Original Paper

Crosstalk of NF- κ B/P65 and LncRNA HOTAIR-Mediated Repression of MUC1 Expression Contribute to Synergistic Inhibition of Castration-Resistant Prostate Cancer by Polyphyllin 1–Enzalutamide Combination Treatment

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Key Words

PPI • Enzalutamide • CRPC cells • NF- κ B/p65 • HOTAIR • MUC1

Abstract

Background/Aims: Polyphyllin I (PPI), one of the steroidal saponins in *Paris polyphylla*, reportedly exhibits antitumor effects. However, the detailed mechanism underlying PPI, particularly in enhancing the effect of the androgen receptor inhibitor enzalutamide in controlling castration-resistant prostate cancer (CRPC) has not been explored. **Methods:** Cell viability and cell cycle distribution were measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and flow cytometry assays, respectively. Long non-coding RNA (lncRNA) HOX transcript antisense RNA (HOTAIR) expression was measured by quantitative real time-PCR (qRT-PCR). Western blot analysis was performed to determine the protein expression levels of MUC1, p65, and p50. Silencing of HOTAIR was evaluated using the siRNA procedure. The promoter activity of the MUC1 gene was determined using Secrete-Pair Dual Luminescence Assay Kit. Exogenous expression of HOTAIR, p65, and MUC1 was conducted by transient transfection assay. A xenograft tumor model in nude mice was used to further evaluate the effect of the combination of PPI and enzalutamide *in vivo*. **Results:** We showed that PPI significantly inhibited growth and induced cell cycle arrest in CRPC cells. PPI also decreased p65 and MUC1 protein expression and reduced HOTAIR expression. Exogenously expressed p65 resisted the PPI-inhibited expression of HOTAIR, whereas silenced HOTAIR reduced MUC1 protein but exerted no effect on the expression of p65 and p50 proteins. Conversely, exogenously expressed HOTAIR resisted the PPI-inhibited MUC1 protein expression, and excessive expression of MUC1 antagonized the PPI-inhibited cell growth. S. Xiang and P. Zou contributed equally to this work.

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Notably, PPI combined with enzalutamide exerted a synergistic effect. Consistent with this finding, PPI inhibited tumor growth, HOTAIR expression, as well as p65 and MUC1 protein expressions *in vivo*. **Conclusions:** Our results indicate that PPI inhibits the growth of CRPC cells by inhibiting p65 protein and concomitantly reducing HOTAIR expression, thereby suppressing MUC1 gene expression. The novel regulatory interaction of p65 and HOTAIR converge in the inhibition of MUC1 expression and overall PPI response. The combination of PPI and enzalutamide exhibits synergy. This study reveals a novel mechanism underlying the synergistic inhibitory effect of PPI and enzalutamide on the growth of CRPC cells.

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Introduction

Prostate cancer is the most common cancer in men worldwide. With a wide array of clinical presentations and high prevalence, the identification and characterization of the malignancy have increasingly improved through risk stratification and advances in multiple management options [1, 2]. Androgen deprivation therapy (ADT) for advanced prostate cancer exerts superior anticancer effects; regardless, most cases of prostate cancer treated with ADT eventually recurs to develop into castration-resistant prostate cancer (CRPC) [3, 4]. Persistent androgen signaling is functionally significant in CRPC and is a validated therapeutic target. However, other pathways also contribute to castration resistance. With limited therapeutic strategies available, treatment of CRPC remains considerably difficult [5, 6]. New drugs that prolong overall survival in patients with metastatic CRPC have been approved in recent years. These drugs include enzalutamide, a nonsteroidal second-generation androgen receptor inhibitor with heightened binding specificity that has been shown to be an effective and well-tolerated therapeutic option in both post-docetaxel and chemotherapy-naïve settings [7]. This compound provides a substantial survival benefit; however, not all patients with CRPC respond to this treatment [8]. This challenge has led to the pursuit of new treatment modalities. Thus, the search for novel options to enhance therapeutic efficacy in patients with CRPC is strongly urged.

Polyphyllin I (PPI), an active component obtained from *Rhizoma Paridis* saponins, has been reported to exhibit various biological activities against many types of cancer [9–12]. In one study, PPI inhibited tumor growth and induced apoptosis in human breast cancer cells, and these effects were enhanced by activating the PTEN-induced kinase 1 signaling pathway [11]. PPI also demonstrated anti-tumor activity on ovarian cancer *in vivo* via modulation of caspase-9, c-Jun, and secreted glycoprotein Wnt5a [13]. Moreover, PPI suppressed growth, induced apoptosis, and inhibited the invasion and migration of osteosarcoma cells by inactivating the Wnt/ β -catenin pathway both *in vitro* and *in vivo* [14]. We previously showed that PPI inhibited the growth of human lung cancer cells via stress-activated protein kinase/c-Jun N-terminal kinase-mediated inhibition of the protein expression of the nuclear factor- κ B (NF- κ B) subunit p65 and DNA methyltransferase 1. This action reduced the enhancement of zeste homolog 2 gene expression in human lung cancer cells [15]. These results suggest the therapeutic potential of PPI in cancer. However, few studies have been conducted on the effects of PPI on prostate cancer, particularly CRPC. Thus, the underlying molecular mechanisms for targeting prostate cancer by PPI remain largely undetermined.

Long noncoding RNAs (lncRNAs) represent a novel class of noncoding RNAs that are longer than 200 nucleotides without protein-coding potential. Accumulating evidence has shown that lncRNAs are dysregulated and involved in various biological processes, such as proliferation, apoptosis, mobility, and invasion [16–19]. Among these lncRNAs, lncRNA HOX transcript antisense intergenic RNA (HOTAIR) acts as an oncogene and is aberrantly expressed in multiple types of cancer. It has also been considered as a predictive factor for poor prognosis in various types of cancer, including prostate cancer [20, 21]. Another study showed that HOTAIR regulated multiple signaling and targets via distinct mechanisms, thereby promoting cancer growth and progression [19, 20]. In addition, HOTAIR has been shown to contribute to prostate cancer risk. A recent study demonstrated the association

between HOTAIR polymorphisms (rs12826786, rs1899663, and rs4759314) and risk of prostate cancer in patients with prostate cancer, suggesting that HOTAIR is a risk locus for prostate cancer [22]. Thus, HOTAIR can be regarded as a potential independent prognostic biomarker and a potential therapeutic target for treatment of prostate cancer.

The NF- κ B transcriptional pathway is involved in several fundamental biological processes, such as immunity, inflammation, angiogenesis, tumorigenesis, and cell proliferation/progression. NF- κ B is activated by various extracellular signals and regulates the expression of different genes [23, 24]. Constitutive NF- κ B activity was observed in a large number of human cancers and shown to play an important role in the process from inflammation to carcinogenesis; constitutive NF- κ B activation is a targeted intervention in various malignancies, including prostate cancer [25, 26]. Limited studies have reported the potential role of NF- κ B in mediating the therapeutic effects of PPI [27]. Therefore, the molecular mechanism of this transcription factor complex involved in the anti-cancer effects of PPI remains undetermined.

