Article

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Rhamnazin Inhibits Malignant Progression of Prostate Cancer Cells via DPP4/JAK/STAT Signaling Pathway

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Background: Rhamnazin is a natural dimethoxyflavonoid compound and Rhamnazin has been reported to have antitumor activity. This work is performed to study the function of Rhamnazin in prostate cancer (PCa) cells and its mechanism.
Methods: Rhamnazin (20 μM) was used to treat 22Rv1 and C4-2B cells, and the cells treated with dimethyl sulfoxide (DMSO) were set as the control group. To investigate the role of dipeptidyl peptidase 4 (*DPP4*) in mediating the biological effects of Rhamnazin, *DPP4* overexpression plasmids were transfected into the PCa cell lines. Cell counting kit-8 method was employed to detect the proliferation; apoptosis and cell cycle were detected by flow cytometry. the target genes of Rhamnazin were predicted in Traditional Chinese Medicine Systems Pharmacology (TCMSP) database. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analysis of target genes of Rhamnazin was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID database). Human Protein Atlas database was utilized to identify genes associated with poor PCa prognosis. GEPIA database was used to validate the expression and prognosis of *DPP4* in PCa. The LinkedOmics database was used to analyze the signaling pathway related to *DPP4* in PCa. Quantitative polymerase chain reaction was performed to detect *DPP4* mRNA levels. Western blot assays were performed to detect the expression levels of DPP4, phosphorylated (p-) Janus kinase 1 (JAK1) and p-signal transducer and activator of transcription 3 (STAT3).
Results: Functional assays confirmed that Rhamnazin inhibited the proliferation of PCa cells ($p < 0.05$), and promoted apoptosis ($p < 0.05$) and blocked the cell cycle progression ($p < 0.05$). In addition, Rhamnazin significantly inhibited the expression of *DPP4* ($p < 0.05$), and up-regulating *DPP4* reversed the effects of Rhamnazin ($p < 0.05$). It was further found that *DPP4* was associated with JAK/STAT signaling and Rhamnazin inhibited the expression of p-JAK1 ($p < 0.05$) and p-STAT3 ($p < 0.05$) through *DPP4*.
Conclusion: Rhamnazin has the potential to kill PCa cells via DPP4/JAK/STAT axis.

Keywords: prostate cancer; Rhamnazin; DPP4; JAK/STAT pathway

Background

Worldwide, prostate cancer (PCa) is a common malignancy, with about 1,600,000 new cases and about 366,000 deaths per year [1]. Its morbidity and mortality in China is rising significantly in recent years [2]. Most patients with advanced PCa have a poor prognosis due to tumor metastasis [3,4]. Therefore, it is important to develop new treatments and drugs for PCa.

In recent years, herbal medicines have been reported to significantly inhibit the progression of a variety of tumors, including PCa [5]. *G. lucidum* (*Ganoderma lucidum*) has been shown to inhibit angiogenesis of tumor by regulating MAPK and Akt signaling [6]. *S. baicalensis* (*Scutellaria baicalensis*) is found to inhibit the growth of PCa cells, and also inhibits prostate-specific antigen production and induces cell cycle arrest; *in vivo* experiments demonstrate that S. baicalensis significantly reduces tumor volume [7]. It is also found that *Ganoderma tsugae* ethanol extract reduces cell cycle protein expression in PCa cells by regulating PI3K/Akt and MAPK/ERK, and it also causes cell cycle arrest and induces cystein-dependent apoptosis [8].

Rhamnazin is a natural flavonoid obtained from some plants (e.g., *Physalis alkekengi L. var. franchetii*) [9]. Rhamnazin is demonstrated to have anti-angiogenic activity and tumor-suppressive properties [10]. Specifically, it potently represses the activity of vascular endothelial growth factor receptor (VEGF) signaling by suppressing the phosphorylation of vascular endothelial growth factor receptor 2 (*VEGFR2*) [10]. A recent work also reports that Rhamnazin represses the expression of programmed cell death 1 ligand 1 (PD-L1, *CD274*) of lung cancer cells, suggesting it may help block the immune evasion of cancer cells [11]. In some developing countries including China, *Physalis alkekengi L. var. Franchetii* is used as alternative drug in cancer treatment. However, the role of Rhamnazin in PCa is blurred. Herein, PCa cell lines were treated with Rhamnazin to study the effects of Rhamnazin on PCa. Then, we screened the target genes of Rhamnazin through bioinformatics and preliminarily explored the mechanism by which Rhamnazin kills PCa cells.

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Materials and Methods

Cell Culture and Rhamnazin Preparation

22Rv1 and C4-2B cells (American Type Culture Collection) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 µg/mL penicillin and 100 U/mL streptomycins (Gibco). Mycoplasma test was also performed to confirm that there was no cell contamination. The authenticity of the cells were verified by STR profile, to confirm that the cells were not mixed with other cell strains. The dishes were maintained at 37 °C in an incubator containing 5% CO_2. Rhamnazin ($C_{17}H_{14}O_7$, Molecular weight: 330.29, purity ≥99%, Cat. 93815, Sigma) was dissolved in dimethyl sulfoxide (DMSO, Sigma), and diluted with medium to treat PCa cells.

