location of each differential protein. Those less than 1% of the subcellular location genes were eliminated, and the rest was made a pie chart.

Protein domains and enrichment analysis. To assess the degree of
enrichment of a particular functional domain, we studied the distribution of differential proteins across the entire protein collection. Differential proteins were analyzed for enrichment using the enrichplot package of R studio. All proteins in the pathway with the highest DEPs enrichment were analyzed in combination with the proteins in the DEPs list to obtain the target protein.

**Protein interaction analysis.** We uploaded the DEPs preserved in the previous step to the String (https://cn.string-db.org/) to obtain the protein network interaction map and entered the obtained file into Cytoscape (V3.9.1). Selection of key proteins based on Degree algorithms.

**Survival analysis and expression analysis.** Survival analysis and expression analysis of the screened key proteins were performed using the GEPIA2 (http://geopia2.cancer-pku.cn/) website.

**Upstream transcription factor prediction and molecular docking validation.** Predicted upstream transcription factors for key proteins at CHEA3 (http://maayanlab.cloud). The 2-dimensional structure of Puerarin was constructed using chemdraw and then imported into ChemDraw 3D, where the MM2 module was used to minimize the energy and preserve the advantageous conception. The molecular structure of the target protein was downloaded from the PDB database and visualized using pymol, then the charge was calculated using Mgo tools 1.5.6, and the ligand and receptor were preserved by de-watering and hydrogenation. The ligand and receptor were docked using Autodock vina 1.1.2 and the higher-scoring conception was taken and visualized using PYMOL and Discovery Studio.

**Western blot.**

**Cell grouping and culture.** Cells were divided into a control group and an experimental group, the control group was without any interference measures, and the experimental group added 200 µmol/L concentration of Puerarin treatment, each group of three samples, each cultured in 75 cm² cell culture flasks.

**Protein extraction and quantification.** The ice machine made ice and cytosolic lysate was configured on ice. Add grinding beads, 200 µL cytoplasmic lysate in each cell sample, symmetrically put into the ultrasonic lyser, 60 Hz lysis for 30 s, put the sample tube in a centrifuge, 10,000 rpm, 4 °C centrifugation for 10 min, discarding the supernatant, and configure the nuclear lysate. Add 100 µL of nuclear lysate to each sample tube, put the sample tube in ultrasonic lyser again, 60 Hz lysis for 30 s, ice bath for 15 min, centrifugation at 10,000 rpm, 4 °C for 10 min, take the supernatant for protein quantification. Add 20 µL of protein up-sampling buffer to each sample tube and heat it in an iron bath at 95 °C for 5 min.

**SDS-PAGE electrophoresis, membrane transfer, primary antibody incubation.** Prepare electrophoresis solution, put the pre-made plate into the electrophoresis tank, add protein samples and marker, then add electrophoresis solution to the appropriate scale, constant pressure 160 V, electrophoresis for 35 min, put the PVDF membrane into methanol to activate for 1 min, then put it into the equilibrium of the membrane activation solution for 10 min, and put it into the wet rotating machine to rotate the membrane. After membrane transfer, add protein blocking solution for 15 min, add primary antibody and internal reference, and incubate at 4 °C overnight.

**Incubate the secondary antibody and develop the image.** Recover primary antibody and internal reference, wash with TBST (Servexbio, Wuhan, China) three times, add secondary antibody, incubate for 1 h, wash with TBST for three times, add developer solution, and place in the developer for imaging. Analyze the gray value of protein with Image J software.

**Statistical methods.** IBM SPSS Statistics 27 was used to carry out the statistical analyses. To compare the differences between the two groups, a Student’s t-test was used. The cutoff for statistical significance was P < 0.05.

**Results.**

**Puerarin inhibited the proliferation of BLCA T24 and 5637 cells.** In the present study, Puerarin was found to have an inhibitory effect on BLCA T24 and 5637 cells, which was positively correlated with drug concentration. The previous study found that the 50% inhibitory concentration (IC50) curve of Puerarin was 218 µmol/L in BLCA T24 cells (Figure 1a) [15]. This study found that the IC50 of Puerarin was 198 µmol/L in BLCA 5637 cells (Figure 1b).