Mucin 1 (MUC1) is a heterodimeric transmembrane protein that is aberrantly expressed and plays a central role in malignant transformation and disease evolution, such as cell proliferation, survival, self-renewal, and metastatic invasion [28, 29]. Consequently, MUC1 has been a target for tumor therapeutic approaches. Reports have demonstrated the prognostic value of MUC1 detection in patients with various types of cancer [30, 31]. A study also showed that high MUC1 expression in primary prostate tumor and/or lymph node metastases correlated with unfavorable tumor features and patient survival [32]. We previously showed that solamargine, a major steroidal alkaloid glycoside purified from *Solanum nigrum* L., inhibited the growth of CRPC cells via AMP-activated protein kinase α (AMPK α)-mediated inhibition of the NF- κ B subunit p65, followed by the reduction of MUC1 expression both *in vitro* and *in vivo* [33]. These findings indicated the potential role of this molecule in the occurrence and progression of prostate cancer. Limited information is available on the association between HOTAIR and MUC1; however, other studies have reported on the involvement of the links of HOTAIR and MUC1 to NF- κ B were found in oncogenic signaling pathways and tumor progression [34, 35].

In the present study, we further demonstrated that PPI inhibited the growth of CRPC cells via inhibition of NF- κ B/p65 protein expression and concomitant reduction of HOTAIR expression, hence the suppression of MUC1 expression.

Materials and Methods

Reagents and cell culture

Monoclonal antibodies specific to the NF- κ B subunits p65 and p50 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-MUC1 antibody, which detects full-length MUC1 and the 17 kDa C-terminal subunit (subunit beta), was provided by Abcam (ab109185, Cambridge, MA, USA). Lipofectamine 3000 was supplied by Life Technologies (AB & Invitrogen, Carlsbad, CA, USA). PPI was purchased from Chengdu Must Biotechnology (Chengdu, China). The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) power was purchased from Sigma-Aldrich (St. Louis, Mo, USA). The cell cycle staining kit was supplied by MultiSciences Biotech Co. (Hangzhou, China). Geneticin (G-418 Sulfate) was purchased from Life Technologies (Grand Island, NY, USA). D-luciferin was provided by PerkinElmer (Waltham, MA, USA). The CRPC cell lines DU145 and PC3 were obtained from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (Shanghai, China). Cells were grown in an F-12K (1:1) medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 μ g/mL streptomycin, and 100 U/mL penicillin. The DU145-Luc medium was added with G-418 sulfate at a concentration of 300 μ g/mL. When the cells reached 70%–75% confluence, they were digested with 0.25% trypsin for passage for subsequent experiments. PPI was dissolved in a small amount of dimethylsulfoxide [DMSO, maximum concentration, 0.1% (v/v)], which was then added to complete the cell culture medium. Cells treated with vehicle only (DMSO, 0.1% in medium) served as the control.

Cell viability assay

Cell viability was measured using the MTT method as described in a previous study [36]. The CRPC cells were seeded into a 96-well plate at 5×10^3 cells/well and incubated at 37 °C with 5% CO₂ for 24 h before they were treated with increasing concentrations of PPI for up to 72 h. In separate experiments, CRPC cells were transfected with the control, pCMV6-MUC1 for 24 h before the cells were exposed to PPI for an additional period of 24 h. Absorbance at 570 nm was determined using the ELISA reader (PerkinElmer, VICTOR X5, Waltham, MA, USA) to estimate MTT-formazan production after incubation for 24, 48, and 72 h. Cell viability (%) was calculated as follows: (absorbance of test sample/absorbance of control) $\times$ 100%. Combination-induced synergy was analyzed and expressed as a combination index (CI) by using CompuSyn (ComboSyn, Inc., Paramus, NJ, USA), following the method developed by Chou and Talalay [37].

Cell cycle analysis

This procedure was conducted as described in a previous study [38]. Cells were cultured in 6-well culture plates at 3×10^5 cells/well and then treated with increased doses of PPI for 24 h. The cells were washed and resuspended in PBS and then incubated with 0.05 mg 0.1% sodium citrate containing propidium iodide and 50 µg RNase for 1 h at room temperature. The cells were washed and subjected to FACSCalibur flow cytometric analysis (FC500, Beckman Coulter, FL, USA), and the proportions of cells within the G0/G1, S, and G2/M phases of the cell cycle were analyzed using the MultiCycle AV DNA Analysis software (Phoenix Flow Systems, Inc. San Diego, CA, USA).

Transient transfection assays

The procedure was conducted as described in a previous study [39]. Cells were seeded at a density of 2.5×10^5 cells/well in 6-well dishes and grown to 60% confluence. For each well, 2 µg of the control or wild-type pEZX-PG04-HOTAIR, or MUC1 promoter constructs (purchased from GeneCopoeia, Inc., Rockville, MD, USA) with or without 0.2 µg of the internal control secreted alkaline phosphatase (SEAP) were co-transfected into the cells with Lipofectamine 3000. The preparation of cell extracts and measurement of luciferase activities were determined using Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia, Inc., Rockville, MD, USA) and was normalized with SEAP activity within each sample. In a separate experiment, 2 µg of the control (pCMV6) and expression constructs of human HOTAIR and MUC1 genes obtained from OriGene Technologies, Inc. (Rockville, MD, USA), and the control (pCMV4) and p65 overexpression vectors obtained from Addgene (Plasmid #21966, Cambridge, MA, USA) [40] at a final concentration of 2 µg/mL were transfected into the cells with Lipofectamine 3000. Cells were incubated for 24 h at 37 °C and then treated with PPI with or without enzalutamide for the indicated time for all other experiments.

Quantitative real-time PCR (qRT-PCR)

Total RNA from cells was extracted using TRIzol Reagent (Invitrogen), and cDNA was synthesized with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). The primers used in this study were designed as follows: MUC1 forward 5'-ACGTCAGCGTGAGTGATGTG-3'; reverse 5'-GACAGACAGCCAAGGCAATG-3'; HOTAIR forward 5'-GGTAGAAAAAGCAACCACGAAGC-3'; HOTAIR reverse 5'-ACATAAACCTCTGTCTGTGAGTGCC-3'; GAPDH forward 5'-AAGCCTGCCGGTGACTAAC-3'; reverse 5'-GCGCCCAATACGACCAAATC-3'. QRT-PCR was performed in a 20 µL mixture containing 2 µL of the cDNA preparation, 10 µL 2 $\times$ SYBR Green Premix ExTaq, and 10 µM primers on an ABI 7500 Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA). The PCR conditions were as follows: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each sample was tested in triplicate. Threshold values were determined for each sample/primer pair, and the average and standard errors were calculated. The relative expression levels of MUC1 and HOTAIR were normalized to that of GAPDH. The data were analyzed using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method.