Quantitative Polymerase Chain Reaction (qPCR) Experiment

Total RNA was extracted from 22Rv1 and C4-2B cells using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using Reverse Transcription Kit (GeneCopoeia). Subsequently, qPCR analysis was conducted using GoTaq qPCR Master Mix (Promega), Dipeptidyl peptidase 4 (*DPP4*) relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method with *GAPDH* expression as a control. The primer sequences are as follows: *DPP4* (Forward, 5′-AAGGTTCTTCTGGGACTGC-3′; Reverse, 5′-TGTAGCATCATCTGTGCCT-3′); *GAPDH* (Forward, 5′-GGGTGTGAAACCATGAGAAGT-3′, Reverse, 5′-CAGTGATGGCATGGACTGTG-3′).

Cell Transfection

The *DPP4* overexpression plasmid (DPP4) was constructed by VectorBuilder, and when cell fusion reached 70–80%, the above plasmid was transfected into 22Rv1 and C4-2B cells using Lipofectamine 3000 (Invitrogen).

Cell Counting Kit-8(CCK-8) Assay

The CCK-8 kit (KeyGene BioTECH) was used to detect cell proliferation. 22Rv1 and C4-2B cells were inoculated in 96-well plates at 5000 cells per well and incubated for 2 h at 37 °C with 5 % CO_2 after 0, 24, 48, 72 h of cell culture by adding 10 µl CCK-8 reagent. A microplate reader (Bio-Rad) was adopted to detect optical density (OD) values at 450 nm per well. Cell inhibition rate was calculated based on the OD values, with the cells treated with DMSO as the controls.

Cell Cycle Analysis

22Rv1 and C4-2B cells were washed with phosphate buffer saline (PBS) and subsequently fixed in 75% prechilled ethanol at –20 °C for 24 h. Next, 22Rv1 and C4-2B cells were washed twice with PBS and stained with propidium iodide (PI) by cycletest plus DNA kit (BD Biosciences) for 30 min. Cellular DNA content will then be measured by flow cytometry (BD Biosciences), and the plots were generated by Cell Quest Modfit software (Verity Software House, Topsham, ME, USA).

Detection of Apoptosis

22Rv1 and C4-2B cell apoptosis was examined using a Annexin V-FITC apoptosis detection kit (BD Biosciences). Cells were collected after Rhamnazin treatment and then resuspended in 1×binding buffer, followed by staining in the darkness. After washing, the stained cells were detected using a flow cytometer (ACCURI C6 PLUS, BD Biosciences). Flowjo software (TreeStar) was for gating and data analyzing.

Western Blot Assay

Proteins were extracted from 22Rv1 and C4-2B cells using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology), and proteins were quantified using a bicinchoninic acid (BCA) kit (KeyGene BioTECH). Forty µg of protein samples were added to each well and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to separate the proteins, which were subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). PVDF membranes were blocked with 5% skimmed milk for 2 h at room temperature, followed by incubation of the membranes with primary antibody at 4 °C overnight. The primary antibodies are as follows: DPP4 (ab231973, 1:1000, Abcam), phosphorylated (p-) Janus kinase 1 (JAK1) (ab138005, 1:1000, Abcam), JAK1 (ab133666, 1:1000. Abcam), p-transducer and activator of transcription 3 (STAT3) (ab267373, 1:1000, Abcam), STAT3 (ab109085, 1:1000, Abcam), β-actin (ab8227, 1:1000, Abcam). The membrane was then incubated with secondary antibody (ab6721, 1:2000, Abcam) at room temperature for 2 h. The protein strips were shown using ECL luminescence reagent (Amersham Pharmacia Biotech) and relative levels were analyzed using ImageJ software (V.1.8.0) (NIH, Bethesda, MD, USA) was used for analysis.

Bioinformatics Analysis

To obtain the potential target genes of Rhamnazin, Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (old.tcmsp-e.com/tcmsp.php) was searched. Gene enrichment analysis [gene ontology (GO) or Kyoto Encyclopedia of Genes and Genomes (KEGG)] was performed with Database for Annotation, Visualization and Integrated Discovery (DAVID database) (https://david.ncifcrf.gov/). Human protein atlas (HPA) database (www.proteinatlas.org/) was used for evaluating DPP4 protein expression in human tissue samples. Gene Expression Profiling Interactive Analysis (GEPIA) database (gepia.cancer-pku.cn/) was applied for the analysis of *DPP4* expression and survival analysis based on RNA sequencing data based on The Cancer Genome Atlas (TCGA).