**Puerarin mainly blocked the G1 phase of BLCA T24 and 5637 cells.** By flow cytometry, we found that Puerarin could inhibit the cell cycle of BLCA T24 cells and 5637 cells. As shown in the Figure 2a, 2b, the G1 phase of Puerarin-treated T24 cells and 5637 cells was significantly prolonged, while the S phase was significantly shortened (P < 0.05). This suggests that Puerarin would inhibit the proliferation of BLCA cells by suppressing their G1 phase.

**Acquisition of differential protein list.** As shown in Figure 3a, the electrophoretic bands are clear and the total amount meets two or more experiments and the quality meets the experimental requirements.

The software Mascot (V2.6) and Proteome Discoverer (V2.2) were used for library search identification and quantitative analysis. To ensure the credibility of the results, we set the number of protein-specific peptides must be ≥ 2. Supplementary Table S1, S2 showed that the marking efficiency was 100% and the mass spectra were all evaluated as A grade. According to our differential protein screening criteria, a total of 1,385 differential proteins, including 793 up-regulated proteins and 592 down-regulated proteins, were screened by comparing the proteome profiles of control and genocide group cells, and these results were visualized in Figure 3b.

![Figure 1 CCK-8 results.](https://www.tmjournals.com/tmr)
Figure 2 Flow cytometry results. (a) Shows the flow cytometry results of T24 cells and 5637 cells in the Puerarin group and the control group. (b) Shows the proportion of cells in each period of G1, G2, and S. In T24 cells, there was a significant difference in the number of cells in the G1 and S phases between the geranylgeranyl group and the control group (" P < 0.0001), and there was no significant difference in the G2 group (P = 0.9814). 5637 cells, the difference in the number of cells in the G1 phase between the geranylgeranyl group and the control group was statistically significant (" P = 0.0004), and the difference in the S phase was also significant (P = 0.0039), and the difference in the G2 phase was not statistically significant (P = 0.7624). G2 phase difference was not statistically significant (P = 0.7624).

Figure 3 The quality control of TMT. (a) Shows the results of the SDS-PAGE test. (b) Volcano plot for showing the significant difference between the two data samples. The blue color on the left side represents down-regulated genes and the orange color on the right side represents up-regulated genes. (c) The results of Cluster analysis. The three green columns on the left are the control group and the three red columns on the right are the Puerarin group. (d) The score chart of principal component analysis. Red is the control group, green is the Puerarin group.
The results of the clustering heat map showed that the differences between the control and control groups were large, while the differences within each group were small, and the results are shown in Figure 3c.

The results of the principal component (Figure 3d) analysis showed that the protein expression profiles were well separated in the Puerrarin group compared to the control group.

The target protein was mainly enriched in DNA replication and cell cycle.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed using the enrichplot package of R studio. The approach of annotating genes and gene products for molecular functions (MF), biological pathways (BP), and cellular components (CC) is known as GO analysis. For the systematic examination of gene functions and associated high-level genomic functional data, KEGG is a helpful tool.

As shown in Figure 4a, the first ten BPs are highly related to the cell cycle, such as chromosome segregation, DNA replication, DNA-dependent DNA replication, sister chromatid segregation, and mitotic and nuclear division. CC shows that they are mainly nuclear and intranuclear substances, and MF is also mostly a functional term related to the cell cycle.

In terms of pathway analysis, the target proteins were involved in a total of 18 KEGG pathways (Figure 4b). The pathways with the highest enrichment of target proteins were related to the regulation of cell cycle and DNA replication, repair of DNA damage, and regulation of cell carcinogenesis, namely DNA replication, mismatch repair, cell cycle, p53 signaling pathway, and AMPK signaling pathway.

Key proteins: CDK1, PLK1, CCNB1, and target proteins were mainly localized in the nucleus

After importing the list of target genes into Cytoscape, according to the cytoHubba plugin, we selected the top 50 proteins according to the degree value to draw a protein interaction network map. The size and color of the circles represent the criticality of the protein in the network diagram, and the darker the color and the larger the circle, the more critical the gene is. As shown in Figure 5a, we suggest that

![Image](https://www.tmrjournals.com/tmr)

**Figure 4** The results of enrichment analysis. (a) Shows the top 10 results of MF, BP, and CC ranked by $-\log_{10}P$. (b) Shows the target proteins were involved in a total of 18 KEGG pathways. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

![Image](https://www.tmrjournals.com/tmr)

**Figure 5** Protein-protein interaction (PPI) network and subcellular localization. (a) The PPI network of the top 50 genes in degree and the darker the color and the larger the circle, the more critical the gene is. (b) The pie chart for subcellular localization.
cycin-B-cyclin dependent kinase 1 (CDK1), cyclin B1 (CCNB1), and polo-like kinase 1 (PLK1) are the key proteins of Puerarin to inhibit BLCA.

