Blockage of MDM2-mediated p53 ubiquitination by yuanhuacine restrains the carcinogenesis of prostate carcinoma cells by suppressing LncRNA LINC00665

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Abstract

Prostate cancer (PCa) is known as a challenging issue for the global men health due to its uncontrolled proliferation and high metastatic potential. Increasing evidence has supported plant extracts and plant-derived natural derivatives as a promising anti-tumor therapeutics as their less toxic side effects. Yuanhuacine is an active component isolated from *Daphne genkwa* and can effectively suppress tumorigenesis of several cancers. However, its role in PCa is still unclear. In this study, yuanhuacine dose-dependently inhibited PCa cell proliferation and induced cell apoptosis. Moreover, yuanhuacine treatment also restrained PCa cell invasion and migration. Mechanically, yuanhuacine decreased p53 protein ubiquitination, degradation and ultimately increased p53 levels, which was regulated by inhibiting phosphorylation and total protein levels of Mouse Double Minute 2 (MDM2). Moreover, elevation of MDM2 reversed the suppressive efficacy of yuanhuacine in PCa cell viability, invasion and migration. The network pharmacology and bioinformatics analysis confirmed that MDM2 might be a common target of *Daphne genkwa* and LINC00665. Furthermore, yuanhuacine reduced LINC00665 expression. Up-regulation of LINC00665 inverted yuanhuacine-mediated inhibition in MDM2 protein expression and suppressed p53 levels by enhancing its ubiquitination in yuanhuacine-treated cells. Importantly, the inhibitory effects of yuanhuacine on cell viability and metastatic potential were offset after LINC00665 elevation. Together, the current finding highlight that yuanhuacine may possess its tumor-suppressive efficacy by inhibiting LINC00665-mediated MDM2/p53 ubiquitination signaling. Therefore, this data indicate that yuanhuacine may be a promising candidate for the treatment of PCa.

Introduction

Prostate cancer (PCa) constitutes a major public health problem for men worldwide and is the second leading cause of cancer-related death. Epidemiologic statistical assay reveals that PCa accounts for 54% of all cancer incident cases in men [1]. Currently, the incidence of PCa remains stable during 2014 through 2018; whilst the proportion of PCs diagnosed at a distant stage continues increase from 3.9–8.2% over the past decade[2]. Approximately 268,000 new cases and 34,500 new death of PCa are predicted in the United States in 2020 [2]. In China, there is a rapid increase incidence and burden of PCa [3]. Although recent advances in the diagnosis and treatment of PCa, the prognosis for men with advanced PCa is poor and less than a third surviving 5 years after diagnosis [4]. Therefore, a better understanding of the molecular events underlining PCa progression is urgently needed for developing effective therapeutic strategy.

Increasing researches have supported a notion that the wide utility of natural agents opens up a novel avenue for cancer therapy as their ability to target multiple cancer-related pathway [5]. Yuanhuacine is a daphnane diterpenoid (Fig. 1A) traditional herbal medicine from the flowers of *Daphne genkwa* (Thymelaeaceae) and has a range of biological activities, including abortifacient, anti-virus and anti-inflammation [6, 7]. In recent years, increasing interests have focused on the pharmacological activities of yuanhuacine, especially its anti-tumor activity [8], including lung cancer [9], colon cancer [10], breast cancer [11]. For instance, yuanhuacine inhibits human lung cancer cell proliferation and exhibits little
cytotoxicity to normal lung epithelial cells [9]. Moreover, administration with yuanhuacine suppresses tumor growth in nude mouse model in non-small cell lung cancer [12]. Furthermore, yuanhuacine also exerts the potential anti-tumor efficacy in triple negative breast cancer [11]. However, its role in PCa remains unclear.

P53 is a proverbial tumor suppressor by regulating diverse signaling pathway involved in cell growth, invasion, migration and chemoresistance [13, 14]. Noticeably, the levels and activity of p53 are usually controlled by the ubiquitin proteinligase Mouse Double Minute 2 (MDM2). Convincingly, targeting the MDM2/p53 axis is a promising challenges for the successful clinical therapy for cancers [13, 15]. Intriguingly, we predicted MDM2 as a potential target of yuanhuacine using the HERB database. Therefore, the present study sought to investigate the function of yuanhuacine in carcinogenesis of PCa. Moreover, the involvement of MDM2/p53 signaling during this process was further explored.