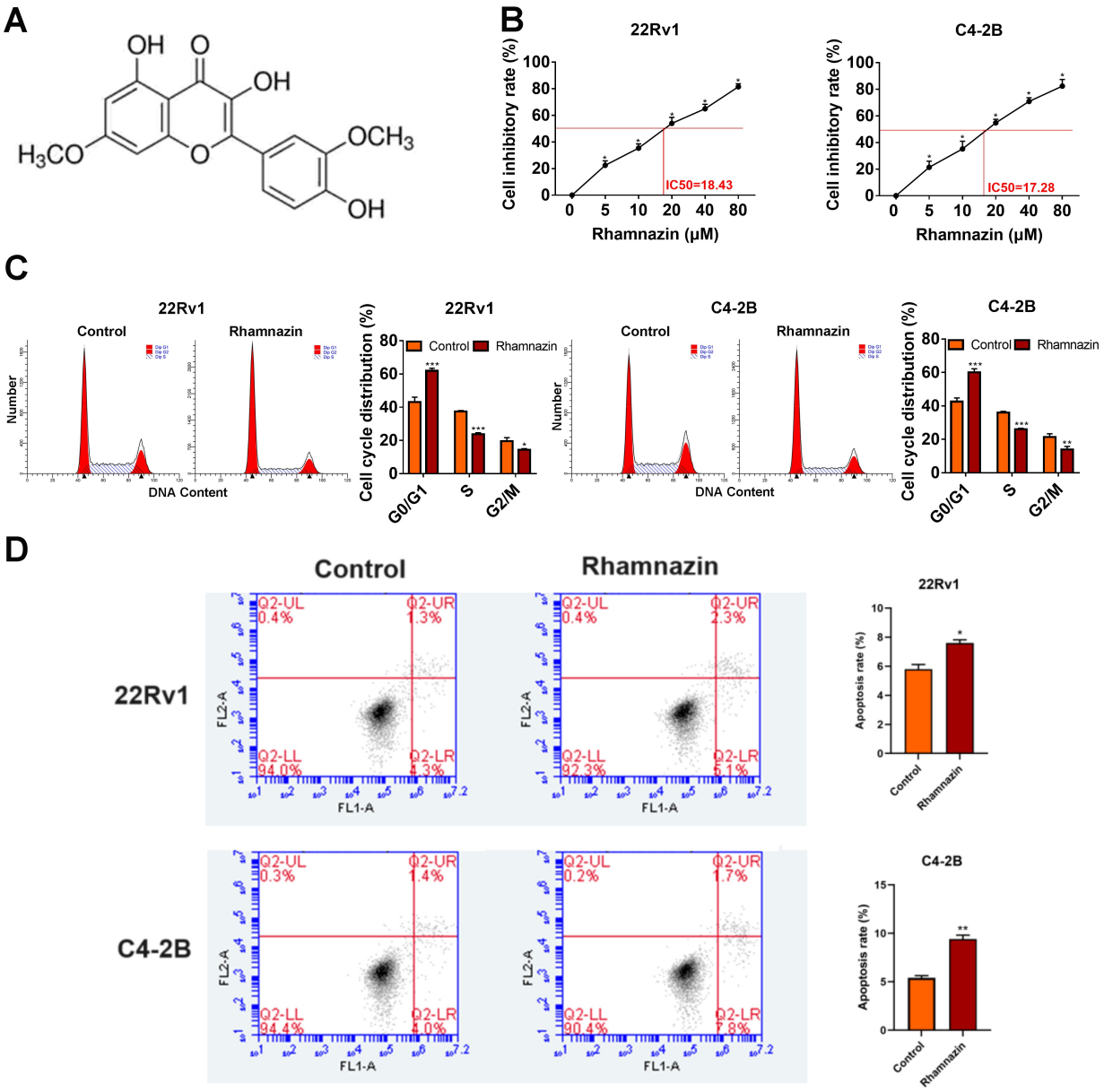

Fig. 1. Rhamnazin inhibits the proliferation of prostate cancer (PCa) cells and promotes apoptosis. (A) Chemical structure formula of Rhamnazin. (B) Cell counting kit-8 (CCK-8) assay was used to detect the inhibition rate of PCa cells (22Rv1 and C4-2B) treated with different concentrations of Rhamnazin. (C) Flow cytometry was employed to analyze the cell cycle distribution after Rhamnazin treatment of PCa cells. (D) The apoptosis of PCa cells treated with Rhamnazin was detected by flow cytometry. All of the experiments were performed in triplicated and repeated for at least 3 times. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Statistical Analysis

SPSS software (version 24.0) (IBM, Chicago, IL, USA) was applied for statistical analysis, and results, with normal distribution, were expressed as mean ± standard deviation, and comparisons between groups were made using Student's *t*-test or one-way ANOVA with Tukey's post-hoc test. $p < 0.05$ signified statistical significance.

Results

Rhamnazin Shows the Potential to Kill PCa Cells

First of all, we treated 22Rv1 and C4-2B cells with Rhamnazin (Fig. 1A) (0, 5, 10, 20, 40, and 80) for 24 h. Subsequently, we measured the cell viability. As shown, cell inhibition rate was significantly higher after Rhamnazin treatment, which indicated that Rhamnazin suppressed PCa cell proliferation in a dose-dependent manner, and the IC_{50} values of treated 22Rv1 and C4-2B cells were 18.43 μM and