HOTAIR siRNA transfection

HOTAIR small interfering RNA (HOTAIR siRNA) and "scramble" siRNA (control siRNA) were purchased from GenePharma (GenePharma, Shanghai, China). The target sequences for HOTAIR siRNAs were as follows: si-HOTAIR1, 5'-GCCUCCUUAUAAGCUCGU-3', 5'-ACGAGCUUAUAAGGAAGGC-3'; si-HOTAIR2, 5'-CAAUAUAUCUGUUGGGCGU-3', 5'-ACGCCCAACAGAUUAUUG-3'; si-HOTAIR3, 5'-GGAAGCUCUUGAAGGUUCA-3', 5'-UGAACCUUCAAGAGCUUCC-3'. Cells (2×10^5 /well) were

transfected with a final concentration of 50 nM siRNAs targeting HOTAIR and control for 24 h by using Lipofectamine 3000 (Invitrogen, CA, USA) in accordance with the instructions provided by the manufacturer. Following the transfection procedures, cells were harvested for all other experiments.

Western blot analysis

The procedure was conducted as described in a previous study [36]. Whole cell lysates either from cytosol or nuclear portions, or both containing the same amount of protein were separated on SDS polyacrylamide gels. Isolation of cytoplasmic and nuclear portions was conducted using the Nuclear and Cytoplasmic Protein Extraction Kit according to the instructions provided by the manufacturer (Beyotime Institute of Biotechnology, Shanghai, China). Membranes (Millipore, Billerica, MA, USA) were incubated with p50, p65, and MUC1 (1:1000) antibodies for 2 h, washed and incubated with a secondary antibody raised against rabbit IgG conjugated to horseradish peroxidase (1:3000, Cell Signaling, Beverly, MA, USA), and transferred to a freshly prepared enhanced chemiluminescence solution (Immobilon Western; Millipore, Billerica, MA, USA). The signals were documented using the Gel Image System (Bio-Rad, Hercules, CA, USA). The software Image J (NIH, Bethesda, MD, USA) was used to compare the gray values between the proteins of interest and the internal control.

Prostate xenograft tumor model

Animal studies were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Guangdong Provincial Hospital of Chinese Medicine (Ethics Approval Number 2017037) and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). A total of 32 female nude mice (aged 4–6 weeks) purchased from Beijing Vital River Experimental Animal Co. Ltd. (Beijing, China) were maintained at the Animal Center of Guangdong Provincial Hospital of Chinese Medicine in a specific pathogen-free environment, with food and water provided. DU145 prostate cancer cells carrying the luciferase report gene (DU145-Luc, obtained from Guangzhou Land Technology Co., Guangzhou, China) (1×10^6 cells) in 100 μ L PBS were implanted via subcutaneous injection into the flanks of the mice. Xenografts were allowed to grow for over 1 week when the initial measurement was made with calipers. Mice were randomly divided into the control, PPI (3 mg/kg) [15], enzalutamide (10 mg/kg) [41, 42] or combination groups, which were given once a day via intraperitoneal injection for up to 27 days ($n=8$ /group).

For bioluminescence imaging (BLI), mice were anesthetized by inhalation of 2% isoflurane and substrate D-luciferin (Caliper Life Sciences, Hopkinton, MA, USA) was injected into the peritoneal cavity with a dose of 150 mg/kg in approximately 100 μ L solution. The intensity of the BLI signal was determined using the IVIS-200 Imaging System (Xenogen/Caliper, Alameda, CA, USA). Tumor volume measurements were calculated using the formula for an oblong sphere: $\text{volume} = (\text{width}^2 \times \text{length}) \times 2$. Quantification of bioluminescence was reported as photons/sec. The body weights of the mice were measured once a week. All mice were euthanized on day 27 in accordance with the Guide for the Care and Use of Laboratory Animals. The tumors were excised from the site of injection and processed for p65 and MUC1 proteins and HOTAIR expression levels by Western blot and qRT-PCR, respectively.

Statistical analysis

All data were expressed as mean $\pm$ SD of 3 independent experiments. Differences between groups were assessed by 1-way ANOVA, and the significance of differences between particular treatment groups was analyzed using Tukey's Multiple Comparison Test for multiple comparisons involved using GraphPad Prism version 5.0 (GraphPad Software, Inc. La Jolla, CA, USA). The results in most graphs were presented relative to the control. Asterisks in figures indicate significant differences in experimental groups, compared with the corresponding control condition. Statistical significance was set at P value < 0.05 .

Results

Combination of PPI and enzalutamide further inhibited growth and induced cell cycle arrest of human CRPC cells

Previous studies, including ours, showed that PPI inhibited growth and induced cell cycle arrest, and apoptosis of human cancer cells [12, 14, 15]. In the current study, we

examined the potential synergy of the combination of PPI and enzalutamide in this process. We found that while enzalutamide alone exerted a slight effect, the combination of PPI and enzalutamide further inhibited growth in the human CRPC cells PC3 and DU145 by MTT assay (Fig. 1A). The CI values were 0.49110 and 0.54560 in PC3 and DU145 cells, respectively, which indicated moderate synergy. Subsequently, in examining the nature of cell cycle arrest, we observed that the combination of PPI and enzalutamide for 24 h led to a further decrease in the proportion of cells at G0/G1 phases, relative to that of PPI alone (Fig. 1B) as detected by flow cytometry. Concomitantly, the population of PC3 and DU145 cells at the S phase was significantly induced after treatment with PPI alone and in combination with enzalutamide (Fig. 1B). These results suggested that the combination of PPI and enzalutamide exerted enhanced effects on inhibiting the proliferation of CRPC cells.

PPI decreased the protein expression levels of p65 and lncRNA HOTAIR

We searched for potential molecular targets that might be involved in the inhibitory effect of PPI with or without enzalutamide on cell growth. Given the tumor promoter roles of NF- κ B [25, 26] and lncRNA HOTAIR [20, 21], we evaluated the effects of PPI with or without enzalutamide. We showed that PPI decreased NF- κ B subunit p65 protein expression (Fig. 2A) and HOTAIR levels in PC3 and DU145 cells (Fig. 2B). As expected, the combination of PPI and enzalutamide further reduced p65 protein mainly in the cytoplasm; however, a similar effect was also observed in the nuclear area (Fig. 2C) and HOTAIR levels in PC3 and DU145 cells (Fig. 2B). Notably, silencing of HOTAIR was found to reduce cell growth in PC3 and DU145 cells (Fig. 2D). We further examined the interaction between p65 and HOTAIR and observed that while silencing of HOTAIR exerted no effect on p65 and p50 proteins (Fig.

