

Polyphyllin II inhibits breast cancer cell proliferation via the PI3K/Akt signaling pathway

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Received July 4, 2024; Accepted September 5, 2024

DOI: 10.3892/mmr.2024.13348

Abstract. Paridis Rhizoma saponins (PRS) are significant components of Rhizoma Paridis and have inhibitory effects on various tumors, such as bladder, breast, liver and colon cancer. Polyphyllin II (PPII), one of the PRS, has an unclear effect on breast cancer. The present study aimed to explore the effect and mechanism of PPII in breast cancer. A network pharmacology approach was employed to predict the core components and breast cancer-related targets of PRS. Moreover, a xenograft tumor model was established to determine the anti-breast cancer effect of PPII *in vivo*. The viability of MDA-MB-231 cells was determined by a Cell Counting Kit-8 assay. Apoptosis was analyzed using annexin V/PI double staining. Additionally, Transwell and scratch assays were performed to evaluate invasion and migration. The potential mechanism was predicted by Kyoto Encyclopedia of Genes and Genomes enrichment analysis and molecular docking analysis and verified by western blot analysis. The effect of PPII on aerobic glycolysis in breast cancer cells was detected by lactic acid and pyruvate kits and Western blotting of glycolytic rate-limiting enzymes. Network pharmacology analysis revealed 26 core targets involved in breast cancer and that PPII was the core active component of PRS. The *in vivo* studies showed that PPII could inhibit the growth of breast cancer in mice. *In vitro* experiments confirmed that PPII induced cancer cell apoptosis and inhibited invasion and migration. Furthermore, PPII was capable of suppressing the expression of key proteins

in the PI3K/Akt signaling pathway, reducing the generation of aerobic glycolytic products, and diminishing the protein expression levels of hexokinase 2 and pyruvate kinase M2. The results indicated that PPII inhibited aerobic glycolysis in breast cancer cells through the PI3K/Akt signaling pathway, thereby inhibiting breast cancer growth.

Introduction

Breast cancer is the most common cancer in women worldwide and a leading cause of cancer-related death (1). A recent study by the National Cancer Center of China revealed that the incidence of breast cancer is the highest in Chinese women (2). Furthermore, the incidence of breast cancer in young women is increasing annually, between 1990 and 2021, the annual growth rate of breast cancer incidence among young women worldwide was 0.82%, which seriously affects their physical and mental health (3). At present, the main treatment methods for breast cancer include surgery, chemotherapy, radiotherapy, endocrine therapy and targeted therapy, which can prolong the survival of patients (4). However, numerous patients do not effectively respond to these treatments, and their prognosis remains poor (5). Therefore, the development of novel therapeutic drugs for breast cancer is urgent. In previous years, traditional Chinese medicine monomers have attracted widespread attention in the field of anticancer therapy because they can effectively inhibit the proliferation and induce the apoptosis of breast cancer cells by regulating targeted genes, signaling pathways and the cancer microenvironment (6-8).

Rhizoma Paridis is a common traditional Chinese medicine used in traditional anticancer prescriptions (9). Modern pharmacology studies have confirmed that Rhizoma Paridis has antitumor, anti-inflammatory, antioxidant, antibacterial and immunomodulatory effects (10,11). At present, there are antitumor Chinese medicines containing Rhizoma Paridis on the market, including Lou-Lian capsules, Jin-Fu-Kang oral liquid (12) and Ruan-Jian oral liquid (13). Furthermore, studies have confirmed that Rhizoma Paridis extract has certain inhibitory effects on bladder (14), breast (15), liver (16) and colon (17) cancer. Paridis Rhizoma saponins (PRS) are important components of Rhizoma Paridis and have become a hotspot in current anticancer research (18,19). Polyphyllin

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Key words: polyphyllin II, breast cancer, apoptosis, PI3K/Akt signaling pathway, aerobic glycolysis

II (PPII) is one of the PRS (20), however, its influence on breast cancer and the mechanism of action are still unclear. The present study aimed to reveal the molecular mechanism by which PPII inhibits the proliferation of breast cancer cells to provide a theoretical basis for its clinical application.

Materials and methods

Screening the potential targets of *Paridis Rhizoma* for breast cancer treatment. The main active ingredients of *Paridis Rhizoma* were obtained through searching the literature. The PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) was used to obtain the canonical simplified molecular-input line-entry system strings of the active ingredients, the targets of active ingredients were predicted with the Swiss Target Prediction database (<http://www.swisstargetprediction.ch/probability>0>) and PharmMapper database (<http://lilab-ecust.cn/pharmmapper/index.html>) (21,22), and the target names were converted into gene symbols using UniProt (<https://www.uniprot.org/>). The GeneCards database (<https://www.genecards.org/>), Comparative Toxicogenomics Database (<https://ctdbase.org/>) and Online Mendelian Inheritance in Man database (<https://omim.org/>) were used to identify breast cancer-related target genes using the keyword 'breast cancer'. Intersecting targets between active ingredients of *Paridis Rhizoma* and breast cancer were identified using the Venny online platform (<https://bioinfo.gp.cnb.csic.es/tools/venny/>).

KEGG pathway enrichment analysis. The ClusterProfiler package (version 3.16.1) in R software (version 4.0.2) was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis with a *q* value <0.05 to identify the cancer-related pathways affected by PRS. The DAVID tool (<https://david.ncifcrf.gov/>) was used to obtain the KEGG enrichment information of the targets. The top 15 KEGG pathways were selected for further analysis.

Molecular docking simulation. The protein structures were obtained from the Protein Data Bank (<https://www.rcsb.org/>) and the compound structures were downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). PyMol (<https://pymol.org/2/>) was used to remove water and solvent molecules from proteins, and process receptors and ligands further using AutoDock Tool 1.5.7 before docking was performed. Using PyMol, the docking data with the lowest binding energies was chosen. The binding sites were visualized using a protein-ligand interaction profiler (<https://projects.biotec.tu-dresden.de/plip-web/plip/index>).

Cell lines and cell culture. MDA-MB-231 human breast cancer cells were obtained from a cell bank/stem cell bank (Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences) and cultured in Leibovitz's L-15 medium (Adamas-Beta, Ltd.) supplemented with 10% FBS (Adamas-Beta, Ltd.) and 1% penicillin/streptomycin (Adamas-Beta, Ltd.), with 100% air in an incubator with 90±5% humidity at 37°C.

