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Original Research

Rubimaillin suppresses proliferation, migration and invasion of prostate cancer cells via the Notch-1/MMP signaling pathway

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Abstract: This study was aimed to explore the effect of Rubimaillin on the survival, migration, and invasion of prostate cancer cell lines DU145 and PC3, and its mechanism. CCK-8 method was used to detect the effects of different concentrations of rubs (0, 5, 10, 20, 40 and 80 μM) on the activity of DU145 cells and PC3 cells. Transwell cell lab test was used to detect the migration and invasion of cells. Western blot was used to detect Notch-1, MMP-2, MMP-9 and Hes-1 protein levels. The CCK-8 assay showed that Rub inhibited the activity of Du145 and PC3 cells in a concentration dependent manner. When the concentration reached 40 μM, the inhibition reached the maximum. After Rub intervention, the migration and invasion ability of Du145 and PC3 cells decreased significantly, while the expression levels of Notch-1, MMP-2, MMP-9 and Hes-1 protein decreased significantly. Rub can inhibit the growth, migration and invasion of prostate cancer cell line DU145 and PC3. The mechanism may be related to the inhibition of Notch-1, MMP-2, MMP-9 and Hes-1 by Rub.

Key words: Rubimaillin; Prostate cancer cells; Cell migration; Cell invasion; Notch-1/MMP signaling pathway.

Introduction

Prostate cancer is a common malignant tumor of the urinary system in men. Its incidence ranks first amongst all cancers in European and American countries. It is the second most fatal malignant tumor in men of European and American descent. There are more than 220,000 newly diagnosed cases and 27,000 prostate cancer-related deaths every year. In addition, although the incidence of prostate cancer is lower in developed countries than in developing countries, the incidence of the disease has increased significantly in developed countries as a result of improvements in standards of living and environmental changes (1-4). Androgen deprivation therapy (ADT), first proposed by Charles Huggins and others in their pioneering research, was very effective in controlling metastatic prostate cancer (5). However, most patients may develop resistance (6, 7). Subsequently, docetaxel (DTX) was used as the standard treatment for drug-resistant patients (8). In 2015, two trials demonstrated that DTX and ADT improve the survival of men with untreated metastatic prostate cancer (9, 10). So far, DTX chemotherapy is regarded as the main treatment option for prostate cancer. However, resistance to DTX still occurs after multiple treatment cycles, usually leading to treatment failure. The mechanism involved in DTX resistance is very complex and has not been fully understood. Therefore, it is necessary to evolve newer and more effective drugs for treating PCA.

Traditional Chinese medicine (TCM) plays an important role in the treatment of cancer because of its natural components, low toxicity and minimal side effects, multiple-drug targets, wide treatment orientation, and the advantages of non-susceptibility to drug resistance. Research has shown that traditional Chinese medicine improves the immune function of cancer patients, reduces the side effects of radiotherapy and chemotherapy, and improves the tolerance of cancer patients to radiotherapy and chemotherapy, thereby improving cancer treatment outcomes.

Rubia cordifolia L. is a plant of the Rubiaceae family which is widely distributed world-wide. It is used in treating ailments associated with blood circulation, hemostasis, *meridian circulation*, cough and phlegm, and it has significant therapeutic effect on tumors (11, 12). The main phytochemical and medicinal components of Rubia are water-soluble cyclohexylpeptides, fat-soluble anthraquinone and its glycosides, reducing naphthoquinone and its glycosides, polysaccharides, terpenes, microelements, alizarin, and oxalic acid (13). Studies have shown that rubimaillin (Rub) is the main bioactive component of *Rubia cordifolia*. Rubimaillin possesses various biological characteristics such as anti-colon cancer, anti-mutation, anti-leukemia, anti-inflammatory and anti-allergic effects (14-16). At present, there are no existing reports in the literature about the effect of Rub on prostate cancer cells. Therefore, this study was aimed at investigating the effect of Rub on prostate cancer cell line viability, migration and invasion, and the pathways involved.