**Materials And Methods**

**Cell culture and treatment**

Human normal prostate stromal immortalized cell line (WPMY-1) and two PCa cell lines (LNCaP and PC-3) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in in RPMI 1640 medium plus 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 mg/ml streptomycin. Fur culture, all cells were stimulated with yuanhuacine (>98% purity; HAS biotech, Shenzhen, China) ranged from 1-50 μM, and then were housed in humidified incubator containing 5%CO₂ at 37°C.

**Construction of recombinant MDM2 plasmids**

The cDNA encoding MDM2 was prepared by PCR amplification using cDNA template that was synthesized via a SuperScript II First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). After treatment with restriction enzymes, the MDM2 cDNA was subcloned into the pcDNA3.1 (Thermo Fisher Scientific, Waltham, MA, USA), and subsequently named pcDNA-MDM2.

**Cell transfection**

PCa cells were seeded in per well of six-well plate, and then were transfected with overexpression plasmids of LINC00665 (GenePharma Biotechnology, Shanghai, China), pcDNA-MDM2 and empty vectors. During this process, Lipofectamine™ 3000 (Invitrogen) was applied. Approximately 48 h later, the expression of MDM2 and LINC00665 were then analyzed by qRT-PCR and western blotting.

**CCK-8 assay**

For cell viability analysis, cells (1 × 10⁴ cells) were placed in 96-well plates in culture medium. Subsequently, cells were treated with LINC00665, pcDNA-MDM2 and yuanhuacine. Then, 10 μl of CCK-8 solution (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was added for further incubation.
After reaction for 4 h at 37°C, the absorbance (OD value) of each sample well at 450 nm was determined to analyze cell viability using a microplate reader (Thermo Electron Corporation, MA, USA).

**Flow cytometry**

Cell apoptosis was evaluated using Annexin V-FITC/PI staining kit (Nanjing Jiancheng Bioengineering Institute). Briefly, cells under LINC00665, pcDNA-MDM2 and yuanhuacine conditions were collected and centrifuged at 1000 g for 5 min. After re-suspending in 500 μl binding buffer, 10 μl of Annexin V-FITC and 5 μl of PI were added for further incubated at temperature for 10 min under dark. Then, all specimens were subjected into a flow cytometer (CytoFlex, Beckman Coulter, Fullerton, CA, USA).

**Transwell invasion assay**

Transwell chamber (8-μm pore filters; Corning, Corning, NY, USA) was applied to assess cell invasion ability. PCa cells under various treatments were adjusted to 1 × 10^5 cells/ml, and then were seeded into the upper chamber. Then, the lower chamber was filled with 400 μl complete medium supplemented with 10% FCS that was used as a chemoattractant. After a 48 h incubation and removal of cells on the bottom surface of filter, 4% paraformaldehyde was adopted for fixing the invaded cells, following staining with 0.1% crystal violet solution. Finally, a microscope was applied to count cell invasion ability at 200× magnification by choosing five randomly fields.

**Wound healing analysis**

Cell migration was analyzed using a conventional scratch test. Cells post-treatment were placed in 6-well plates with the concentration of 1 × 10^5/well and allowed to grown into 80–90% confluence. Then, a cross-shaped scratch in the middle of each well were implemented with a sterile 200 μL plastic pipette tips. After an incubation in an air atmosphere of 37 °C for 24 h, non-adherent cells were gently rinsing and images were then photographed over time using an inverted microscope (IX71; Olympus, Tokyo, Japan). The migration area was calculated as (%) = ((original gap area – gap area at X h)/original gap area) × 100%.