According to the results of subcellular localization (Figure 3b), target proteins are widely distributed in the nucleus, cytoplasm, extracellular matrix, and mitochondria.

PLK1, CCNB1 significantly affected the prognosis of BLCA patients and its expression was inhibited by Puerarin

Survival analysis curves showed that PLK1 and CCNB1 significantly affected the disease-free survival of BLCA patients (Figure 6a). Expression analysis showed that the expression of all three key proteins was up-regulated in BLCA (Figure 6b). However, the expression of PLK1 and CCNB1 was reduced after Puerarin intervention, and we believe that these two are the key proteins that play a role.

Centromere protein A (CENPA) was an upstream transcription factor for key proteins and binded well to Puerarin molecules

The top 5 upstream transcription factors predicted based on CHEA were CENPA, zinc finger protein 695 (ZNF695), forkhead box protein M1 (FOXM1), MYB Proto-Oncogene Like 2 (MYBL2), and E2F transcription factor 1 (E2F1) (Table 1). The first-ranked CENPA was selected for subsequent validation. The binding energy of Puerarin to

![Figure 6](https://www.tmrjournals.com/tmr)

**Figure 6 Survival analysis and expression analysis.** (a) Shows the result of survival analysis demonstrating the difference in disease-free survival between high and low expression of CDK1, CCNB1, and PLK1 in BLCA patients. (b) Shows the comparison of CENPA, CDK1, CCNB1 and PLK1 expression in BLCA cells and normal cells. CENPA, centromere protein A; CCNB1, cyclin B1; CDK1, cyclin-B-cyclin dependent kinase 1; PLK1, polo-like kinase 1.

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<td>CDK1; CCNB1; PLK1</td>
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</tr>
<tr>
<td>4</td>
<td>MYBL2</td>
<td>26.5</td>
<td>CDK1; CCNB1; PLK1</td>
<td>Literature ChIP-seq, 4; ARCHS4 Coexpression, 66; ENCODE ChIP-seq, 38; Enrichr Queries, 46; ReMap ChIP-seq, 2; GTEx Coexpression, 3</td>
</tr>
<tr>
<td>5</td>
<td>E2F1</td>
<td>37.5</td>
<td>CDK1; CCNB1; PLK1</td>
<td>Literature ChIP-seq, 16; ARCHS4 Coexpression, 61; ENCODE ChIP-seq, 59; Enrichr Queries, 55; ReMap ChIP-seq, 28; GTEx Coexpression, 6</td>
</tr>
</tbody>
</table>

CENPA, centromere protein A; ZNF695, zinc finger protein 695; FOXM1, forkhead box protein M1; MYBL2, MYB Proto-Oncogene Like 2; E2F1, E2F transcription factor 1; CCNB1, cyclin B1; CDK1, cyclin-B-cyclin dependent kinase 1; PLK1, polo-like kinase 1.

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CENPA was ~6.3 kcal/mol, which proved to have a good binding effect (Figure 7a). The benzene ring of Puerarin can form PI-Pi stacking conjugation interactions with amino acid phenylalanine101 (PHE101) of CENPA, and the carbon atoms in the compound form hydrophobic interactions with phenylalanine67 (PHE67), Alanine71 (ALA71), Leucine94 (LEU94), Alanine98 (ALA98), and Leucine102 (LEU102) of the protein.

Puerarin inhibited the expression of CENPA

Compared with the control group (Figure 7b, 7c), the expression of CENPA protein in the experimental group was significantly reduced ($P = 0.0142$). Whereas CENPA was highly expressed in BLCA (Figure 5b), the expression was reduced after Puerarin intervention, which was consistent with the conjectural results.