Fig. 1. Combination of PPI and enzalutamide further inhibited growth and induced cell cycle arrest of human CRPC cells. A, CRPC cells PC3 and DU145 were treated with PPI (1.2 μ M) in the presence or absence of enzalutamide (30 μ M) for up to 48 h. Afterwards, the cell viability and the potential synergy of PPI and enzalutamide were determined using the MTT assay and the Chou-Talalay method expressed as CI values as described in the Materials and Methods section, respectively. B, CRPC cells PC3 and DU145 were treated with PPI (1.2 μ M) in the presence or absence of enzalutamide (30 μ M) for up to 48 h. The cells were collected and processed for analysis of the cell cycle distribution. Cell cycle was analyzed by flow cytometry after propidium iodide (PI) staining, and the percentages of the cell population in each phase (G0/G1, S and G2/M) of cell cycle were analyzed by Multicycle AV DNA Analysis Software. Data are expressed as a percentage of total cells. Values are given as the mean $\pm$ SD from three independent experiments and expressed as a percentage of total cells. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the PPI treated alone ($P < 0.05$).

Fig. 2. PPI decreased the protein expression levels of p65 and lncRNA HOTAIR. A, CRPC cells PC3 and DU145 were treated with increased doses of PPI for up to 24 h. The cell lysate was harvested and the expression of p65 and p50 proteins were measured by Western blot. GAPDH was used as loading control. B-C, CRPC cells PC3 and DU145 were treated with PPI (1.2 μ M) in the presence or absence of enzalutamide (30 μ M) for up to 48 h. Afterwards, the HOTAIR levels, the nuclear and cytoplasmic portions of p65, and total p50 protein expressions were examined by qRT-PCR and Western blot, respectively. D, CRPC cells PC3 and DU145 were transfected with the control or HOTAIR siRNAs for 24 h. Afterwards, the cell viability was examined by MTT assays as described in the Materials and Methods section. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the PPI treated alone ($P < 0.05$).

Fig. 3. Interaction and regulation of p65 and HOTAIR attributed to effect of PPI. A, CRPC cells PC3 and DU145 were transfected with the control or HOTAIR siRNAs for 24 h. Afterwards, the p65 and p50 protein expressions were examined by Western blot. B, PC3 and DU145 were transfected with the control or the overexpression vectors of p65 for 24 h before exposure of cells to PPI for an additional 24 h. Afterwards, HOTAIR levels were examined by qRT-PCR. C, PC3 and DU145 were transfected with the control or overexpression of HOTAIR vectors for 24 h in the absence of PPI, or before exposure of cells to PPI for an additional 24 h. Afterwards, HOTAIR levels, total p65 and p50 protein expressions were examined by qRT-PCR and Western blot, respectively. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the PPI treated alone ($P < 0.05$).

3A), overexpression of p65 resisted the effect of PPI on HOTAIR expression (Fig. 3B). By contrast, exogenously expressed HOTAIR exerted no significant effect on PPI-reduced p65

protein levels (Fig. 3C). High expression of HOTAIR following transfection was observed, as determined by qRT-PCR (Fig. 3C). This result indicated that the combination of PPI and enzalutamide further inhibited p65 protein and HOTAIR levels; more importantly, p65 regulated HOTAIR expression and the regulatory loop of p65 and HOTAIR contributed to the effects of PPI in this process.

PPI decreased protein, mRNA expression, and promoter activity of MUC1 by repression of p65 and HOTAIR

We further explored the functional relevance of p65 and HOTAIR expressions and the potential downstream target. We evaluated the role of MUC1, a tumor promoter and oncogenic factor reported to be highly expressed in prostate cancer [32]. We found that PPI decreased protein, mRNA levels, and the promoter activity of MUC1 in PC3 and DU145 cells (Figs. 4A–4C). As expected, the combination of PPI and enzalutamide further reduced MUC1 protein expression (Fig. 4D). Given the decreases in p65 and HOTAIR, we determined the roles of p65 and HOTAIR in MUC1 regulation in this process. We showed that overexpressed p65 resisted PPI-reduced MUC1 protein expression (Fig. 5A). Interestingly, while silencing of HOTAIR inhibited MUC1 (Fig. 5B), excessive expression of HOTAIR neutralized the PPI-inhibited MUC1 protein expression (Fig. 5C) and promoter activity (Fig. 5D). Together, these results indicated that p65 and HOTAIR were upstream of MUC1 and that reductions in the expression levels of p65 and HOTAIR were involved in the inhibition of MUC1 expression affected by PPI.

Exogenously expressed MUC1 antagonized PPI-inhibited cell growth but exerted no effect on NF- κ B/p65 and HOTAIR

To further gain insight into the molecular mechanism by which the interactions among NF- κ B/p65, HOTAIR, and MUC1 contributed to the overall response of PPI in this setting, we transfected the MUC1 expression plasmids into the cells. As shown in Figures 6A–6B,

Fig. 4. PPI decreased protein, mRNA expression, and promoter activity of MUC1. A, PC3 and DU145 cells were exposed to increased concentrations of PPI for 24 h. Afterwards, the expression of MUC1 protein were detected by Western blot. B, PC3 and DU145 cells were exposed to PPI (1.6 μ M) for 24 h, followed by measuring the mRNA levels of MUC1 by qRT-PCR. C, PC3 and DU145 cells were transfected with the wild type human MUC1 promoter reporter construct ligated to the luciferase reporter gene and the internal control for 24 h, followed by treating with PPI for an additional 24 h. Afterwards, the promoter activities were determined using the Secrete-Pair Dual Luminescence Assay Kit as described in the Materials and Methods section. D, PC3 and DU145 cells were treated with PPI (1.2 μ M) in the presence or absence of enzalutamide (30 μ M) for up to 24 h. Afterwards, MUC1 protein expression was examined by Western blot. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the PPI treated alone ($P < 0.05$).

Values in bar graphs were given as the mean $\pm$ SD from three independent experiments. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the PPI treated alone ($P < 0.05$).

Fig. 5. PPI decreased protein expression of MUC1 through repression of p65 and HOTAIR. A, PC3 and DU145 were transfected with the control or the overexpression vectors of p65 for 24 h before exposure of the cells to PPI for an additional 24 h. Afterwards, MUC1 protein expression was examined by Western blot. B, PC3 and DU145 were transfected with the control or HOTAIR siRNAs for 24 h. Afterwards, MUC1 protein was examined by Western blot. C, PC3 and DU145 were transfected with the control or HOTAIR expression vectors for 24 h before exposure of the cells to PPI for an additional 24 h. Afterwards, MUC1 protein was examined by Western blot. D, PC3 and DU145 were transfected with the control or HOTAIR expression vectors, the wild type human MUC1 promoter reporter construct ligated to the luciferase reporter gene, and the internal control secreted alkaline phosphatase for 24 h, followed by treating with PPI for an additional 24 h. Afterwards, the promoter activities of MUC1 gene were determined using the Seclite-Pair Dual Luminescence Assay Kit as described in the Materials and Methods section. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments performed in triplicate. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the PPI treated alone ($P < 0.05$).