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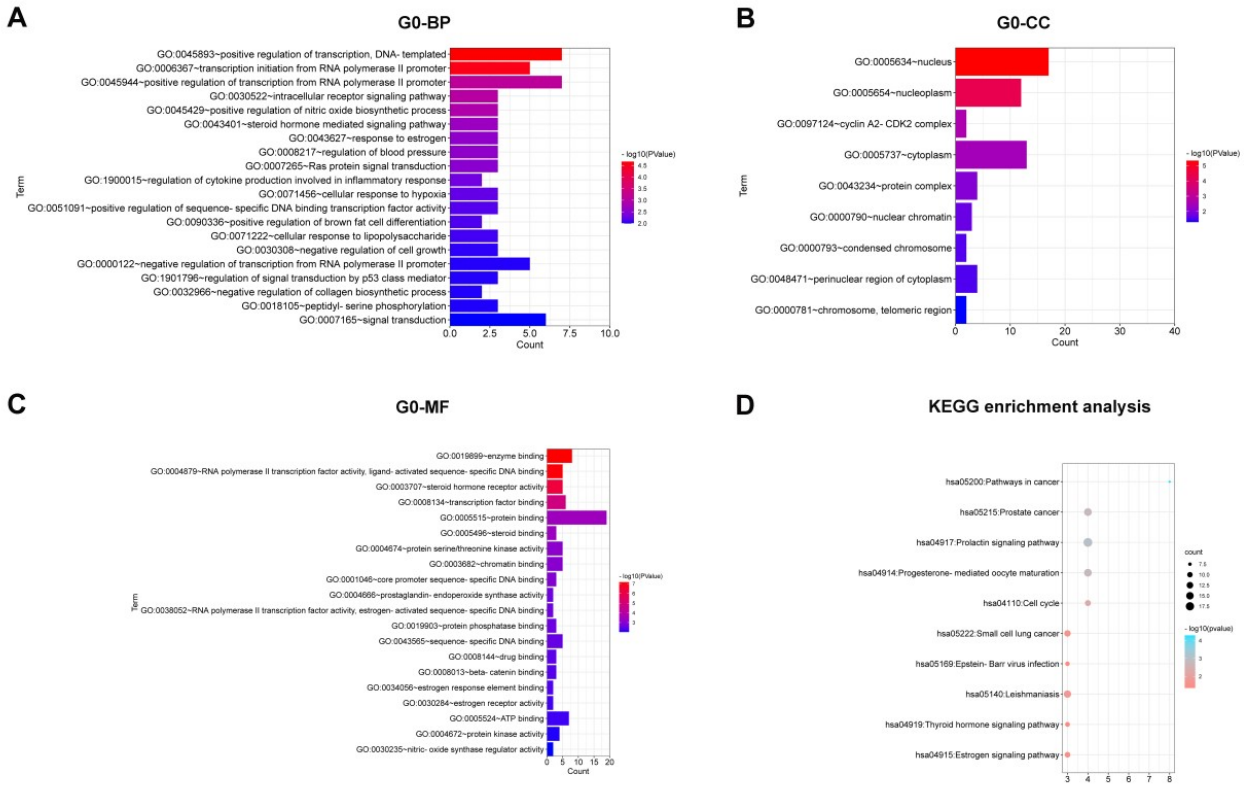

Fig. 2. Functional enrichment analysis of Rhamnazin target genes. (A–C) The target genes of Rhamnazin were identified in the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database and gene ontology (GO) analysis of the target genes was performed using the DAVID database. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed on the target genes of Rhamnazin using the Database for Annotation, Visualization and Integrated Discovery (DAVID database), and a bubble chart was plotted.

17.28 μM, respectively (Fig. 1B). Therefore, we used 20 μM as the experimental concentration of Rhamnazin. Subsequent flow cytometry results showed a larger number of PCa cells in G0/G1 phase and a significantly reduced number of PCa cells in S and G2/M phases in the Rhamnazin-treated group ($p < 0.05$) (Fig. 1C). Additionally, the apoptosis rate was higher in the Rhamnazin-treated group ($p < 0.05$) (Fig. 1D). The above results suggested that Rhamnazin has the potential to kill 22Rv1 and C4-2B cells.

Functional Enrichment Analysis of Rhamnazin Target Genes

Then, TCMSP database was used to identify the target genes of Rhamnazin, and the results showed that there are 23 target genes of Rhamnazin. We then performed functional enrichment analysis of these 23 target genes through the DAVID database, and GO analysis revealed significant enrichment in: Biological Process (BP): negative regulation of cell growth, regulation of blood pressure, cellular response to hypoxia (Fig. 2A); Cellular Component (CC): nucleus, cytoplasm, condensed chromosome (Fig. 2B); Molecular Function(MF): prostaglandin-endoperoxide synthase activity, nitric-oxide synthase regulator activity, drug binding (Fig. 2C). KEGG enrichment analysis manifested that the targets of Rhamnazin were mainly enriched in the Cell cycle, Estrogen signaling pathway, Thyroid hormone signaling pathway (Fig. 2D).