Discussion

Chinese herbs and their active ingredients have been proven to treat many malignancies especially, with minimal side effects and synergistic results [16]. Puerarin has a core three-carbon structure that connects two benzene rings. Numerous studies demonstrate that this structure can suppress the expression of Rass, block the cell cycle, downregulate mutant p53 protein, and improve anticancer characteristics [17]. However, this structure also makes Puerarin less water-soluble and fat-soluble, leading to poor oral absorption and low bioavailability, which limits its wide clinical application [18]. Recent studies have found that Puerarin combined with nanomolecular systems can solve these limitations. Puerarin can be embedded or dissolved in this system and its poor hydrophilicity is improved. In addition, nanoparticles can improve targeting and reduce toxicity and side effects [19]. The solubility of geranylgeranyl can also be significantly enhanced by co-crystallization with L-proline [20]. The potential of polyamide-amine dendritic polymers as solubility enhancers and oral drug delivery systems could increase the solubility and bioavailability of Puerarin [21]. This is a promising strategy for the oral drug delivery of Pueraria Mirifica.

Through CCK-8 and flow cytometry experiments we found that geranool could inhibit the proliferation of BLCA cells by suppressing their cell cycle. The results of enrichment analysis showed that the screened differential proteins were enriched in cell cycle-related pathways such as chromosome segregation, DNA-dependent DNA replication, DNA replication, sister chromatid segregation, mitotic karyokinesis, and sister chromatid mitosis; and the key proteins obtained by FPI were also cycle-related. The cell cycle can be divided into the following consecutive phases: G0/G1, S, and G2/M phases, with a transition period between the phases to ensure the completion of the cell cycle. The successful transition from one phase to the next is controlled by several specific factors called cell cycle proteins and cell cycle protein-dependent kinases [22]. The key proteins obtained from our screening are all cell cycle proteins, from this we concluded that Puerarin may regulate CENPA to control the expression of CCNB1 and PLK1 to affect the cell cycle BLCA cells to inhibit their proliferation. Then we conducted functional experiments to verify the hypothesized results and found that Puerarin could significantly inhibit the expression of CENPA and thus inhibit the proliferation of BLCA.

Centromere proteins (CENPs) are a class of mitosis-associated proteins, including CENPA, CENPB, and CENPC, which play important roles in the regulation of mitosis formation, chromosome segregation, cell division, and cell cycle [23]. Among them, CENPA is the main component of the CENP family, which has two important functions. On the one hand, it provides the necessary conditions for the formation and maintenance of mitotic grains, and on the other hand, it serves as a platform for the formation of mitotic assembly and mediation of chromosome segregation [24]. The current study suggests that aberrant expression of CENPs during cell division may affect cell cycle progression and normal segregation of sister chromatids, leading to chromosomal instability, which is an important factor in tumorigenesis and development [25]. Overexpression of CENPA increases ectopic deposition of CENPA at high histone turnover sites and blocks CCCTC binding, and CENPA overexpression leads to epithelial-mesenchymal transition, which is an important precursor for tumor cell invasion and metastasis [26]. Zhang et al. demonstrated that knockdown of CENPA reduced cell proliferation, blocked the G1 phase cell cycle, and induced apoptosis in HepG2 cells.