Materials and Methods

Experimental reagents

Rubimaillin (purity > 98%, Shanghai TITAN Technology Co. Ltd.), heavy trypsin, MTT, Ripa lysate, sample buffer, BCA protein concentration test kit and all secondary antibodies were purchased from Biyuntian Biotechnology Co. Ltd. Phosphate buffer was purchased from Chongqing Haiyun Biotechnology Co. Ltd.; dimethyl sulfoxide was supplied by Chongqing Haiyun Biotechnology Co. Ltd., while sodium dodecyl sulfate (SDS), concentrated hydrochloric acid, Tween 20, sodium chloride, Tris buffer, acrylamide and ammonium persulfate were purchased from Biotechnology (Shanghai) Co. Ltd.. Luminous solution was bought from Beijing Yingen Biotechnology Co. Ltd.; skimmed milk powder was purchased from Inner Mongolia Yili Industrial Group Co. Ltd., while Notch-1, MMP-2, MMP-9, HES-1 and GAPDH antibodies were products of American cell Signaling Technology.

Cell culture

Prostate cancer cell lines DU145 and PC3 were purchased from Shanghai Bangjing Industry Co. Ltd., RPMI1640 culture medium and fetal bovine serum were obtained from Hyclone Company (USA). The DU145 and PC3 cells were inoculated in 25-ml glass flasks and cultured in RPMI1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin mixture at 37°C, 5% CO_2 and saturated humidity. When the cells grow to 80% confluence, they are passed on 1:3, and the cells in the logarithmic growth period were used for the subsequent experiments.

CCK-8 assay for cell proliferation

The DU145 and PC3 cells were subjected to trypsin digestion. After counting the cells, the cell concentration was adjusted to 5×10^4 - 8×10^4 cells/ml with complete cell culture medium containing 10% serum and 1% penicillin/streptomycin mixtures. Then, the cells were inoculated into 96-well plates at a volume of 100uL single cell suspension per well, followed by culturing in incubators at 37°C, 5% CO_2 and saturated humidity. When the cells were attached to the wall on the next day, they were treated with varying Rub concentrations i.e. 5, 10, 20, 40 and 80 μM, each in a final volume of 100uL. Each concentration was set up in triplicate wells. Then, 10 μL CCK-8 was added to each well after 12, 24 and 48 hours in an incubator at 37°C, 5% CO_2 and saturated humidity, followed by culturing for 4h. Thereafter, the culture medium was discarded. The absorbance (OD) of each well was measured at 450 nm (17).

Transwell cell migration experiment

Cells at logarithmic growth period were subjected to digestion with 0.25% trypsin, and the supernatant was discarded after centrifugation. The cell density was adjusted to 2×10^8 cells/L, and 200 μL cell suspension was added to the upper chamber, while 500 μL RPMI-1640 medium containing 10% fetal bovine serum was added to the lower chamber. The cells in Transwell chambers were incubated at 37°C and 5% CO_2 for 24 hours. Then, the cells in upper chamber were wiped off with cotton swab, and the cells were fixed in 4% POM for 15 min, washed 3 times with PBS, and stained with 0.1% crystal violet for 3 min. This was followed by rinsing with distilled water, drying, observation and photographing under the microscope, and cell counting.

Transwell chamber invasion experiment

Before the experiment, 50 mg/L Matrigel was diluted with diluent (1:8), and then coated on the upper chamber surface of Transwell chamber bottom membrane, followed by drying at 4°C. The last step followed the same procedure as indicated in "Transwell cell migration experiment".

Western blotting for protein expressions

After incubation of cells with different concentrations of Rub for 48 hours, the cells were lysed with RIPA cell lysate, and the total protein was extracted and quantified using BCA protein assay test kit. Equal amounts of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis, and the protein bands were transferred to nitrocellulose membrane. Non-specific binding was blocked with 5% skimmed milk at room temperature for 1 hour, followed by incubation with anti-bodies for Notch-1, MMP-2 and MMP-9 (1:1000 dilution); as well as HES-1 and GAPDH (1:500 dilution) at room temperature for 1 hour. Then, the membrane was washed and incubated with horse radish- conjugated secondary antibody (1:1000 dilution) at room temperature for 1 hour. The blots were subjected to enhanced chemiluminescence for color development, and scanned with image scanner. The gray value of film and image strip was determined using ImageJ software.

Statistical analysis

Measurement data are expressed as mean ± standard deviation (SD). Single factor analysis of variance was used for comparison between groups, while the Dixon's *q*-test was used for the comparison between multiple means. All statistical analyses were done with GraphPad prism 7.0 statistical software. Values of $p < 0.05$ were statistically significant.