Rhamnazin Inhibits the Expression of DPP4

The poor prognosis genes in PCa were identified using the HPA database, and 133 prognosis-related genes were subsequently plotted in a Venn diagram against the 23 target genes of Rhamnazin in the TCMSP database, showing that *DPP4* was located within the intersection (Fig. 3A). Immunohistochemical staining in the HPA database showed that *DPP4* expression was promoted in PCa (Fig. 3B). Meanwhile, the data of the GEPIA database demonstrated that *DPP4* was not only up-regulated in PCa tissues ($p < 0.05$) but also associated with worse prognosis in PCa patients ($p = 0.025$) (Fig. 3C–D). To verify whether Rhamnazin can regulate *DPP4* expression in PCa cells, we treated the cells with Rhamnazin and examined the expression level of DPP4 protein using Western blot assay, and as expected, the *DPP4* expression level was reduced in the Rhamnazin-treated group ($p < 0.05$) (Fig. 3E–F).

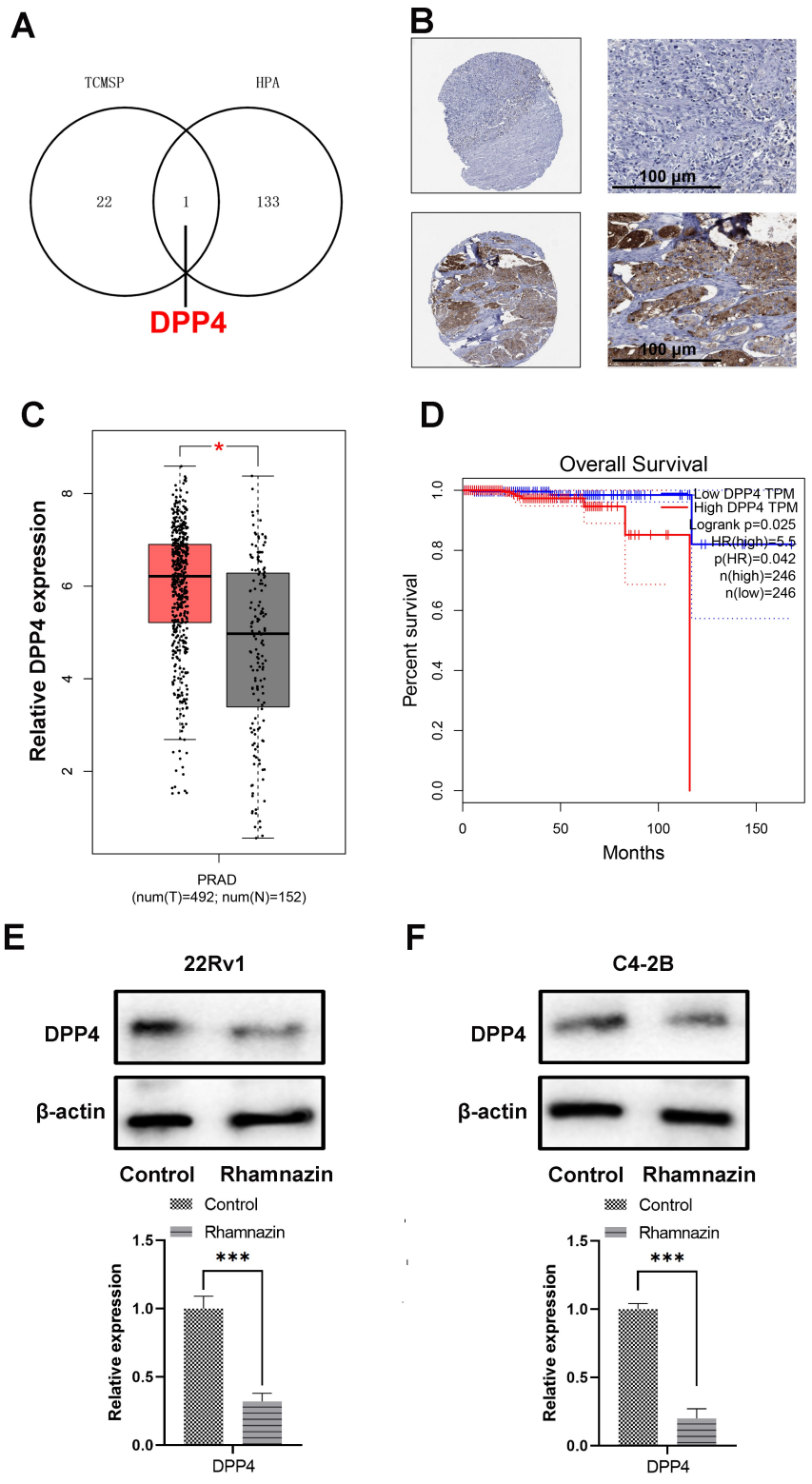

Fig. 3. Rhamnazin inhibits the expression of DPP4. (A) The Rhamnazin target genes screened in the TCMSP database and the poor prognosis genes for PCa in the HPA database were plotted in a Venn diagram. (B) HPA database showed the representative immunohistochemical staining of DPP4 in PCa tissues (below) and non-cancerous tissues (upper). (C) Characteristics of *DPP4* expression in normal and PCa tissues were presented by GEPIA database. (D) Analysis of overall survival of PCa patients with different *DPP4* expression in the GEPIA database. (E–F) Western blot assay was performed to detect *DPP4* expression in PCa cells after Rhamnazin treatment. All of the experiments were performed in triplicated and repeated for at least 3 times. ***$p < 0.001$.