Establishment and treatment of xenograft tumor model mice. All mouse experiments were carried out according to protocols authorized by The Scientific Research Ethics Committee

of Shanghai University of Medicine and Health Sciences (Shanghai, China; approval no. 2023-XKY-07-320924198803 08342X) and in accordance with UK guidelines (23). A total of ten 8-week-old female BALB/cA nude mice (19±3 g; Shanghai Jiesijie Laboratory Animal Co., Ltd.) were maintained in an SPF setting (22±2°C and 55±5% humidity) on a 10 h light, 14 h dark cycle with food and water supplied *ad libitum* throughout the experimental period.

MDA-MB-231 cells (5×10⁶ cells) suspended in 100 µl L-15 media were injected into the mice through the subcutaneous axilla. When the tumor volume reached 50-100 mm³, the mice were randomly divided into two groups (n=5/group): i) The PPII group [0.5 mg/kg/day; CAS: 50773-42-7; Beijing Solarbio Science & Technology Co., Ltd.; high-performance liquid chromatography ≥98%, dissolved in DMSO (Beijing Solarbio Science & Technology Co., Ltd.) and diluted in complete media to the final concentration]; and ii) the control group (0.9% saline). The mice were administered PPII or 0.9% saline every other day for 14 days by intraperitoneal injection. Tumor growth was calculated according to the following equation: Volume=(width² × length)/2 and the maximum tumor volume permitted in the tumor-bearing mice was ≤1,500 mm³. On the 14th day, the mice were euthanized by cervical dislocation, and the tumors were removed for further analysis. Tumor growth inhibition of tumor weight (TGI_{tw}) was assessed using the following formula: TGI_{tw}=(tumor weight of control group-tumor weight of PPII group)/tumor weight of control group ×100%.

For immunohistochemistry, tumor tissues were fixed with 4% paraformaldehyde at room temperature for 24 h (cat. no. P0099; Beyotime Institute of Biotechnology). After fixation, the tissues were dehydrated in ethanol with different concentrations (70, 80, 90 and 100%; Sinopharm Chemical Reagent Co., Ltd.) successively for 10 min each. After which, the tissues were soaked in paraffin (Sinopharm Chemical Reagent Co., Ltd.) with a temperature not exceeding 60°C for 1-2 h, and cut into 3 µm-thick sections. The sections were baked at 60°C for 20 min, infiltrated with xylene twice (10 min each time; Sinopharm Chemical Reagent Co., Ltd.), and placed into ethanol of different concentrations (100, 95 and 85%; Sinopharm Chemical Reagent Co., Ltd.) for infiltration (5 min each time). The sections were rinsed with distilled water 5 min for rehydration, and in a pressure cooker antigen retrieval was performed on the sections with citric acid repair solution (cat. no. P0081; Beyotime Institute of Biotechnology) for 2.5 min. Subsequently, the tissues were blocked with 5% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd.) at 37°C for 10 min. Then the sections were incubated with Ki67 antibody (1:200; cat. no. AF0198; Affinity Biosciences) and Caspase 3 antibody (1:200; cat. no. AF6311; Affinity Biosciences) overnight at 4°C. After which the samples were incubated with Goat Anti-Rabbit IgG (H+L) HRP (1:200; cat. no. S0001; Affinity Biosciences) at 37°C for 1 h and stained with DAB Horseradish Peroxidase Color Development Kit (cat. no. P0203; Beyotime Institute of Biotechnology) and redyed with hematoxylin (cat. no. C0107; Beyotime Institute of Biotechnology). Images were obtained under a fluorescence inverted microscope (IX73P2F; Olympus Corporation) and quantified using ImageJ 1.48V (National Institutes of Health).

Cell viability assay. A Cell Counting Kit-8 (CCK-8; cat. no. C8022-500T; Adamas-Beta, Ltd.) was used to evaluate the cytotoxic effect of PPII on MDA-MB-231 cells, and all steps were performed according to the manufacturer's instructions. MDA-MB-231 cells (6×10^3 cells/well) were seeded in 96-well plates. After overnight incubation, the culture media were changed to new media containing different concentrations of PPII (0, 0.09765625, 0.390625, 1.5625, 6.25, 25 or 100 $\mu\text{mol/l}$) and 0.1% DMSO, and the cells were then incubated for 0, 12, 24, 36, 48 or 72 h at 37°C in an incubator. Afterwards, the culture media were replaced with 100 $\mu\text{l/well}$ 10% CCK-8 solution, and the cells were incubated for 2 h at 37°C. Subsequently, the absorbance values were measured at 450 nm on a microplate reader (MULTISKAN FC; Thermo Fisher Scientific, Inc.). The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = $[1 - (A_{\text{PPII}} - A_{\text{Blank}}) / (A_{\text{Control}} - A_{\text{Blank}})] \times 100\%$.

Flow cytometry. MDA-MB-231 cells were seeded in 6-well plates (6×10^5 cells/well) and incubated until they adhered to the plate. Subsequently, the cells were treated as follows: The control group was treated with 0.1% DMSO and the PPII group was treated with PPII (2 $\mu\text{mol/l}$) for 48 h at 37°C. The supernatant was discarded and the cells were washed three times with PBS and digested with 0.25% trypsin (Adamas-Beta, Ltd.). The cells were centrifuged at $300 \times g$ for 10 min at 4°C, the supernatant was discarded and PBS was added for washing three times. A total of 100 μl binding buffer was added to each tube to prepare the cell suspension. The cells were stained singly with annexin V-FITC and doubly with PI, and incubated at room temperature away from the light for 15 min. After the incubation, 400 μl binding buffer was added to each tube. Cell apoptosis was detected by flow cytometry (CytoFLEX S; Beckman Coulter, Inc.) using an Annexin V-FITC Apoptosis Detection Kit (cat. no. C1062M; Beyotime Institute of Biotechnology). CytExpert 2.4 (Beckman Coulter, Inc.) was used to evaluate the ratios of apoptotic and non-apoptotic cells in each of the four populations: i) Live cells, annexin V-negative and PI-negative; ii) early apoptotic cells, annexin V-positive and PI-negative; iii) late apoptotic or dead cells, annexin V-positive and PI-positive; iv) and dead non-apoptotic cells, annexin V-negative and PI-positive. Apoptosis rate = ratios of early apoptosis + ratios of late apoptosis.