Fig. 6. Exogenously expressed MUC1 antagonized PPI-inhibited cell growth but exerted no effect on NF- κ B/p65 and HOTAIR. A, PC3 and DU145 cells were transfected with the control or MUC1 expression vectors for 24 h before exposure of the cells to PPI for an additional 24 h. Afterwards, the p65 and MUC1 expression levels were examined by Western blot. B-C, PC3 and DU145 cells were transfected with the control or MUC1 expression vectors for 24 h before exposure of the cells to PPI for an additional 24 h. Afterwards, the expression levels of HOTAIR and the cell viability were determined using the qRT-PCR and MTT assays as described in the Materials and Methods section. Inserts in B and C were the protein levels of MUC1 as determined by Western blot. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments performed in triplicate. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the PPI treated alone ($P < 0.05$).

overexpression of MUC1 exerted no effect on p65 and HOTAIR expression affected by PPI. However, exogenously expressed MUC1 could resist PPI-reduced cell growth (Fig. 6C). The aforementioned findings further implied that NF- κ B/p65 and HOTAIR were upstream of MUC1 and that reduction of MUC1 was required in mediating the inhibitory effect of PPI on CRPC cell growth.

Xenograft tumor model

To extend these results obtained from *in vitro* experiments, we evaluated the effect of PPI with or without enzalutamide in a prostate cancer xenograft mouse model. Mice bearing xenograft CRPC were treated with the control, PPI (3 mg/kg) [15], enzalutamide (10 mg/kg) [41, 42] or the combination treatment, given once a day via intraperitoneal injection for up to 27 days (n=8/group), followed by intraperitoneal injection of D-luciferin. Compared with the control group, the PPI-treated mice demonstrated significant growth inhibition as assessed using the Xenogen IVIS200 System (Fig. 7A). Even greater growth inhibition was observed in the combination treatment group (Fig. 7A). In addition, we found significant reductions in tumor weight and size (volume) in the PPI-treated group relative to that in the control group (Figs. 7B–7D). Greater reductions in tumor weight and size were observed in the combination treatment group (Figs. 7B–7D). Moreover, consistent with the results from the *in vitro* data, reductions in the expression levels of p65, MUC1, and HOTAIR in fresh

Fig. 7. Xenograft tumor model. Mice (n=8/group) were divided into 4 groups [Con (saline), PPI (3 mg/kg), enzalutamide (10 mg/kg) and PPI plus enzalutamide] and given by intraperitoneal injection for up to 27 days. A, The xenografts were assessed by *in vivo* bioluminescence imaging at the fifth and end of the experiments (on day 5 and 27, respectively). The tumor growth was monitored by injecting D-luciferin in the mice followed by measuring bioluminescence and analyzed as described in the Materials and Methods section. Representative images are shown. B and C, The xenografts were harvested on day 27, and the size and weight of tumors were measured. The bar graphs represented the tumor weight and size of mice results expressed as mean $\pm$ SD. D, The photographs of PPI, or enzalutamide alone and PPI plus enzalutamide, and vehicle-treated xenografts derived from nude mice are shown. E–F, At the end of the experiments, xenografted tumors were isolated from the individual animals and the expressions of p65, p50 and MUC1 proteins and HOTAIR were detected by Western blot and qRT-PCR as described in the Materials and Methods sections. GAPDH was used as loading control for Western blot. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments. *indicates the significant difference from untreated control (p<0.05). **indicates significant difference from PPI treated alone (P<0.05). G, The diagram shows that PPI inhibits the growth of CRPC cells via a reduction in p65 protein levels and concomitant inhibition of lncRNA HOTAIR. These actions lead to the suppression of MUC1 gene expression. The novel regulatory interaction of p65 and HOTAIR also converges in the inhibition of MUC1 expression and the overall response of PPI. The combination of PPI and enzalutamide exhibits synergy.

alone and PPI plus enzalutamide, and vehicle-treated xenografts derived from nude mice are shown. E–F, At the end of the experiments, xenografted tumors were isolated from the individual animals and the expressions of p65, p50 and MUC1 proteins and HOTAIR were detected by Western blot and qRT-PCR as described in the Materials and Methods sections. GAPDH was used as loading control for Western blot. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments. *indicates the significant difference from untreated control (p<0.05). **indicates significant difference from PPI treated alone (P<0.05). G, The diagram shows that PPI inhibits the growth of CRPC cells via a reduction in p65 protein levels and concomitant inhibition of lncRNA HOTAIR. These actions lead to the suppression of MUC1 gene expression. The novel regulatory interaction of p65 and HOTAIR also converges in the inhibition of MUC1 expression and the overall response of PPI. The combination of PPI and enzalutamide exhibits synergy.

tumors harvested from the aforementioned experiment were observed in the PPI group, compared with that in the control by Western blot analysis and qRT-PCR (Figs. 7E–7F). Notably, the combination treatment group exhibited enhanced effects (Figs. 7E–7F).

Discussion

Despite advances in diagnosis and management, morbidity from prostate cancer remains high, particularly for the CRPC type. In recent years, newer agents have been introduced, targeting some of these mechanisms of resistance to provide better survival. These agents include AR signaling inhibitors, such as enzalutamide and abiraterone acetate, a cytochrome P450 17A1 inhibitor. However, these agents can eventually fail to control patients with CRPC because of both *de novo* and acquired resistance [43]. Several studies showed that natural medicine such as PPI could be considered as a potential agent for the treatment of cancer in humans. However, the current data were limited, and the exact mechanisms involved in the anti-cancer activities of PPI had not been well elucidated. In the present study, significant cell growth inhibition in CRPC cells was demonstrated, and an even greater response to PPI combined with enzalutamide was observed. These findings suggested the possibility of the anti-prostate cancer activity of PPI. In this study, the PPI concentrations found to inhibit cell growth and induce cell cycle arrest in a dose-dependent manner were consistent with those of other studies, or even lower, without toxicities [27, 44, 45]. Mechanistically, our results indicated that inhibition of p65 protein and concomitant reduction of HOTAIR expression result in the suppression of MUC1 gene expression; all of these processes were involved in the inhibition of CRPC cell growth by PPI with or without enzalutamide.