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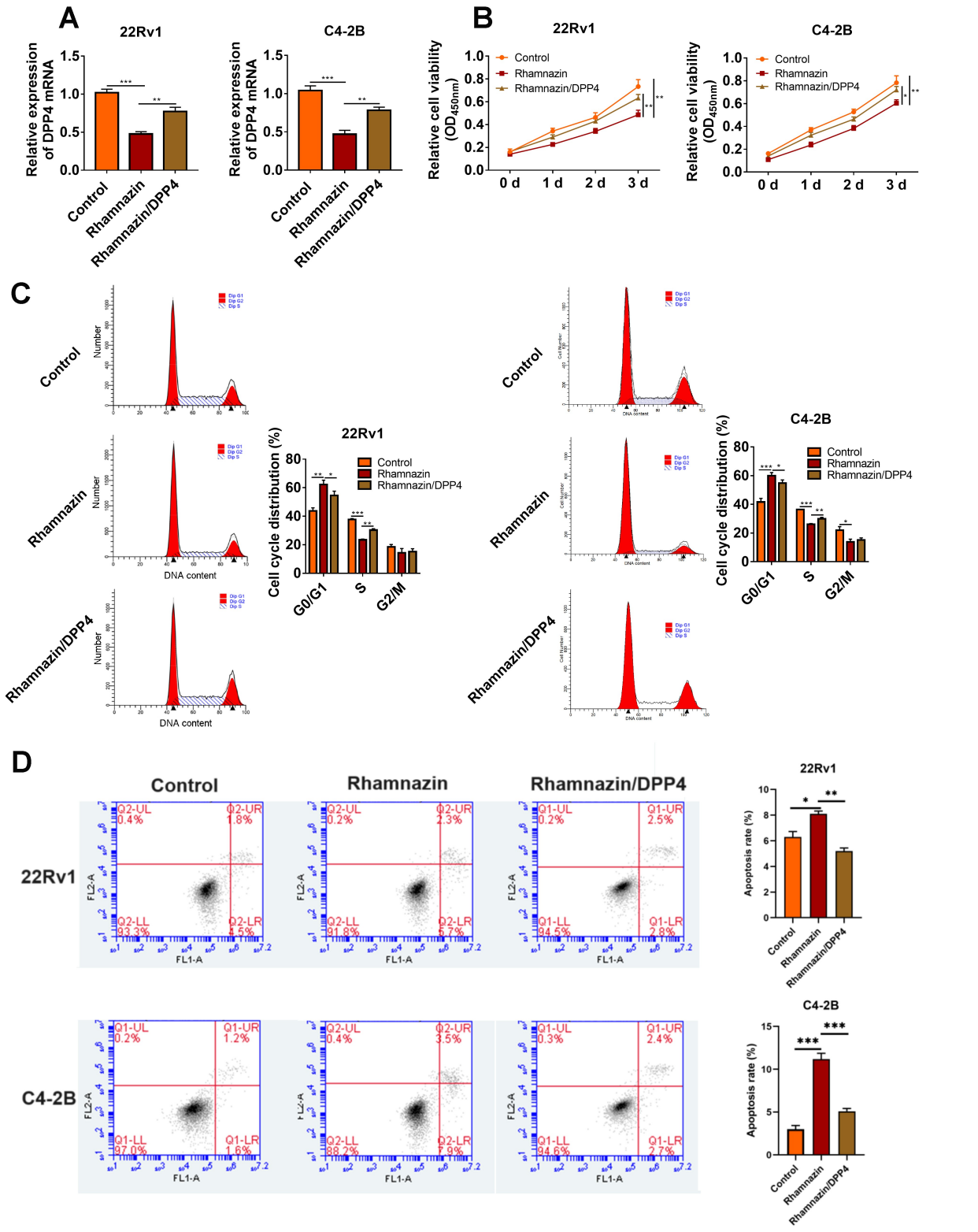

Fig. 4. Exogenous DPP4 expression reverses the effect of Rhamnazin on PCa cells. (A) The *DPP4* overexpression plasmid was transfected into 22Rv1 and C4-2B, and the efficiency was verified by qPCR assay. (B) CCK-8 assay was used to detect the viability of 22Rv1 and C4-2B cells treated with Rhamnazin and transfected with *DPP4* overexpression plasmid. (C) Flow cytometry analysis of 22Rv1 and C4-2B cell cycle distribution after Rhamnazin treatment and transfection of *DPP4* overexpression plasmid. (D) Flow cytometry was utilized to detect the apoptosis of 22Rv1 and C4-2B cells treated with Rhamnazin and transfected with *DPP4* overexpression plasmid. All of the experiments were performed in triplicated and repeated for at least 3 times. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