Results

Effect of different concentrations of Rub on the viabilities of DU145 cells and PC3 cells

Compared with the control group, the viabilities of DU145 and PC3 cells were dose-dependently decreased by Rub. However, as shown in Figure 1, when Rub concentration was 40 μM, there was no significant difference in cell viability, when compared with the viability at Rub concentration of 80 μM after 48 hours of cell culture. Therefore, in subsequent experiments, the cells

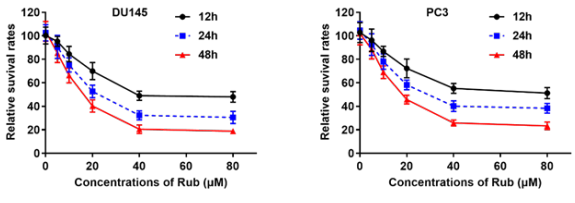

Figure 1. Effect of Rub on the viabilities of prostate cancer lines DU145 and PC3. Data are shown as mean ± SD (n =3).

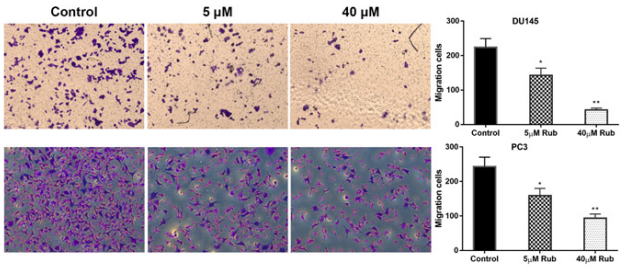

Figure 2. Effect of Rub on the migration ability of prostate cancer cell lines Du145 and PC3. Data are shown as mean ± SD (n = 3). *p < 0. 05, **p < 0. 01, compared with control group.

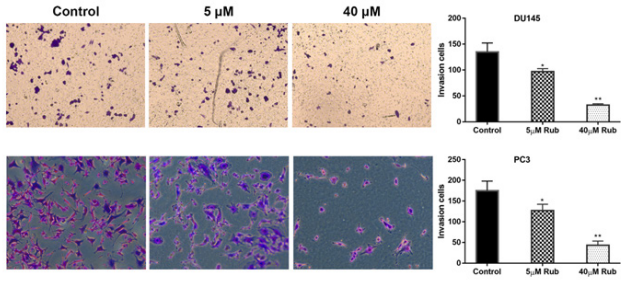

Figure 3. Effect of Rub on the invasion abilities of the prostate cancer cell lines Du145 and PC3. Data are shown as mean ± SD (n = 3). *P < 0. 05, **p < 0. 01, compared withs Control group.

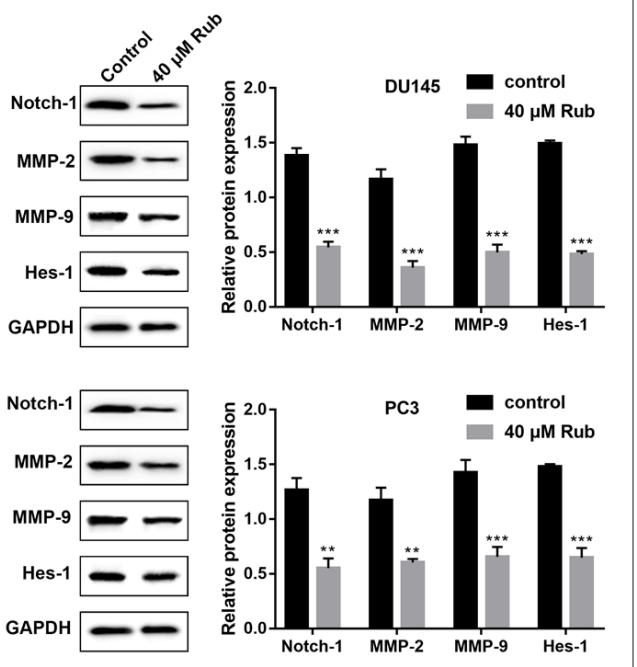

Figure 4. Effect of Rub on the protein expressions of Notch-1, MMP-2, MMP-9 and Hes-1 in Du145 cells and PC3 cells. Results are shown as mean ± SD (n = 3. *P <0.05, *** P <0.01, ***P <0.0001 compared with Control group.

were cultured for 48 hours with 40 μM Rub.