Our study demonstrated the roles of p65 and HOTAIR inhibition in mediating the effect of PPI. As a critical transcription factor, constitutive NF- κ B activity was observed in a larger number of human cancers and promoted growth and progression, including that in prostate cancer [25, 26, 46]. In addition, HOTAIR expression was associated with induced proliferation and aggressive behavior of cancer cells, resulting in poor prognosis [19–21]. Thus, targeting NF- κ B/p65 and HOTAIR may provide therapeutic benefits for patients with malignancies, including prostate cancer. Our results also implied the potential interaction and regulatory loop of p65 and HOTAIR. In line with this, studies have revealed connections between these 2 molecules [35, 47, 48]. One study demonstrated that HOTAIR positively induced phosphorylation of p65 and activation of NF- κ B and that production of HOTAIR-induced tumor necrosis factor alpha (TNF- α) was blocked by the NF- κ B inhibitor. This finding suggested that NF- κ B/p65 was involved in the production of HOTAIR-induced TNF- α in cardiomyocytes in a lipopolysaccharide-induced mouse model [47]. In addition, knockdown of HOTAIR inhibited growth and induced cell cycle arrest and apoptosis *in vitro* partly via the NF- κ B signaling pathways in large B cell lymphoma cells [48]. Our findings provided a novel insight into the relation between NF- κ B/p65 and HOTAIR affected by PPI, as well as emphasized the tumor promoter roles of NF- κ B/p65 and HOTAIR involved in the anti-tumor effects of PPI. Further studies need to be conducted to elucidate the physical interaction between p65 and HOTAIR by using methods such as chromatin immunoprecipitation–qRT-PCR or/and RNA immunoprecipitation assays.

Our results also suggested that inhibition of p65 and HOTAIR contributed to the PPI-inhibited expression of the MUC1 gene. As an oncogenic factor, overexpressed MUC1 was observed in several epithelial cancers, including prostate cancer [32, 49]. The regulation and interaction between p65 and MUC1 were also demonstrated in other studies [34, 50, 51]. By binding to the promoter regions, the MUC1/NF- κ B complex could promote certain gene expression in different cancer cells [34, 52]. Thus, targeting NF- κ B and MUC1 could be involved in the anti-cancer mechanism of PPI. Notably, no studies on the potential connection between HOTAIR and MUC1 have thus far been reported. Therefore, how HOTAIR regulates MUC1 expression has yet to be determined. Our results implied that HOTAIR, as an upstream regulator, controlled MUC1 expression. More experiments need to be conducted

to determine whether HOTAIR interacts with MUC1 or epigenetically regulates the MUC1 promoter, thereby regulating MUC1 gene expression and influencing downstream biological effects.

Our data demonstrated a potentially synergistic inhibitory effect of the combination of PPI and enzalutamide on CRPC cell growth and relevant molecular targets. Enzalutamide combined with other agents exhibited even greater inhibitory effects on CRPC cells via multiple molecular mechanisms [53–56]. In addition, PPI was reported to reverse epithelial–mesenchymal transition, decrease the expression levels of interleukin-6 and signal transducer activator of transcription 3 (STAT3) in the epidermal growth factor receptor tyrosine kinase inhibitor erlotinib-resistant lung cancer cells. This enhanced the effect of erlotinib on lung tumor growth both *in vitro* and *in vivo*. Thus, combined PPI and erlotinib strengthened drug response and provided a novel therapeutic for patients with lung cancer [57]. Our findings suggested that PPI sensitized or enhanced the effect of enzalutamide on controlling CRPC cell growth or *vice versa* by targeting p65, HOTAIR, and MUC1 regulatory signaling axis, implying a potential synergy between PPI and enzalutamide in anti-androgen-resistant ways. This finding also emphasized an intriguing therapeutic target for the treatment of CRPC in the clinical setting. Regardless, future studies are required to explore the in-depth mechanism of these combination effects on the inhibition of prostate cancer growth.

Moreover, our *in vivo* data were consistent with the findings *in vitro*, verifying the effect of PPI combined with enzalutamide on the inhibition of prostate cancer growth and the regulation of p65, MUC1 protein, and HOTAIR expression. The given doses of PPI and enzalutamide were similar to the doses in other studies demonstrating substantial anti-tumor effects in the inhibition of various cancer, including CRPC [15, 41, 42]. Future studies still need to be conducted to elucidate the precise role of MUC1 by using stable cells transfected with expression vectors containing the coding region of the MUC1 gene in the nude mice model.

In summary, our results indicate that PPI inhibits the growth of CRPC cells via a reduction in p65 protein levels and concomitant inhibition of lncRNA HOTAIR. These actions lead to the suppression of MUC1 gene expression. The novel regulatory interaction of p65 and HOTAIR also converges in the inhibition of MUC1 expression and the overall response of PPI. The combination of PPI and enzalutamide exhibit synergy. This study reveals a novel mechanism for the regulation of MUC1 gene expression in response to PPI with or without enzalutamide and proposes a strategy for a CRPC-associated therapy.

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Disclosure Statement

The authors declare to have no competing interests.