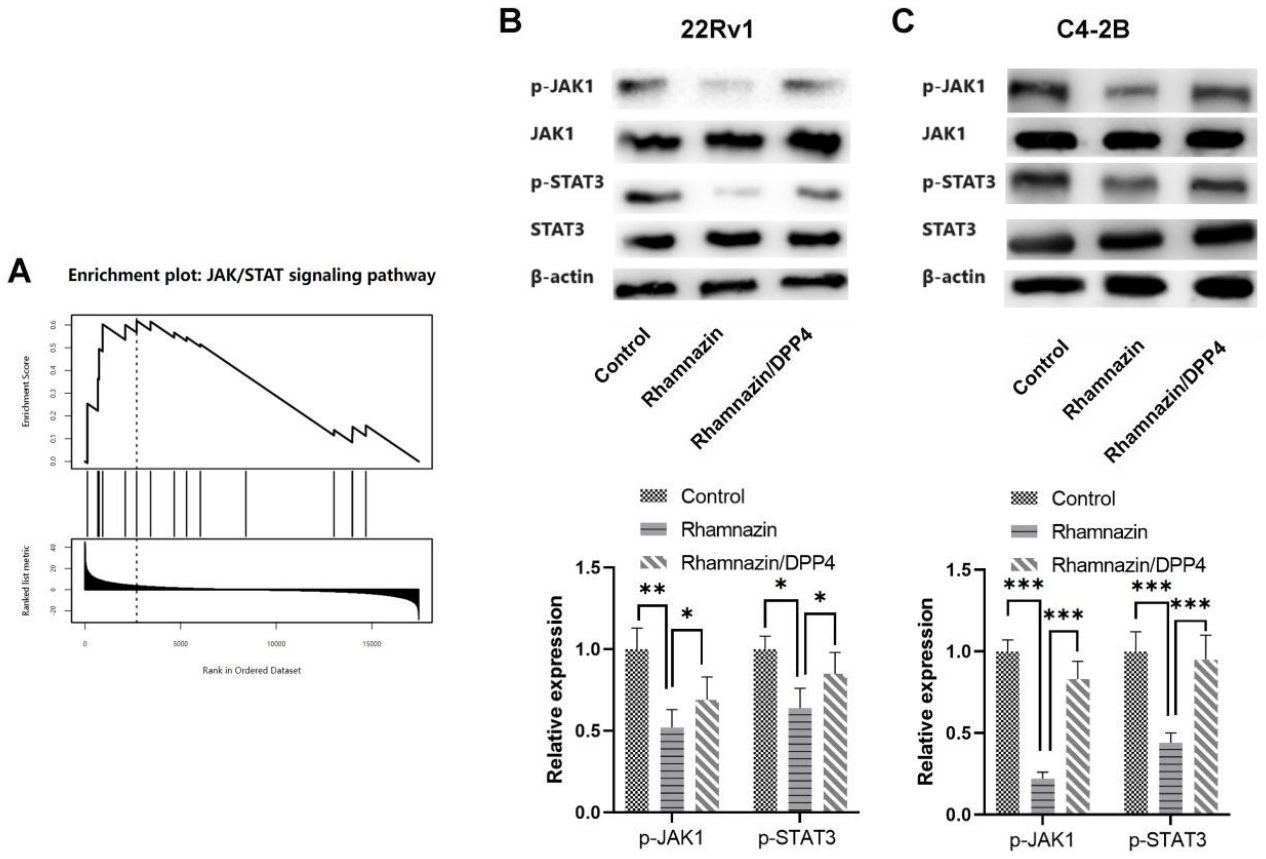

Fig. 5. Rhamnazin regulates the JAK/STAT signaling pathway through *DPP4*. (A) JAK/STAT3 was predicted by GSEA to be regulated by *DPP4* in PCa. (B–C) Western blot assay was performed to detect the expression levels of p-JAK-1 and p-STAT3 in PCa cells after Rhamnazin treatment and transfection with *DPP4* overexpression plasmids. All of the experiments were performed in triplicated and repeated for at least 3 times. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Exogenous DPP4 Reverses the Effect of Rhamnazin on PCa Cells

To verify whether Rhamnazin functions on PCa cells via *DPP4*, given that Rhamnazin regulates the expression of *DPP4*, we transfected the *DPP4* overexpression plasmid into 22Rv1 and C4-2B cells to construct a *DPP4* overexpression model. qPCR experiments validated successful transfection (Fig. 4A). As shown, cell viability was remarkably higher in the group transfected with *DPP4* overexpression plasmid compared to the Rhamnazin-treated group ($p < 0.05$) (Fig. 4B); the number of PCa cells in G0/G1 phase was significantly declined after *DPP4* overexpression plasmid transfection compared to the Rhamnazin-treated group ($p < 0.05$) (Fig. 4C); exogenous *DPP4* significantly reversed the apoptosis-promoting effect of Rhamnazin on 22Rv1 and C4-2B cells ($p < 0.05$) (Fig. 4D). These results supported our hypothesis.

Rhamnazin Regulates the JAK/STAT Signaling Pathway through DPP4

We used gene set enrichment analysis (GSEA) with LinkedOmics database to analyze the pathways probably regulated by *DPP4* in PCa, and as shown high *DPP4* expression was positively correlated with the JAK/STAT signaling pathway (Fig. 5A). We then treated PCa cells with Rhamnazin and transfected with *DPP4* overexpression plasmids, and it showed p-JAK-1 and p-STAT3 expression levels were significantly reduced in the Rhamnazin-treated cells ($p < 0.05$), and overexpression of *DPP4* partially attenuated this inhibitory effect ($p < 0.05$) (Fig. 5B–C).