Cell scratch assay. MDA-MB-231 cells (2×10^5 cells/well) were seeded in a 6-well plate and grown to 90% confluence. A scratch was made in each well using a 200- μl pipette tip, and the cells were subsequently washed with PBS. The cells were treated with 0.1% DMSO and 2 $\mu\text{mol/l}$ PPII in serum-free medium. Wound healing was observed at 0 and 48 h under a microscope (XD-202; Nanjing Jiangnan Novel Optics Co., Ltd.). The areas of the scratches were measured using ImageJ 1.48v software (National Institutes of Health) and the cell migration was calculated according to the following formula: Migration rate (%) = (final scratch area - initial scratch area) / initial scratch area $\times 100\%$.

Invasion assay. The Transwell plate (Costar; Corning, Inc.) insert membrane was coated with Matrigel (BD Biosciences) to assess cell invasion. On ice, the Matrigel was diluted to

1 mg/ml with serum-free medium. After which, 60 μl of diluted Matrigel was evenly spread on the bottom of the Transwell chamber and incubated at 37°C for 3 h. After which, the Matrigel was removed and 100 μl serum-free culture medium was added and incubated at 37°C for 30 min for hydration. Finally, the liquid in the chamber was removed and cell inoculation was carried out. MDA-MB-231 cells (5×10^4 cells/well) were seeded in Transwell chambers, and the cells were starved in serum-free media for 12 h. Subsequently, 700 μl L-15 medium supplemented with 20% FBS (Adamas-Beta, Ltd.) was added to the lower wells, and 500 μl serum-free medium supplemented with 2 $\mu\text{mol/l}$ PPII and 0.1% DMSO were added to the upper Transwell chambers. The cells were incubated for 48 h at 37°C. Subsequently, the cells and Matrigel on the upper chamber were scraped with a cotton swab. The filter was fixed with 4% paraformaldehyde fixative solution (cat. no. P0099; Beyotime Institute of Biotechnology) for 20 min and stained with 0.1% crystal violet (Shanghai Aladdin Biochemical Technology Co., Ltd.) at room temperature for 30 min. Finally, the number of cells that adhered to the lower surface of the insert membranes was counted under a microscope (XD-202; Nanjing Jiangnan Novel Optics Co., Ltd.).

Pyruvate and lactic acid assays. The cell treatment conditions were the same as those for flow cytometry. The intracellular pyruvate and lactic acid levels were detected using pyruvate assay kits (cat. no. BC2205; Beijing Solarbio Science & Technology Co., Ltd.) and lactic acid assay kits (cat. no. A019-2-1; Nanjing Jiancheng Bioengineering Institute), respectively. All steps were performed according to the manufacturer's instructions. Subsequently, the absorbance values were measured at 520 and 530 nm on a microplate reader.

Western blotting. Western blotting was performed according to standard procedures (24). The cell treatment conditions were the same as those for flow cytometry. Cells were collected and lysed in lysis buffer supplemented with protease inhibitors (cat. no. BL504A; Biosharp Life Sciences) to extract proteins. The protein concentration was measured using a BCA Protein Assay kit (cat. no. G2026-1000T; Wuhan Servicebio Technology Co., Ltd.). Proteins were separated by 10% SDS-PAGE (cat. no. Ba1012; Wuhan Baiqiandu Biotechnology Co., Ltd.) with a protein loading amount of 40 $\mu\text{g/lane}$ and transferred to a PVDF (cat. no. IPVH00010; MilliporeSigma) membrane. After blocking the membranes with 5% nonfat milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with the primary antibodies, followed by incubation with the HRP-Goat anti Rabbit and HRP-Goat anti Mouse at room temperature for 1 h. β -actin was used as the internal control. Finally, proteins were detected with ECL reagent (cat. no. MA0186; Dalian Meilun Biology Technology Co., Ltd.) and the membranes were developed by the image analyzer (cat. no. 4800; Tanon Science and Technology Co., Ltd.).

The antibodies in the present experiments included: Akt (1:2,000; cat. no. 60203-I-Ig; Wuhan Sanying Biotechnology, Inc.), phosphorylated (p-)Akt (1:10,000; cat. no. T40067F; Abmart Pharmaceutical Technology Co., Ltd.), PI3K (1:2,000; cat. no. 60225-1-Ig; Wuhan Sanying Biotechnology, Inc.), p-PI3K (1:1,000; cat. no. 341468; Chengdu Zen-Bioscience Co., Ltd.), hexokinase 2 (HK2; 1:2,000; cat. no. DF6176;