References

- 1 Siegel RL, Miller KD, Jemal A: Cancer statistics, 2016. *CA Cancer J Clin* 2016;66:7-30.
- 2 Attard G, Parker C, Eeles RA, Schroder F, Tomlins SA, Tannock I, Drake CG, de Bono JS: Prostate cancer. *Lancet* 2016;387:70-82.
- 3 Nakano K, Komatsu K, Kubo T, Natsui S, Nukui A, Kurokawa S, Kobayashi M, Morita T: External validation of risk classification in patients with docetaxel-treated castration-resistant prostate cancer. *BMC Urol* 2014;14:31.
- 4 Martin SK, Kyprianou N: Exploitation of the androgen receptor to overcome taxane resistance in advanced prostate cancer. *Adv Cancer Res* 2015;127:123-158.
- 5 Armstrong AJ: New treatment options in castration-resistant prostate cancer. *J Natl Compr Canc Netw* 2015;13:690-693.
- 6 Lorente D, Mateo J, Perez-Lopez R, de Bono JS, Attard G: Sequencing of agents in castration-resistant prostate cancer. *Lancet Oncol* 2015;16:e279-e292.
- 7 Rodriguez-Vida A, Galazi M, Rudman S, Chowdhury S, Sternberg CN: Enzalutamide for the treatment of metastatic castration-resistant prostate cancer. *Drug Des Devel Ther* 2015;9:3325-3339.
- 8 Buttiglieri C, Tucci M, Bertaglia V, Vignani F, Bironzo P, Di Maio M, Scagliotti GV: Understanding and overcoming the mechanisms of primary and acquired resistance to abiraterone and enzalutamide in castration resistant prostate cancer. *Cancer Treat Rev* 2015;41:884-892.
- 9 Gu L, Feng J, Xu H, Luo M, Su D: Polyphyllin i inhibits proliferation and metastasis of ovarian cancer cell line ho-8910pm *in vitro*. *J Tradit Chin Med* 2013;33:325-333.
- 10 Shi YM, Yang L, Geng YD, Zhang C, Kong LY: Polyphyllin i induced-apoptosis is enhanced by inhibition of autophagy in human hepatocellular carcinoma cells. *Phytomedicine* 2015;22:1139-1149.
- 11 Li GB, Fu RQ, Shen HM, Zhou J, Hu XY, Liu YX, Li YN, Zhang HW, Liu X, Zhang YH, Huang C, Zhang R, Gao N: Polyphyllin i induces mitophagic and apoptotic cell death in human breast cancer cells by increasing mitochondrial pink1 levels. *Oncotarget* 2017;8:10359-10374.
- 12 Yu S, Wang L, Cao Z, Gong D, Liang Q, Chen H, Fu H, Wang W, Tang X, Xie Z, He Y, Peng C, Li Y: Anticancer effect of polyphyllin i in colorectal cancer cells through ros-dependent autophagy and g2/m arrest mechanisms. *Nat Prod Res* 2017;1-4.
- 13 Gu L, Feng J, Zheng Z, Xu H, Yu W: Polyphyllin i inhibits the growth of ovarian cancer cells in nude mice. *Oncol Lett* 2016;12:4969-4974.
- 14 Chang J, Li Y, Wang X, Hu S, Wang H, Shi Q, Wang Y, Yang Y: Polyphyllin i suppresses human osteosarcoma growth by inactivation of wnt/beta-catenin pathway *in vitro* and *in vivo*. *Sci Rep* 2017;7:7605.
- 15 Li L, Wu J, Zheng F, Tang Q, Wu W, Hann SS: Inhibition of ezh2 via activation of sapk/jnk and reduction of p65 and dnmt1 as a novel mechanism in inhibition of human lung cancer cells by polyphyllin i. *J Exp Clin Cancer Res* 2016;35:112.
- 16 Fu WM, Zhu X, Wang WM, Lu YF, Hu BG, Wang H, Liang WC, Wang SS, Ko CH, Waye MM, Kung HF, Li G, Zhang JF: Hotair mediates hepatocarcinogenesis through suppressing mirna-218 expression and activating p14 and p16 signaling. *J Hepatol* 2015;63:886-895.
- 17 Zhang A, Zhao JC, Kim J, Fong KW, Yang YA, Chakravarti D, Mo YY, Yu J: Lncrna hotair enhances the androgen-receptor-mediated transcriptional program and drives castration-resistant prostate cancer. *Cell Rep* 2015;13:209-221.
- 18 Hu H, Shu M, He L, Yu X, Liu X, Lu Y, Chen Y, Miao X, Chen X: Epigenomic landscape of 5-hydroxymethylcytosine reveals its transcriptional regulation of lncrnas in colorectal cancer. *Br J Cancer* 2017;116:658-668.
- 19 Deng J, Yang M, Jiang R, An N, Wang X, Liu B: Long non-coding rna hotair regulates the proliferation, self-renewal capacity, tumor formation and migration of the cancer stem-like cell (csc) subpopulation enriched from breast cancer cells. *PLoS One* 2017;12:e0170860.
- 20 Lee M, Kim HJ, Kim SW, Park SA, Chun KH, Cho NH, Song YS, Kim YT: The long non-coding rna hotair increases tumour growth and invasion in cervical cancer by targeting the notch pathway. *Oncotarget* 2016;7:44558-44571.
- 21 He H, Wei Z, Du F, Meng C, Zheng D, Lai Y, Yao H, Zhou H, Wang N, Luo XG, Ma W, Zhang TC: Transcription of hotair is regulated by rhoc-mrtf-a-srf signaling pathway in human breast cancer cells. *Cell Signal* 2017;31:87-95.

- 22 Taheri M, Habibi M, Noroozi R, Rakhshan A, Sarrafzadeh S, Sayad A, Omrani MD, Ghafouri-Fard S: Hotair genetic variants are associated with prostate cancer and benign prostate hyperplasia in an Iranian population. *Gene* 2017;613:20-24.
- 23 Wu D, Wu P, Zhao L, Huang L, Zhang Z, Zhao S, Huang J: Nf-kappab expression and outcomes in solid tumors: A systematic review and meta-analysis. *Medicine (Baltimore)* 2015;94:e1687.
- 24 Bradford JW, Baldwin AS: Ikk/nuclear factor-kappab and oncogenesis: Roles in tumor-initiating cells and in the tumor microenvironment. *Adv Cancer Res* 2014;121:125-145.
- 25 Nandakumar N, Muthuraman S, Gopinath P, Nithya P, Gopas J, Kumar RS: Synthesis of coumapherine derivatives: Their nf-kappab inhibitory effect, inhibition of cell migration and their cytotoxic activity. *Eur J Med Chem* 2017;125:1076-1087.
- 26 Yu L, Li L, Medeiros LJ, Young KH: Nf-kappab signaling pathway and its potential as a target for therapy in lymphoid neoplasms. *Blood Rev* 2017;31:77-92.
- 27 Chang J, Wang H, Wang X, Zhao Y, Zhao D, Wang C, Li Y, Yang Z, Lu S, Zeng Q, Zimmerman J, Shi Q, Wang Y, Yang Y: Molecular mechanisms of polyphyllin i-induced apoptosis and reversal of the epithelial-mesenchymal transition in human osteosarcoma cells. *J Ethnopharmacol* 2015;170:117-127.
- 28 Stroopinsky D, Kufe D, Avigan D: Muc1 in hematological malignancies. *Leuk Lymphoma* 2016;57:2489-2498.
- 29 Nath S, Mukherjee P: Muc1: A multifaceted oncoprotein with a key role in cancer progression. *Trends Mol Med* 2014;20:332-342.
- 30 Xu F, Liu F, Zhao H, An G, Feng G: Prognostic significance of mucin antigen muc1 in various human epithelial cancers: A meta-analysis. *Medicine (Baltimore)* 2015;94:e2286.
- 31 Ahmad R, Alam M, Hasegawa M, Uchida Y, Al-Obaid O, Kharbada S, Kufe D: Targeting muc1-c inhibits the akt-s6k1-elf4a pathway regulating tigar translation in colorectal cancer. *Mol Cancer* 2017;16:33.
- 32 Genitsch V, Zlobec I, Thalmann GN, Fleischmann A: Muc1 is upregulated in advanced prostate cancer and is an independent prognostic factor. *Prostate Cancer Prostatic Dis* 2016;19:242-247.
- 33 Xiang S, Zhang Q, Tang Q, Zheng F, Wu J, Yang L, Hann SS: Activation of ampkalpha mediates additive effects of solamargine and metformin on suppressing muc1 expression in castration-resistant prostate cancer cells. *Sci Rep* 2016;6:36721.
- 34 Mori Y, Akita K, Tanida S, Ishida A, Toda M, Inoue M, Yashiro M, Sawada T, Hirakawa K, Nakada H: Muc1 protein induces urokinase-type plasminogen activator (upa) by forming a complex with nf-kappab p65 transcription factor and binding to the upa promoter, leading to enhanced invasiveness of cancer cells. *J Biol Chem* 2014;289:35193-35204.
- 35 Ozes AR, Miller DF, Ozes ON, Fang F, Liu Y, Matei D, Huang T, Nephew KP: Nf-kappab-hotair axis links DNA damage response, chemoresistance and cellular senescence in ovarian cancer. *Oncogene* 2016;35:5350-5361.
- 36 Zheng F, Tang Q, Wu J, Zhao S, Liang Z, Li L, Wu W, Hann S: P38alpha mapk-mediated induction and interaction of foxo3a and p53 contribute to the inhibited-growth and induced-apoptosis of human lung adenocarcinoma cells by berberine. *J Exp Clin Cancer Res* 2014;33:36.
- 37 Chou TC: Drug combination studies and their synergy quantification using the chou-talalay method. *Cancer Res* 2010;70:440-446.
- 38 Zhao S, Wu J, Zheng F, Tang Q, Yang L, Li L, Wu W, Hann SS: Beta-elemene inhibited expression of DNA methyltransferase 1 through activation of erk1/2 and ampkalpha signalling pathways in human lung cancer cells: The role of sp1. *J Cell Mol Med* 2015;19:630-641.
- 39 Hann SS, Chen J, Wang Z, Wu J, Zheng F, Zhao S: Targeting ep4 by curcumin through cross talks of amp-dependent kinase alpha and p38 mitogen-activated protein kinase signaling: The role of pgc-1alpha and sp1. *Cell Signal* 2013;25:2566-2574.
- 40 Ballard DW, Dixon EP, Pepper NJ, Bogerd H, Doerre S, Stein B, Greene WC: The 65-kda subunit of human nf-kappa b functions as a potent transcriptional activator and a target for v-rel-mediated repression. *Proc Natl Acad Sci U S A* 1992;89:1875-1879.
- 41 Lallous N, Leblanc E, Munuganti RS, Hassona MD, Nakouzi NA, Awrey S, Morin H, Roshan-Moniri M, Singh K, Lawn S, Yamazaki T, Adomat HH, Andre C, Dugaard M, Young RN, Guns ES, Rennie PS, Cherkasov A: Targeting binding function-3 of the androgen receptor blocks its co-chaperone interactions, nuclear translocation, and activation. *Mol Cancer Ther* 2016;15:2936-2945.