Discussion

Rhamnazin is a natural compound that can be isolated from various herbal plants [12,13]. Reportedly, Rhamnazin has a series of pharmacological effects including antioxidant, antibacterial, anti-inflammatory and antitumor effects [14]. In rat model with acute lung injury, Rhamnazin exerts antioxidant and anti-inflammatory functions [15]. In mice model, Rhamnazin is found to improve traumatic brain injury by ameliorating neural death, oxidative stress and inflammatory responses [16]. It is also reported that Rhamnazin, as a new inhibitor of *VEGFR2*, significantly inhibits the angiogenesis ability of human umbilical vascular endothelial cells (HUVECs). Additionally, Rhamnazin sig-

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nificantly suppresses the viability of MDA-MB-231 cells [10]. In this work, we confirmed that Rhamnazin inhibited the viability of PCa cells, and this is the first study to resolve the role of Rhamnazin in killing PCa cells.

To analyze the mechanism of Rhamnazin in PCa cells, we identified the target genes of Rhamnazin through the TCMSP database and performed bioinformatics analysis. The obtained target genes were closely associated with cell growth, the cell cycle pathway. We then identified prognosis-related genes in PCa through the HPA database, and a comprehensive analysis revealed that *DPP4*, a target gene of Rhamnazin, was associated with poor prognosis in PCa. DPP4 is a 110 kDa type II transmembrane protein that is expressed in a multiple kinds of cells, and mainly involved in the regulation of glucose and insulin metabolism, and immune response; it is abnormally expressed in some pathological processes including but not limited to inflammation, diabetes, obesity, and cancer [17]. In thyroid carcinoma, *DPP4* is highly expressed in thyroid carcinoma tissues, and it is regarded as a target for immunotherapy [18]. Reportedly, *DPP4* inhibitors significantly prolong the survival of PCa patients [19], suggesting targeting *DPP4* shows exciting clinical application prospect. Here, we found that Rhamnazin significantly inhibited *DPP4* expression, and overexpression of *DPP4* partially attenuated the effects of Rhamnazin on PCa cells, suggesting that Rhamnazin could exert tumor-suppressive effects in PCa cells by inhibiting the expression of *DPP4*. It's worth noting that some studies hold the view that *DPP4* functions as a tumor suppressor in PCa. A previous study demonstrates that *DPP4*, promoted by androgen receptor, mediates the degradation of multiple growth factors; by this mechanism, downregulation of *DPP4* contributes to the resistance of PCa cells to androgen deprivation therapy [20]. This study suggests that the specific role of *DPP4* in PCa awaits further research, and the other targets of Rhamnazin remain to be explored and identified.

Next, we analyzed the pathways probably regulated by *DPP4* in PCa and found that the high *DPP4* expression and JAK/STAT pathway had a positive correlation. JAK/STAT axis is a ubiquitously expressed intracellular pathway that participates in diverse key biochemical processes [21]. STAT3 is a member of STAT protein family. It is stimulated by upstream signaling and growth factors, and can be activated by Janus kinase 2 (JAK2); after STAT3 is phosphorylated in Ser727 and Tyr705, it will be translocated to the nucleus to play a transcriptional regulatory role [22]. JAK/STAT signaling is also one of the deciders in PCa progression. Give an example, benzo(a)pyrene, as an environmental carcinogen, facilitates abnormal proliferation and induces gene mutation via activating JAK2/STAT3 signaling [23]. Tripartite motif containing 66 (*TRIM66*) promotes PCa cell development through JAK/STAT axis [24]. Furthermore, *G. lucidum* inhibits the viability of PCa cells by suppressing JAK1/STAT3 activity [25]. In this work, our *in vitro* data proved that Rhamnazin significantly inhibited the expression levels of p-JAK-1 and p-STAT3, and overexpression of *DPP4* partially counteracted this inhibition, suggesting that Rhamnazin could modulate DPP4/JAK/STAT axis.

This study is the first reports about the tumor-suppressive property of Rhamnazin in treating prostate cancer, and we preliminarily explained the downstream mechanism - Rhamnazin represses the activation of DPP4/JAK/STAT signaling. This is main novelty of the present work. Of course there are some shortcomings in the present work. First of all, only *in vitro* models are applied in the study, and an animal model may further validate the tumor-suppressive role for Rhamnazin. Secondly, the detailed mechanism by which Rhamnzin suppresses DPP4/JAK/STAT pathway is still obscure, and in the future, technologies such as molecular docking may help further clarify this issues. Last but not least, the regulatory effects of Rhamazin on the other malignant biological behaviors such as chemoresistance and resistance to androgen deprivation therapy.

Conclusion

In conclusion, our work confirms that Rhamnazin suppresses PCa cell proliferation and promotes apoptosis by suppressing *DPP4* expression and suppressing expression levels of p-JAK and p-STAT3, thereby inhibiting PCa development. Rhamnazin may be used as a potentially effective natural drug for PCa treatment and also provides new insights into the clinical treatment of PCa.

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