Figure 7 Schematic diagram of molecular docking and results of western blot experiments. (a) Shows the schematic representation of the docking of Puerarin to CENPA protein. (b) Shows the protein expression results of the Puerarin group and the control group. (c) Shows the gray values measured according to the protein expression results were significantly different between the two groups ($P < 0.05$). (d) Shows the mechanism of action of Puerarin in inhibiting BLCA. CENPA, centromere protein A; CCNB1, cyclin B1; PLK1, polo-like kinase 1; BLCA, bladder cancer.
In this study, we found that Puerarin inhibited the expression of CCNB1, which caused cell cycle arrest in G1 phase and reduced the proliferation of tumor cells. CCNB1 is mostly found in the cytoplasm during the G phase before entering the nucleus at the beginning of mitosis and being actively transcribed throughout the mitotic stage [28]. CCNB1 builds up in the S phase and reaches its peak concentration during mitosis. When the cell cycle transitions from metaphase to anaphase, CCNB1 is then quickly destroyed [29]. CCNB1 regulates cell cycle transition, resulting in uncontrolled proliferation [30]. In contrast to normal cells and tissues, CCNB1 is overexpressed in a variety of malignancies, including breast cancer and non-small cell lung cancer [31]. To control the G2/M stages of the mammalian cell cycle, which are crucial for the start of mitosis, CCNB1 and CCNB2 can assemble complexes with CDK1 [32]. Chromosome condensation, spindle pole assembly, and nuclear envelope collapse are all started in tumor cells by the activation and activation of the CCNB1-CDK1 complex [33]. Wang et al. found that after knocking down the expression of CCNB1, 5,637 cells were significantly blocked in the G2/M phase, with a relative decrease in the G0/G1 phase, and the ratio of T24 cells in the G0/G1 phase was reduced. These findings suggest that CCNB1 blocks BLCA cells in G2/M phase and inhibits BLCA cell proliferation [34]. Numerous studies have demonstrated the role of CCNB1 in the p53 signaling pathway. The absence of CCNB1 may suppress proliferation, reduce the proportion of cells in the S-phase, cause apoptosis and senescence, and raise the proportion of cells in the G0/G1 phase, suggesting that these effects may be brought on by the activation of the p53 signaling pathway [35]. Meanwhile, there is evidence that inhibition of CCNB1 expression makes cancer cells more sensitive to the chemotherapeutic drug paclitaxel [36]. This suggests that Puerarin, as an adjuvant to chemotherapy, may increase the ability of chemotherapeutic drugs to kill cancer cells and reduce the dose of chemotherapeutic drugs used, thus reducing toxic side effects.

PLK1 is the principle member of the well-conserved serine/threonine kinase family [37, 38]. It is essential for controlling cell division during mitosis. PLK1 can phosphorylate particular downstream targets and hence regulate mitosis because it is typically abundant in mitotic centrosomes, kinetochores, and the cytoplasmic midbody [29]. Recent data hint at its critical role in the regulation of the G2/M checkpoint, in the response to DNA damage and replication stress, and in the pathways leading to cell death [37]. The activity of the CDK/cyclin B complex, which controls mitotic entrance, is governed by phosphorylation. PLK1 stimulates Cdc25 phosphatase, which then dephosphorylates CDK/cyclin B and inactivates Wee1 to activate CDK/cyclin B [39]. Our expression analysis showed that PLK1 and CCNB1 expression was up-regulated in BLCA cells, while TMT results showed that both key proteins were down-regulated after Puerarin treatment. It is reasonable to hypothesize that these two proteins are the key factors in the inhibition of BLCA cell proliferation by Puerarin. The degradation of CCNB1 by PLK1 inhibition appeared to be a critical promoter of mitotic slippage. In head-and-neck squamous cell carcinoma, PLK1 siRNA significantly increased the CCNB1 mRNA level [40]. In summary, Puerarin inhibits the expression of the upstream transcription factor CCNB1, which in turn inhibits the activity of PLK1, contributing to the reduction of mitosis in tumor cells and achieving tumor cell suppression. Recent data suggest that it plays a key role in regulating the G2/M checkpoint, the response to DNA damage and replication stress, and pathways leading to cell death [37].

To summarize, we found that Puerarin may inhibit the proliferation of BLCA cells by regulating CCNB1 to control the expression of CCNB1 and PLK1, thereby affecting the cell cycle of BLCA cells. At present, Puerarin has a great application prospect in the treatment of BLCA. Puerarin can be used as an adjuvant chemotherapeutic agent after TURBT to enhance the sensitivity of cancer cells to chemotherapeutic agents and, as a natural component of Chinese herbs, it can reduce the toxic side effects of chemotherapeutic agents. But more studies are needed to confirm its efficacy and safety. In this study, we confirmed the inhibitory effect of Puerarin on BLCA and performed preliminary functional experiments to validate the effect, and conducted functional experiments for the BLCA T24 cell line to verify this role, but we believe that the T24 cell line is not a complete surrogate for BLCA, so we need to select a few more BLCA cells to verify this, and this is what we intend to continue to do at a later stage of our study. With the continuous progress of science and technology and in-depth research in the field of medicine, it is believed that Puerarin will play a greater role in the treatment of BLCA in the future, and bring the gospel to BLCA patients.

**Conclusion**

We speculate that Puerarin may further inhibit the proliferation of BLCA cells by regulating the expression of PLK1 and CCNB1 through the modulation of CCNB1, thereby affecting the cell cycle.

**References**


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