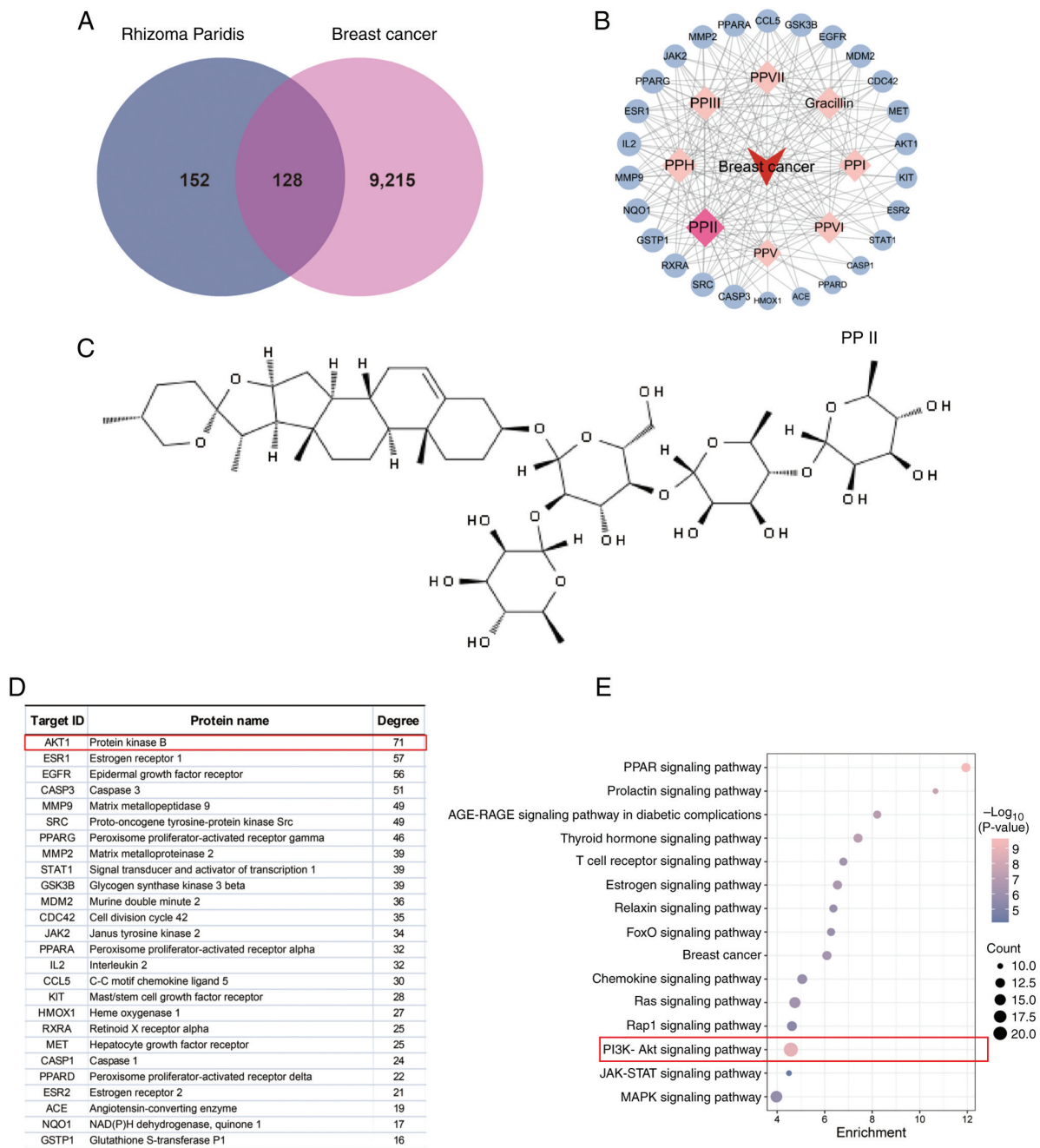


Figure 1. Network pharmacology analysis of the effects of PRS on breast cancer. (A) Venn diagram of predicted 128 targets of PRS. (B) Breast cancer-PRS-targets network, the PPII has more edges than those of the other saponins. (C) The chemical structure of PPII. (D) The top 26 important targets and full names of predicted targets. (E) KEGG pathway enrichment analysis of the top 15 predicted targets of PRS in the treatment of breast cancer. The size of dots indicates the number of genes in the KEGG pathways. The effect of PRS on breast cancer was closely associated with the PI3K/Akt signaling pathway. PP, polyphyllin; KEGG, Kyoto Encyclopedia of Genes and Genomes; PRS, Paridis Rhizoma saponins.

Affinity Biosciences), pyruvate kinase M2 (PKM2; 1:2,000; cat. no. AF5234; Affinity Biosciences), β -actin (1:5,000; cat. no. bs-0061R; BIOSS), HRP-Goat anti Rabbit (1:50,000; cat. no. 5220-0336; SeraCare Life Sciences) and HRP-Goat anti Mouse (1:50,000; cat. no. 5220-0341; SeraCare Life Sciences).

Statistical analysis. The results were analyzed using GraphPad Prism 9.0.0 (Dotmatics). Statistical comparisons between two groups were performed using unpaired t-test and the analysis of the control group and the experimental group at different time points was performed using two-way ANOVA and Sidak

multiple comparisons test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Screening the potential targets of Paridis Rhizoma for breast cancer treatment. Previous studies have demonstrated that polyphyllin I, PPII, polyphyllin III, polyphyllin V, polyphyllin VI, polyphyllin VII, polyphyllin H and gracillin are the important components in Rhizoma Paridis, which have antitumor properties (25-32). A total of 280 potential

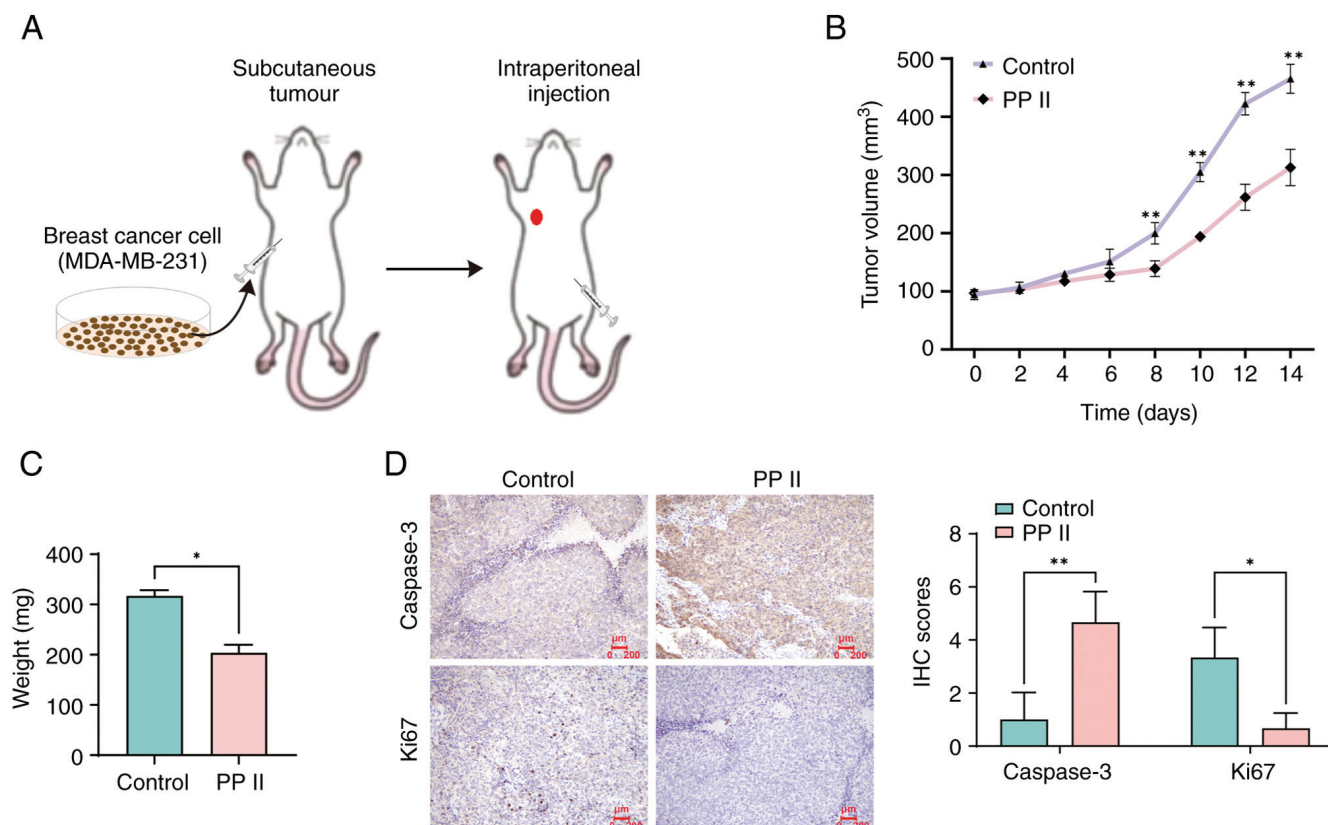


Figure 2. Effect of PPII treatment on breast cancer *in vivo*. (A) Diagram shows the experimental course of the xenograft tumor mouse model. (B) Tumor volumes were measured every two days during the treatment period. (C) Tumor mass was weighed after the tumor tissues were harvested. (D) Ki67 and Caspase 3 expression in tumor xenograft tissues were detected by immunohistochemistry. The data are presented as the mean $\pm$ SEM (n=3). *P<0.05 and **P<0.01 compared with the control. PPII, polyphyllin II.