- 42 Fong KW, Zhao JC, Kim J, Li S, Yang YA, Song B, Rittie L, Hu M, Yang X, Perbal B, Yu J: Polycomb-mediated disruption of an androgen receptor feedback loop drives castration-resistant prostate cancer. *Cancer Res* 2017;77:412-422.
- 43 Chandrasekar T, Yang JC, Gao AC, Evans CP: Mechanisms of resistance in castration-resistant prostate cancer (crpc). *Transl Androl Urol* 2015;4:365-380.
- 44 Kong M, Fan J, Dong A, Cheng H, Xu R: Effects of polyphyllin i on growth inhibition of human non-small lung cancer cells and in xenograft. *Acta Biochim Biophys Sin (Shanghai)* 2010;42:827-833.
- 45 Yue G, Wei J, Qian X, Yu L, Zou Z, Guan W, Wang H, Shen J, Liu B: Synergistic anticancer effects of polyphyllin i and evodiamine on freshly-removed human gastric tumors. *PLoS One* 2013;8:e65164.
- 46 Mendonca MS, Turchan WT, Alpuche ME, Watson CN, Estabrook NC, Chin-Sinex H, Shapiro JB, Imasuen-Williams IE, Rangel G, Gilley DP, Huda N, Crooks PA, Shapiro RH: Dmpt inhibits nf-kappab activity and increases sensitivity of prostate cancer cells to x-rays *in vitro* and in tumor xenografts *in vivo*. *Free Radic Biol Med* 2017;112:318-326.
- 47 Wu H, Liu J, Li W, Liu G, Li Z: Lncrna-hotair promotes tnfr-alpha production in cardiomyocytes of lps-induced sepsis mice by activating nf-kappab pathway. *Biochem Biophys Res Commun* 2016;471:240-246.
- 48 Yan Y, Han J, Li Z, Yang H, Sui Y, Wang M: Elevated rna expression of long noncoding hotair promotes cell proliferation and predicts a poor prognosis in patients with diffuse large b cell lymphoma. *Mol Med Rep* 2016;13:5125-5131.
- 49 Wang J, Ni WH, Hu KB, Zhai XY, Xie F, Jie J, Zhang NN, Jiang LN, Yuan HY, Tai GX: Targeting muc1 and jnk by rna interference and inhibitor inhibit the development of hepatocellular carcinoma. *Cancer Sci* 2017;108:504-511.
- 50 Rajabi H, Alam M, Takahashi H, Kharbanda A, Guha M, Ahmad R, Kufe D: Muc1-c oncoprotein activates the zeb1/mir-200c regulatory loop and epithelial-mesenchymal transition. *Oncogene* 2014;33:1680-1689.
- 51 Li J, Xiang S, Zhang Q, Wu J, Tang Q, Zhou J, Yang L, Chen Z, Hann SS: Combination of curcumin and bicalutamide enhanced the growth inhibition of androgen-independent prostate cancer cells through sapk/jnk and mek/erk1/2-mediated targeting nf-kappab/p65 and muc1-c. *J Exp Clin Cancer Res* 2015;34:46.
- 52 Tagde A, Rajabi H, Stroopinsky D, Gali R, Alam M, Bouillez A, Kharbanda S, Stone R, Avigan D, Kufe D: Muc1-c induces DNA methyltransferase 1 and represses tumor suppressor genes in acute myeloid leukemia. *Oncotarget* 2016;7:38974-38987.
- 53 Fernandez EV, Reece KM, Ley AM, Troutman SM, Sissung TM, Price DK, Chau CH, Figg WD: Dual targeting of the androgen receptor and hypoxia-inducible factor 1alpha pathways synergistically inhibits castration-resistant prostate cancer cells. *Mol Pharmacol* 2015;87:1006-1012.
- 54 Nguyen HG, Yang JC, Kung HJ, Shi XB, Tilki D, Lara PN, Jr., DeVere White RW, Gao AC, Evans CP: Targeting autophagy overcomes enzalutamide resistance in castration-resistant prostate cancer cells and improves therapeutic response in a xenograft model. *Oncogene* 2014;33:4521-4530.
- 55 Wu H, Zhang L, Gao X, Zhang X, Duan J, You L, Cheng Y, Bian J, Zhu Q, Yang Y: Combination of sorafenib and enzalutamide as a potential new approach for the treatment of castration-resistant prostate cancer. *Cancer Lett* 2017;385:108-116.
- 56 Liu C, Armstrong CM, Lou W, Lombard AP, Cucchiara V, Gu X, Yang JC, Nadiminty N, Pan CX, Evans CP, Gao AC: Niclosamide and bicalutamide combination treatment overcomes enzalutamide- and bicalutamide-resistant prostate cancer. *Mol Cancer Ther* 2017;16:1521-1530.
- 57 Lou W, Chen Y, Zhu KY, Deng H, Wu T, Wang J: Polyphyllin i overcomes emt-associated resistance to erlotinib in lung cancer cells via il-6/stat3 pathway inhibition. *Biol Pharm Bull* 2017;40:1306-1313.