**qRT-PCR**

After RNA preparation by TRIzol reagent, cDNA was synthesized via a SuperScript II First Strand Synthesis System (Invitrogen). Then, qRT-PCR was performed to determine the expression of MDM2 and LINC00665 on the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). All protocols were carried out according to the instructions of SYBR® Premix Ex Taq™ (Takara) Kit (TaKara, Dalian, China). The specific primer sequences were as follows: MDM2 (Forward: 5’-GATCCAGGCAAATGTGCAATAC-3’; Reverse: 5’-TGGTCTAACCAGGGTCTTCTT-3’), LINC00665 (Forward: 5’-CCAGGTGAAGTGGGAAGT-3’; Reverse: 5’-CGGTGGACGGATAGAAGACG-3’). Gene expression was then analyzed as normalizing to GAPDH using the 2^−ΔΔCt equation.
Bioinformatics assay online

The correlation between LINC00665 and MDM2 in PCa was predicted by bioinformatics tool GIPA2 (http://gepia2.cancer-pku.cn/#index). The target genes of *Flos Genkwa* and LINC00665 were predicted by HERB database (http://herb.ac.cn/Detail/?v=HERB006773&label=Herb) and AnnoLnc2 database (http://annolnc.gao-lab.org/), respectively. The intersection of *Flos Genkwa* and LINC00665 was analyzed by WENNY (https://bioinfogp.cnb.csic.es/tools/venny/index.html).

Western immunoblot analysis

After treatments, cells were collected and rinsed with cold PBS. Then, cells were treated with lysis buffer (Beyotime, Shanghai, China) and the prepared protein concentrations were quantified using the BCA kit (Beyotime). Equal amounts of protein were then separated on 12% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). Then, 5% non-fat dry milk in TBS was added to block the non-specific binding. The primary antibodies against human MDM2 (1:1000) was supplemented for further incubation at 4°C overnight. Then, membranes were reacted with the horseradish peroxidase-conjugated secondary antibodies after rinsing with TBST. The binding signals were developed using ECL reagent (Beyotime) and quantified by an Image J software.

Ubiquitination assay

Cells were treated with 25 μM MG132 for 4 h. Then, cells were lysed with immunoprecipitation assay lysis buffer (Beyotime). Subsequently, the prepared protein extracts were pre-treated with 20 μl Protein A Agarose beads (Beyotime) to remove the non-specifically adhered proteins and incubated with anti-p53 antibody or control IgG antibody overnight at 4 °C. Then, the precipitated proteins were subject to immunoblotting analysis using the anti-ubiquitin p53 antibody to evaluate the ubiquitination of p53 protein.

Statistical analysis

All quantitative data were analyzed using SPSS 20.0 (SPSS, Chicago, IL, USA). All biological experiments were performed at least three separate experiments and data are shown as mean ± SD. For statistical analysis, Student’s *t*-test or ANOVA with Student-Newman-Keuls test was used for comparisons in two or more groups. A criterion of *P* <0.05 was defined as statistical significance.

Results

**Treatment with yuanhuacine selectively kills PCa cell line but has little cytotoxicity to human normal prostate stromal cells**

Before the investigation of yuanhuacine (YC) potential in the progression of PCa, we first evaluated its cytotoxicity to normal prostate stromal immortalized cell line (WPMY-1) and observed the little toxicity of yuanhuacine to WPMY-1 (Fig. 1B). Further assay corroborated that exposure to yuanhuacine (1-50 μM)
dose-dependently suppressed PCa cell LNCaP (Fig. 1C) and PC-3 (Fig. 1D) viability. The IC50 of yuanhuacine was 17.47 µM and 24.67 µM of LNCaP and PC-3 cells. Furthermore, stimulation with 20 µM yuanhuacine leaded to obvious cell apoptosis in LNCaP and PC-3 cells, respectively (Fig. 1E). These data indicate that yuanhuacine effectively kills PCa cells but has cytotoxicity to normal prostate stromal cells.

**Yuanhuacine restrains the metastatic potential of PCa cells**

We next elaborated the effects of yuanhuacine on PCa cell metastatic potential in vitro. As shown in Fig. 2A and 2B, transwell assay substantiated that 20 µM yuanhuacine exposure notably inhibited the invasiveness of LNCaP cells (Fig. 2A) and PC-3 cells (Fig. 2B). Similarly, compared with control groups, treatment with yuanhuacine prevented LNCaP and PC-3 cell migration in a wound healing assay (Fig. 2C and 2D). Thus, these finding suggest that yuanhuacine may inhibit the metastatic potential of PCa cells.