targets of PRS were identified from databases, and there were 9,343 disease-related targets obtained from the three databases. The comparison of breast cancer-related targets and potential targets of PRS yielded a total of 128 shared targets according to the Venny online platform analysis (Fig. 1A). The protein-protein interaction network of PPII had more edges than that of other saponins, indicating that PPII is a core component in the treatment of breast cancer (Fig. 1B). Fig. 1C shows the chemical structural formula of PPII. Through further analysis, several essential genes were identified. The top 26 important targets (Fig. 1D) included AKT1, ESR1, EGFR, CASP3, MMP9, SRC, PPARG, MMP2, STAT1, GSK3B, MDM2, CDC42, JAK2, PPARG, IL2, CCL5, KIT, HMOX1, RXRA, MET, CASP1, PPARG, ESR2, ACE, NQO1 and GSTP1. Furthermore, the KEGG pathway enrichment analysis revealed that the effect of PRS on breast cancer was closely related to the PI3K/Akt signaling pathway (Fig. 1E).

PPII affects tumor growth *in vivo*. To verify the effect of PPII on breast cancer growth *in vivo*, a xenograft tumor model was established by subcutaneously injecting MDA-MB-231 cells into nude mice. Next, PPII was injected intraperitoneally (Fig. 2A), and the tumor volume was measured every 2 days. The results demonstrated that, compared with that in the control group, the tumor volume growth in the PPII group was slowed down, indicating that PPII inhibited the growth of tumors *in vivo*. Compared with the control group, tumor

growth was significantly inhibited starting from the 8th day (P<0.01; Fig. 2B). After 14 days of PPII intervention, the mice were euthanized by cervical dislocation, and the tumors were removed and weighed. The tumor weight of the PPII group was lower than that of the control group (P<0.05; Fig. 2C), and the TGI_{wt} was 35.74%.

To determine the effects of PPII on the proliferation of cancer cells, immunohistochemical analysis was performed. The Ki67 staining results revealed that the percentage of Ki67-positive cells was decreased in the PPII group compared with the control group (P<0.05). However, the Caspase 3 staining results revealed that the percentage of Caspase 3-positive cells was significantly increased in the PPII group compared with the control group (P<0.01; Fig. 2D).

PPII inhibits the proliferation, migration and invasion of breast cancer cells. The viability of MDA-MB-231 cells treated with PPII at different concentrations for different durations was determined using a CCK-8 assay. PPII effectively decreased the viability of MDA-MB-231 cells, suggesting that PPII could inhibit breast cancer cell proliferation. As shown in Fig. 3A and B, MDA-MB-231 cell viability was inhibited by ~50% at 48 h, and the IC₅₀ was 2.131 μ mol/l. Therefore, the PPII concentration used in subsequent experiments was 2 μ mol/l for 48 h. The apoptotic effect of PPII on breast cancer cells was determined by flow cytometry. The results showed that the percentage of apoptotic cells was 8.98% in