Effects of Rub on migration of DU145 and PC3 cells

The results of Transwell migration experiment showed that 48 hours after treatment with 5 μM or 40 μM Rub, the migration abilities of DU145 and PC3 cells were significantly and dose-dependently smaller than that of the blank control group, as shown in Figure 2.

Effect of Rub on the invasiveness of DU145 and PC3 cells

The results of Transwell invasion experiment showed that 48 hours after treatment with 5 μM or 40 μM Rub, the invasion abilities of D145 and PC3 cell lines were significantly and concentration-dependently smaller than those of the control group. These results are shown in Figure 3.

Effect of Rub on the expression levels of Notch-1/MMP pathway

Results from Western blot showed that the protein expression levels of Notch-1, MMP-2, MMP-9 and HES-1 in DU145 cells and PC3 cells were significantly and concentration-dependently lower than those in the blank control group after 48 hours of treatment with Rub at doses of 5 μM and 40 μM, as shown in Figure 4.

Discussion

Bioactive monomers in natural products have been widely studied in the research for antitumor drugs (18, 19). Some studies have shown that Rub exerts targeted effect in tumor therapy, as well as antitumor effect *in vitro* (16).

In this study, it was found that the viability of prostate cancer DU145 cells was inhibited concentration-dependently by Rub. When the concentration of Rub was 40 μM, the cell survival was only 18.81 ± 2.16%, suggesting that Rub has a significant inhibitory effect on prostate cancer cells.

The Notch receptor is a highly conserved transmembrane protein which is closely related to cell proliferation, differentiation and apoptosis. Dysplasia refers to the generation of interstitial collagen in cancer (20). Matrix metalloproteinase (MMP) is a zinc-dependent proteolytic enzyme which is closely related to tumor metastasis, vascular remodeling and heteromorphic hyperplasia (21, 22). Collagen micronodule deposition has been reported in biopsy and prostatectomy samples from PCA (23). In addition, a strong deplastic activity was observed in intermediate and advanced PCA, as well as increased expressions of vimentin, IGF-1, MMP-2, FGF-2, c-Myc, PSCA and Era (24). It has been reported that stroma can differentiate between benign and malignant prostate tissues (25). These findings highlight the importance of extracellular collagen as a microenvironment component that enhances metastasis. In other words, changes in extracellular matrix (ECM) due to tissue fibrosis promote tumor progression (26). The results obtained in this study suggest that MMP-2 and MMP-9 are important members of the MMPs family (27-29).

It has been reported that MMP-2 promotes tumor cell invasion by hydrolyzing the extracellular matrix (30). Studies have demonstrated that the expression of MMP-9 is increased in cervical cancer, renal cell carcinoma and esophageal squamous cell carcinoma, thereby enhancing the invasion and metastasis of tumor cells through degradation of ECM (31). Local metastasis of tumor is one of the important causes of high mortality in cancer. Loss of integrity of basement membrane and ECM is closely related to tumor metastasis (32, 33). As the downstream target gene of Notch-1 pathway, Hes-1plays an important role in the Notch-1 pathway. In

this study, it was found that the migration and invasion ability of DU145 cells decreased after treatment with Rub, which may be related to the down-regulation of Notch-1, MMP-2, MMP-9 and HES-1 protein expression levels. Thus, it can be reasonably speculated that Rub may play a regulatory role in the growth, migration and invasion of prostate cancer cells through inhibition of the Notch-1 pathway.

In conclusion, this study has demonstrated that Rub inhibits the viability, migration and invasion of prostate cancer cells. Thus, Rub has potential for use as adjuvant therapy for prostate cancer patients. This study provides experimental basis for the application of Rub in prostate cancer management, and also provides new ideas for follow-up drug development.

Acknowledgements
None.

Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Wenbin Liu; Fangzhen Cai, Shuxin Guo, Sihuai Huang, Jianwei Li, Wenbin Liu collected and analysed the data; Fangzhen Cai wrote the text and all authors have read and approved the text prior to publication.

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