**Yuanhuacine facilities p53 ubiquitination and degradation by interfering MDM2 to exert the anti-tumor potential in PCa cells**

Abundant evidence has supported a fact that p53 often act as a well-known tumor suppressor to participate cell apoptosis, epithelial-mesenchymal transition, and metastatic potential [14]. Herein, treatment with yuanhuacine increased p53 protein levels by decreasing p53 ubiquitination (Fig. 3A). Noticeably, p53 levels and activity are majorly controlled by the ubiquitin protein-ligase Mdm2 [13]. We therefore further elaborated the correlation between yuanhuacine and MDM2 and found that yuanhuacine restrained the mRNA levels of MDM2 in LNCaP cells and PC-3 cells relative to control groups (Fig. 3B). Moreover, yuanhuacine treatment dramatically reduced phosphorylation of MDM2 and total MDM2 protein levels (Fig. 3C and 3D), which in turn result in the decrease of p53 degradation. Thus, these results indicate that yuanhuacine may regulate MDM2 expression to regulate p53 ubiquitination and degradation. To further investigate the involvement of MDM2 in yuanhuacine-mediated anti-tumorigenesis potential, we overexpressed MDM2 protein levels (Fig. 3E). Moreover, up-regulation of MDM2 reversed yuanhuacine-induced inhibition of cell viability (Fig. 3F) and cell apoptosis (Fig. 3F). Concomitantly, the suppressive ability of yuanhuacine on LNCaP cell invasion (Fig. 3G) and migration (Fig. 3H) were also offset after MDM2 overexpression.

**Yuanhuacine affects MDM2-mediated ubiquitination of p53 by blocking LINC00665**

Our previous study confirmed the high expression of LINC00665 in PCa tissues that could act as an oncogene to facilitate the progression of PCa [16]. To further decipher the mechanism underlying yuanhuacine-mediated MDM2 downregulation in PCa progression, we explored the involvement of LINC00665 during this process. As shown in Fig. 4A, network pharmacology analysis have identified 112 target genes of *Flos Genkwa* using HERB database. Moreover, we also identified 937 target genes of LINC00665 by AnnoLnc2 database databases and corroborated that there was a common target gene MDM2 between *Flos Genkwa* and LINC00665 (Fig. 4B). qRT-PCR assay found that yuanhuacine treatment decreased LIN0065 expression in PCa cells (Fig. 4C). Transfection with recombinant LINC00665 plasmids enhanced LINC00665 expression (Fig. 4D). Bioinformatics tool GIPA2 substantiated
there was no significant correlation between LINC00665 and MDM2 mRNA (Fig. 4E). Consistent with above prediction, LINC00665 overexpression did not affect the expression of MDM2 mRNA in yuanhuacine-treated PCa cells (Fig. 4F). Intriguingly, up-regulation of LINC00665 antagonized the inhibitory effects of yuanhuacine on MDM2 protein expression and phosphorylation (Fig. 4G and 4H). Furthermore, LINC00665 enhancement inhibited p53 protein levels by increased p53 ubiquitination relative to yuanhuacine-treated groups (Fig. 4I).

**LINC00665 accounts for the anti-tumor efficacy of yuanhuacine in PCa cells**

The above results indicated that yuanhuacine could induced MDM2/p53 axis by inhibiting LINC00665. We next elucidated the role of LINC00665 in yuanhuacine-mediated tumor inhibition in PCa, and found that elevation of LINC00665 overturned yuanhuacine-mediated inhibition of cell viability (Fig. 5A). Moreover, the pro-apoptotic efficacy of yuanhuacine was abrogated after LINC00665 up-regulation (Fig. 5B). Additionally, overexpression of LINC00665 enhanced PCa cell invasion (Fig. 5C) and migration (Fig. 5D) relative to yuanhuacine-treated groups. These data suggest that yuanhuacine may exert the suppressive efficacy in carcinogenesis of PCa by blocking LINC00665.