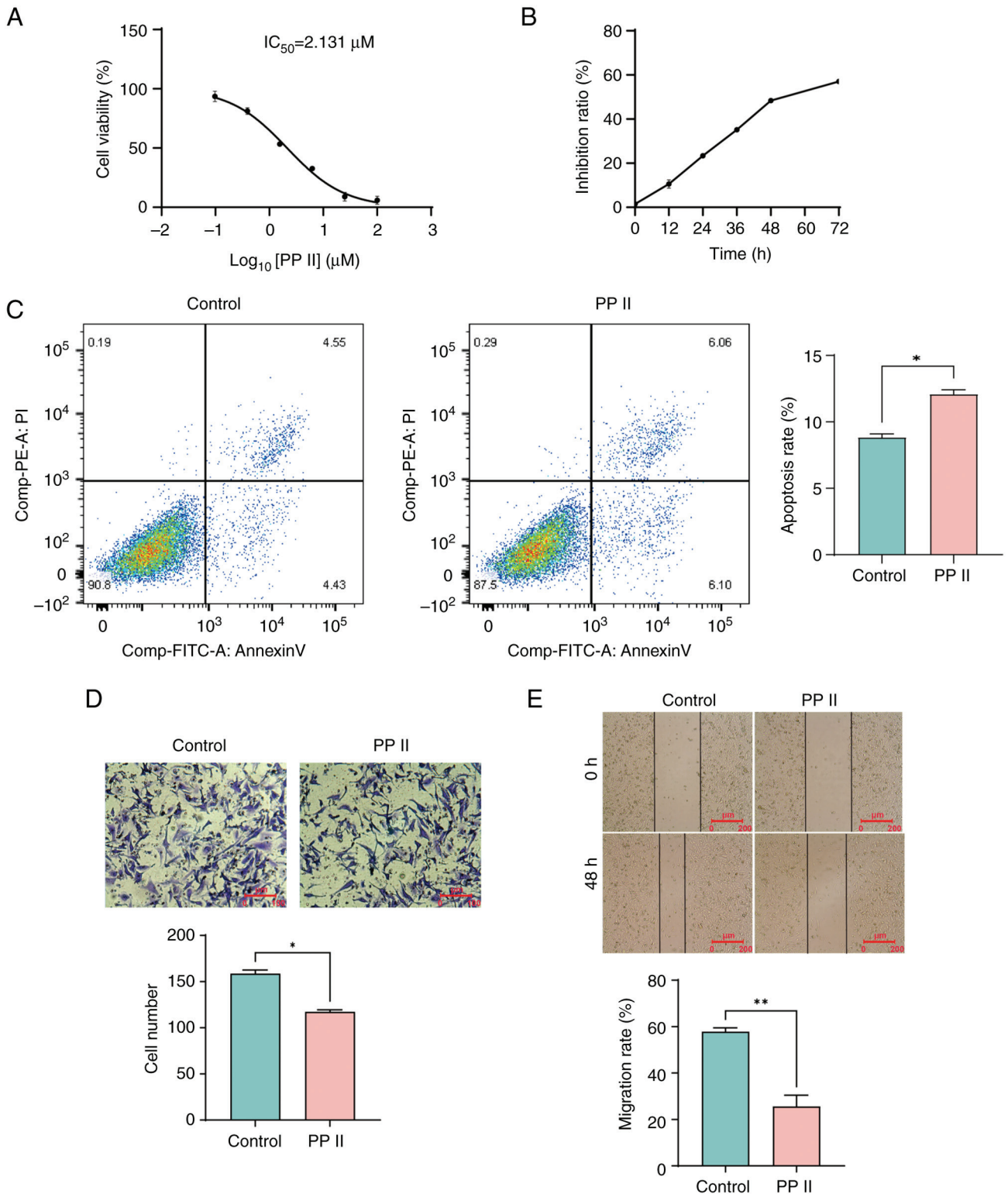


Figure 3. PPII inhibited the proliferation, migration and invasion of breast cancer cells. (A) IC_{50} values of PPII on MDA-MB-231 cells. (B) The cytotoxicity of PPII on MDA-MB-231 cells for different durations. (C) PPII induced apoptosis in MDA-MB-231 cells. (D) Effects of PPII on the invasion of MDA-MB-231 cells. (E) Effects of PPII on the migration of MDA-MB-231 cells. The data are presented as the mean $\pm$ SEM (n=3). * $P<0.05$ and ** $P<0.01$ compared with the control. PPII, polyphyllin II.

the control group and 12.16% in the PPII group. This result indicated that PPII treatment could increase apoptosis in MDA-MB-231 cells ($P<0.05$; Fig. 3C).

Transwell and scratch assays were performed to evaluate the effects of PPII treatment on cell invasion and migration.

When counting the number of cells that migrated across the Transwell insert, the number of invasive cells in the control group was greater than that in the PPII group ($P<0.05$; Fig. 3D). The results of the scratch assay revealed that the migration rate of the control group was higher than that

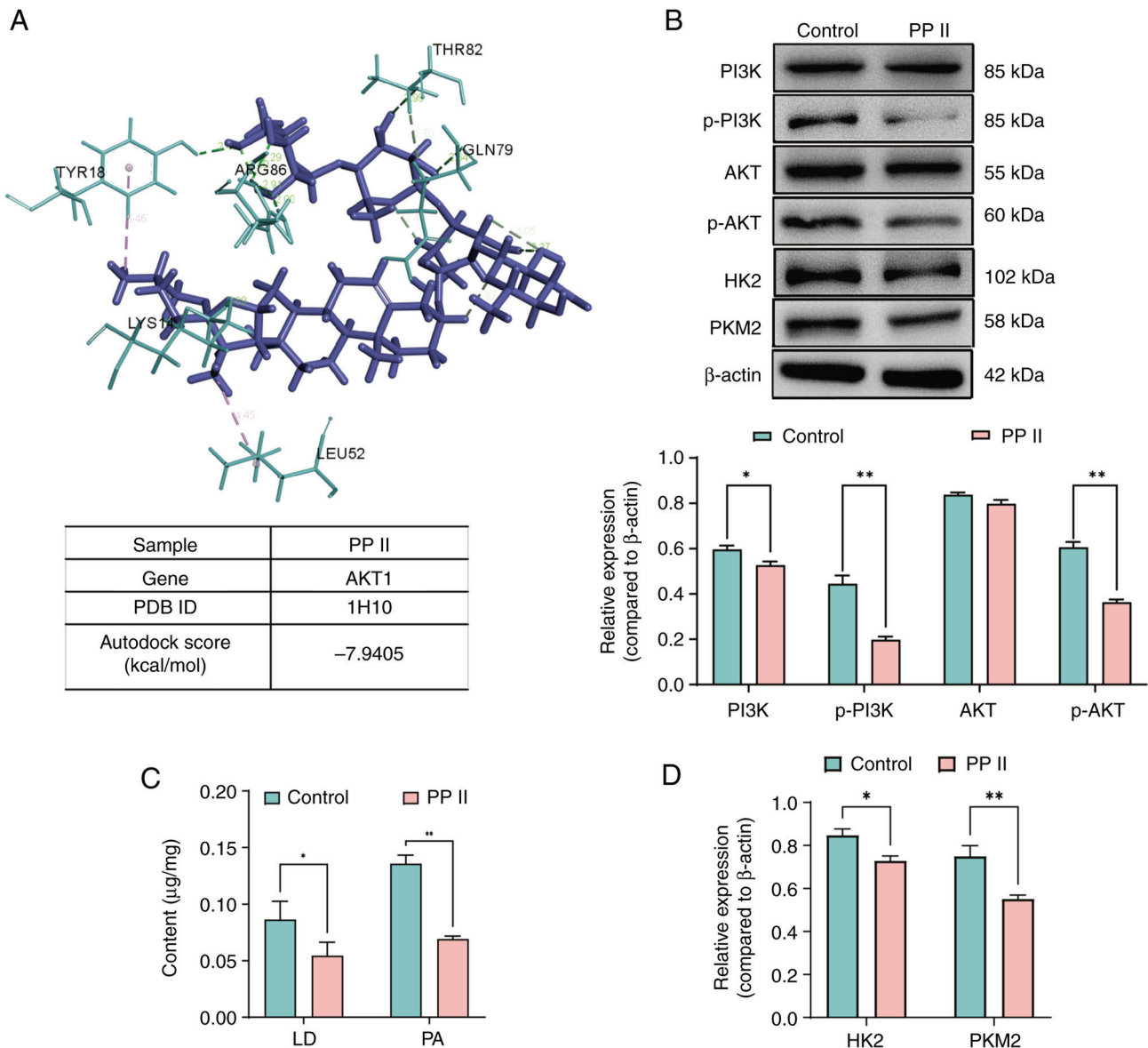


Figure 4. PPII blocked the PI3K/Akt pathway and suppressed aerobic glycolysis in breast cancer cells. (A) Visualization of molecular docking of PPII and Akt. (B) PPII inhibited the activation of PI3K/Akt pathway by suppression of PI3K, p-PI3K and p-AKT in breast cancer cells. (C) PPII inhibited the production of LD and PA, which are the metabolites of aerobic glycolysis. (D) PPII lowered the expression levels of the aerobic glycolysis enzymes HK2 and PKM2. The data are presented as the mean $\pm$ SEM (n=3). *P<0.05 and **P<0.01 compared with the control. LD, lactic acid; PA, pyruvate; PPII, polyphyllin II; HK2, hexokinase 2; PKM2, pyruvate kinase M2; p-, phosphorylated.

of the PPII group (P<0.01; Fig. 3E). These results demonstrated that PPII could inhibit the migration and invasion of MDA-MB-231 cells.