**Discussion**

PCa is known as a challenging issue for the global men health and its increasing burden necessitates seeking novel and alternative therapies. In recent years, increasing evidence has supported plant extracts and plant-derived natural derivatives as a promising anti-tumor therapeutics as their less toxic side effects[5, 17]. Yuanhuacine is an active component isolated from *Daphne genkwa* and has been proved to exert the inhibitory efficacy in several cancer cell growth, including lung cancer, breast cancer and colon carcinoma[8]. Nevertheless, its role in PCa remains elusive. The present study confirmed that yuanhuacine dose-dependent suppressed PCa cell viability and induced cell apoptosis. Moreover, yuanhuacine exhibited little cytotoxicity to normal prostate cells. Analogously, a previous study also corroborated the little toxicity of yuanhuacine to normal lung epithelial cells[9].

Exception to the uncontrolled proliferation, most cancers share a common basic characteristic, the highly metastatic potential. It is generally believed that high invasion and migration usually lead to high metastasis that is correlated with treatment failure and poor prognosis for cancer patients at advanced stage including PCa [18, 19]. Currently, though therapeutic advances in cancer treatment, metastasis is still the principal cause of cancer-related death, which indicates a promising strategy against cancer by blocking cancer cell metastatic potential [20, 21]. Therefore, we next elucidated the role of yuanhuacine in PCa cell metastatic potential and found that treatment with yuanhuacine restrained PCa cell invasion and migration, suggesting a potential to inhibit PCa metastasis.

The p53 protein, a tumor suppressor, is recognized as a central safeguard that protects cells against genome instability and malignant transformation [13, 15]. Given that p53 protein stability is majorly controlled by ubiquitin-dependent degradation pathway. Inducing of p53 protein expression by decreasing its ubiquitination participates the carcinogenesis, including cancer cell growth, chemoresistance,
invasion, migration and metastasis [13]. Generally, E3 ubiquitin ligase MDM2 can act as a suppressor to reduce intracellular p53 levels by inducing p53 ubiquitination and subsequent degradation [15]. To further analyze the mechanism underlying the inhibitory efficacy of yuanhuacine in the carcinogenesis of PCa, we explored the correlation between yuanhuacine and p53 signaling. As except, yuanhuacine treatment inhibited p53 ubiquitination, degradation and ultimately increasing p53 levels. More importantly, this process was regulated by blocking MDM2 phosphorylation. Noticeably, overexpression of MDM2 reversed the suppressive efficacy of yuanhuacine in PCa cell growth and metastatic potential. Thus, yuanhuacine may act as an anti-tumor agent by regulating the MDM2/p53 axis.

Long noncoding RNAs are responsible for the majority of human genome transcriptions and their aberrant expression are correlated with various pathological processes including cancer development [22]. Among them, LINC00665 is located at chromosome 19q13.12 and is abnormally up-regulated in several cancers [23–25]. Our previous finding confirmed that LINC00665 could serve as an oncogene in PCa progression [16]. Intriguingly, evidence from the bioinformatics database predicted MDM2 as a common target of LINC00665 and Daphne genkwa. Moreover, yuanhuacine also inhibited LIN0065 expression in PCa cells. We therefore further investigated the involvement of LINC00665 in yuanhuacine-mediated anti-tumor progression in PCa. Noticeably, elevation of LINC00665 expression overturned yuanhuacine-mediated inhibition of MDM2 expression at post-transcriptional levels to regulate p53 ubiquitination and degradation. Moreover, overexpression of LINC00665 offset yuanhuacine-mediated suppression in PCa cell viability, invasion and migration.

**Conclusions**

Collectively, the current findings first revealed that yuanhuacine could act as the effective suppressor to reduce PCa cell viability and metastatic potential by blocking LINC00665-mediated MDM2/p53 signaling. Therefore, this study may highlight that yuanhuacine may act as a therapeutic agent against PCa. Additionally, the efficacy of yuanhuacine in the development of PCa will be further investigated *in vivo* in our next plan.