PPII blocks the PI3K/Akt signaling pathway. KEGG enrichment analysis of the targets identified by network pharmacology prediction methods demonstrated that the effect of PRS on breast cancer was closely related to the PI3K/Akt signaling pathway, which is an important signaling pathway for tumor development, and can regulate cell survival, metastasis and metabolism (33). To investigate the effect of PPII on the PI3K/Akt signaling pathway in breast cancer cells, the binding of PPII to expected critical targets in the PI3K/Akt signaling pathway was evaluated by molecular docking analysis. The binding of PPII to Akt was evaluated. PyMol was used, and the binding energy

between PPII and Akt was -7.9405 kcal/mol. The molecular docking stability is associated with the binding energy, and it is generally considered that a binding energy <-5 kcal/mol represents stable binding (34). Therefore, the results of the analysis suggested that the binding of PPII to Akt is stable. The binding sites were visualized and the results showed that the binding of PPII and Akt involved hydrogen bond interactions with TYR18, ARG86, THR82, GLN79, LYS14 and LEU52 (Fig. 4A). These docking results indicated that PPII binds to the target via hydrogen bonding. In summary, the KEGG results were further supported by molecular docking analysis results, and PPII likely exerts its anticancer effect via the PI3K/Akt signaling pathway.

To verify the effect of PPII on PI3K/Akt signaling in breast cancer cells, the expression and phosphorylation levels of PI3K and Akt were detected by western blotting. The results

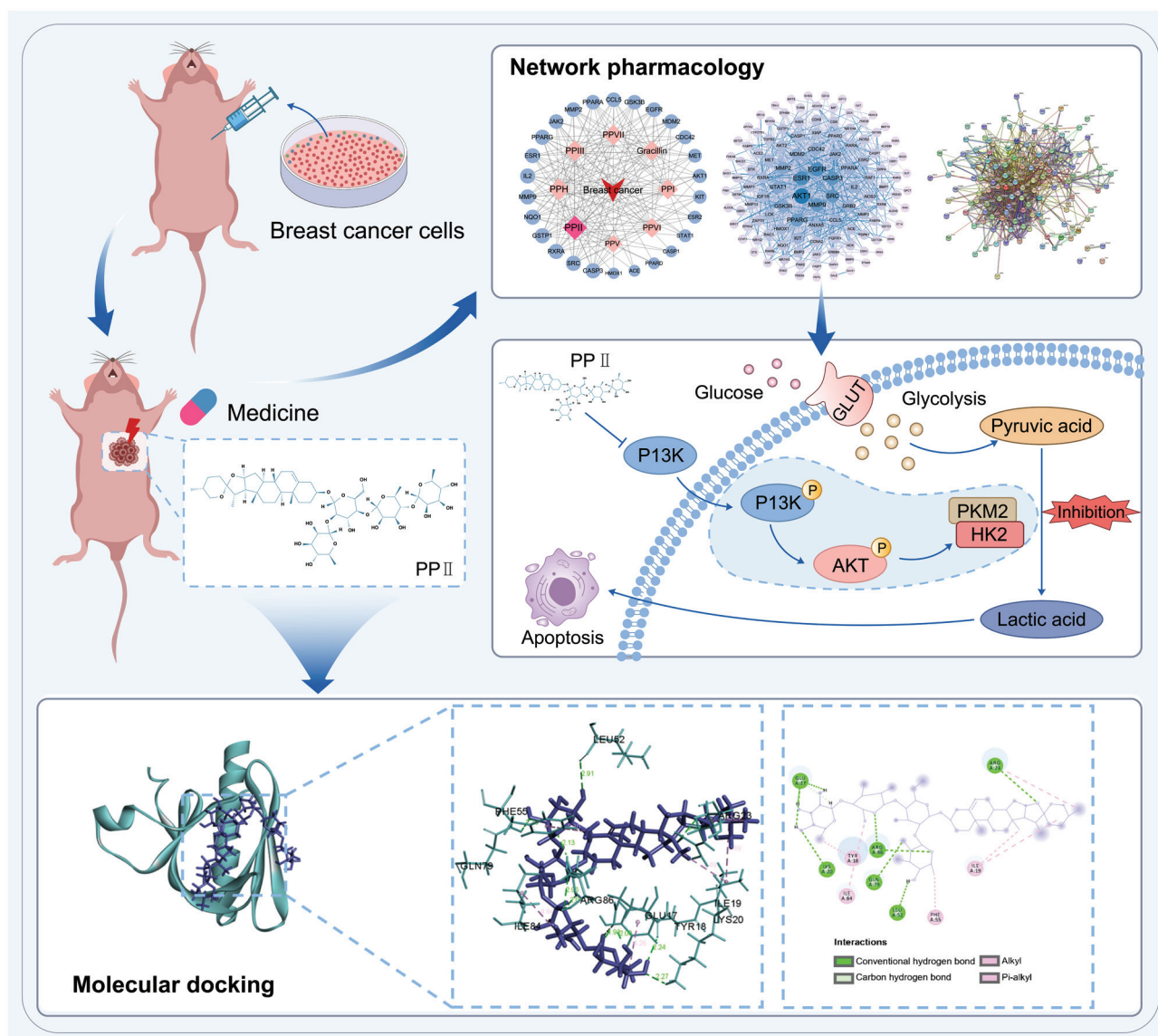


Figure 5. Proposed mechanism responsible for the anti-breast cancer effects of PPII. PPII inhibited aerobic glycolysis in breast cancer cells through the PI3K/Akt signaling pathway, thereby inhibiting breast cancer growth. PPII, polyphyllin II; HK2, hexokinase 2; PKM2, pyruvate kinase M2; p-, phosphorylated.

demonstrated that the levels of PI3K, p-PI3K and p-Akt were decreased (Fig. 4B). These results confirmed that PPII could inhibit breast cancer cell proliferation via the PI3K/Akt signaling pathway.

PPII suppresses aerobic glycolysis in breast cancer cells. Lactic acid and pyruvate are metabolites of aerobic glycolysis. To explore the effect of PPII on aerobic glycolysis in breast cancer cells, the levels of lactic acid and pyruvate were measured with the appropriate detection kits. The results revealed that the level of lactic acid in the control group was 0.086 mmol/gprot and that of pyruvate was 0.136 $\mu\text{g}/\text{mg prot}$. In the PPII group, the lactic acid level was 0.054 mmol/gprot and the pyruvate level was 0.069 $\mu\text{g}/\text{mgprot}$ (Fig. 4C). This indicated that PPII inhibited the production of lactic acid and pyruvate in breast cancer cells.

To further verify the effect of PPII on aerobic glycolysis in breast cancer, the expression levels of the key enzymes involved in aerobic glycolysis, namely HK2 and PKM2, were

evaluated. The results indicated that the expression levels of HK2 and PKM2 were decreased (Fig. 4D). Overall, PPII was capable of inhibiting the expression of key enzymes and downregulating aerobic glycolysis.

Discussion

Rhizoma Paridis refers to the dried root of *Paris polyphylla* Smith var. *yunnanensis* (Franch.) Hand.-Mazz. and *Paris polyphylla* Smith var. *chinensis* (Franch.) Hara (35). Traditional Chinese medicines containing Rhizoma Paridis have been extensively utilized for the treatment of cancer (9). PPII is one of the most prominent active components isolated from Rhizoma Paridis, exhibiting antitumor activity against a wide range of tumor cells such as melanoma, liver cancer, bladder cancer and colon-rectal cancer (36-41). Furthermore, PPII effectively inhibited the growth of melanoma xenograft tumors and also inhibited the invasion and migration of B16F10 cells through a mechanism related to autophagy

and epithelial-mesenchymal transition (EMT) (36). PPII can suppress the proliferation and metastasis of human head and neck squamous cell carcinoma cells by inhibiting the nitric oxide metabolism pathway (37). Additionally, PPII inhibits the proliferation and induces the apoptosis of HepG2 and BEL7402 cells, and low doses of PPII also restrain hepatocellular carcinoma cell migration and invasion (38). Furthermore, PPII inhibits the proliferation of NCI-H460 and NCI-H520 cells, and activates cell apoptosis and autophagy (39). PPII might inhibit the migration and invasion of bladder cancer cells by regulating the expression of EMT-related genes and matrix metalloproteinases (40). PPII also reduced the size of colorectal cancer xenograft tumors, induced apoptosis, and inhibited colony formation in HT29 and HCT116 cells (41). In the present study, a total of 26 core targets of PRS for breast cancer treatment were identified through network pharmacology analysis. Furthermore, PPII was capable of mediating more targets compared with other saponins. Thus, PRS could be utilized to treat breast cancer, and PPII is the core component of the treatment. In *in vivo* experiments, the rate of tumor volume growth in the PPII group was decreased and the tumor weight inhibition rate was as high as 35.74%. Immunohistochemistry indicated that PPII reduced the positive expression of Ki67 and enhanced the expression of Caspase 3, suggesting that PPII achieved the anti-breast cancer effect by suppressing the proliferation of tumor cells and promoting their apoptosis. Furthermore, *in vitro* MDA-MB-231 cell experiments also demonstrated that PPII possessed robust anti-breast cancer cell activity, promoted the apoptosis rate of breast cancer cells, and inhibited migration and invasion.

KEGG enrichment analysis of the core targets of PRS for breast cancer revealed that the effect of PRS on breast cancer was closely associated with the PI3K/Akt signaling pathway. Clinical studies have demonstrated that dysregulation of the PI3K/Akt signaling pathway is particularly prevalent in breast cancer (42-44). The PI3K/Akt signaling pathway is the core signaling axis implicated in the transduction of multiple signaling pathways, and abnormal activation of this pathway assumes a crucial role in cell survival, proliferation, growth and metabolism (33). *In vitro* and *in vivo* experiments have indicated that targeting of the PI3K/Akt signaling pathway was able to inhibit breast cancer proliferation (45,46). The present study revealed that the levels of PI3K, p-PI3K and p-Akt in breast cancer cells were diminished following PPII treatment, suggesting that PPII could restrain the growth of breast cancer by modulating the PI3K/Akt signaling pathway.

Current studies have demonstrated that the PI3K/Akt signaling pathway is capable of inhibiting the growth of cancer by regulating the aerobic glycolysis pathway that cancer cells adopt to sustain rapid proliferation (47,48). Hexokinase (HK) and pyruvate kinase (PK) are rate-limiting enzymes in the process of aerobic glycolysis and their expression changes largely influence the progression of cancer cells (49). Among the four subtypes of HK, HK2 is the key enzyme catalyzing the first step of glycolysis (50). While PK is the key enzyme catalyzing the last step of glycolysis, among the four isoforms of PK, PKM2 is the main type expressed and upregulated in cancer (51). Targeting HK2 and PKM2 can inhibit the growth of breast cancer (52,53). In the present study, PPII reduced the production of pyruvate and lactic acid, which are the aerobic

glycolysis metabolites in breast cancer cells (54), and lowered the expression levels of the aerobic glycolysis enzymes HK2 and PKM2. Consequently, it reduces the aerobic glycolysis of breast cancer cells and inhibits the growth of breast cancer.

The present study revealed that PPII could reduce the expression of enzymes in the glycolysis pathway and the generation of pyruvate and lactic acid by influencing the PI3K/Akt signaling pathway, blunting the aerobic glycolysis of breast cancer cells, thereby promoting cell apoptosis, inhibiting cancer proliferation, invasion and migration (Fig. 5). The present study provides a basis for the clinical application of PPII.

Acknowledgments

Not applicable.

Funding

No funding was received.

Availability of data and materials

The data generated in the present study are included in the figures of this article.

Authors' contributions

JG conceptualized the study, carried out the study methodology and supervised the project. WM carried out the experiments and data curation, wrote the original draft of the manuscript, reviewed and edited the manuscript and performed the formal analysis. ZW carried out scheme design, statistical analysis and data interpretation. YO participated in the conception and design of the study, verified the feasibility of the study, reviewed the manuscript and performed project management. ZW and JG confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal experiments were carried out according to protocols authorized by The Shanghai University of Medicine and Health Sciences Animal Care and Use Committee (approval no. 2023-XKY-07-32092419880308342X; Shanghai, China) and in accordance with UK guidelines (23).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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