**Declarations**

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**Competing interests**

The authors declare that they have no competing interests.

**Author contributions**
Miao Yan: Data curation; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft. Xin Li: Data curation; Investigation; Methodology; Formal analysis; Data curation; Resources; Software; Supervision; Validation; Writing - review & editing. Jinbao Gu: Data curation; Methodology; Investigation; Formal analysis; Data curation; Validation; Resources. Guojun Gao: Data curation; Software; Validation, Methodology; Formal analysis. Ziyu Wu: Conceptualization; Data curation; Methodology; Investigation; Supervision; Software; Validation, Formal analysis. Peng Xue: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Software; Validation.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

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No.

Ethics approval and consent to participate

Not applicable.

Consent to participate

Not applicable.

Consent to Publish

Not applicable.

References


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**Figures**

![Figure 1](image-url)

**Figure 1**
Yuanhuacine selectively kills PCa cells. (A) Chemical structure of yuanhuacine (YC) (2D structure). (B) Normal prostate stromal immortalized cell line (WPMY-1) was stimulated with the indicated doses of yuanhuacine (1-50 μM) for 24 h. Then, cell toxicity was evaluated by CCK-8 assay. (C, D) After treatment with yuanhuacine for 24 h, cell viability of LNCaP (C) and PC-3 (D) were analyzed. (E) Following the exposure to 20 μM yuanhuacine, cells were then stained with AnnexinV-FITC and PI. Flow cytometry was then used to determine cell apoptosis. *P < 0.05 vs. control group.
Figure 2

Treatment with yuanhuacine inhibits PCa cell invasion and migration. (A) LNCaP (A) and PC-3 cells (B) were treated with 20 µM yuanhuacine. Transwell assay was then performed to detect cell invasion. (C, D) Cell migration ability was evaluated by wound healing analysis. \(^* P < 0.05\) vs. control group.

Figure 3

Yuanhuacine antagonizes carcinogenesis of PCa cells by regulating MDM2-mediated p53 ubiquitination and degradation. (A) After treatment with yuanhuacine, ubiquitination status of p53 was then analyzed in LNCaP and PC-3 cells. (B) The mRNA levels of MDM2 were determined by qRT-PCR. (C, D) Western blotting was applied to detect the protein levels of MDM2 and p-MDM2. The corresponding quantified analysis of binding signals were analyzed by Imag J software. (E) LNCaP cells were transfected with pcDNA-MDM2 plasmids or empty vectors. The protein expression of MDM2 was then detected. (F-) Cells were treated with pcDNA-MDM2 and yuanhuacine. Then, cell viability (F), apoptosis (G), invasion (H) and migration (I) were further assessed. \(^* P < 0.05\) vs. control group. \(^\# P < 0.05\) vs. YC-treated group.
Figure 4

Yuanhuacine regulates MDM2-mediated ubiquitination of p53 by blocking LINC00665. (A) Network pharmacology analysis revealed target genes of Flos Genkwa using HERB database. A red circle was used to mark MDM2. (B) The Venn diagram shows the intersection of Flos Genkwa and LINC00665. (C) The expression of LINC00665 was determined in LNCaP cells under yuanhuacine exposure. (D) The effects of LINC00665 plasmids on its expression. (E) The potential correlation between LINC00665 and MDM2 mRNA was analyzed using a bioinformatics tool GIPA2. (F-H) Cells were treated with LINC00665 plasmids and yuanhuacine. Then, the mRNA levels (F), MDM2 and p-MDM2 protein expression (G and H) were determined. (I) The subsequent effects on p53 ubiquitination were explored. *P < 0.05 vs. control group. #P < 0.05 vs. YC-treated group.
LINC00665 involves in Yuanhuacine-mediated suppression of carcinogenesis in PCa cells. Cells transfected with LINC00665 vectors were exposed to yuanhuacine. Then, cell viability (A), apoptosis (B), invasion (C) and migration (D) were analyzed by CCK-8, flow cytometry, Transwell and wound healing analysis, respectively. *P < 0.05 vs. control group. #P < 0.05 vs. YC-treated group.