

Huaier suppresses proliferative and metastatic potential of prostate cancer PC3 cells via downregulation of Lamin B1 and induction of autophagy

AILIN YANG¹, YANAN ZHAO¹, YING WANG², XIAOJUN ZHA³, YUNFANG ZHAO¹,
PENGFEI TU¹ and ZHONGDONG HU¹

¹Modern Research Center for Traditional Chinese Medicine, School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing 100029; ²Department of Molecular Orthopaedics, Beijing Institute of Traumatology and Orthopaedics, Beijing Jishuitan Hospital, Beijing 100035;

³Department of Biochemistry and Molecular Biology, School of Basic Medicine, Anhui Medical University, Hefei, Anhui 230032, P.R. China

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Abstract. Prostate cancer is one of the most common malignancies occurring in males. Although large advances have been made in the pathogenesis of prostate cancer, the development of drugs with high efficacy and low toxicity for the treatment of prostate cancer is urgently needed. Recently, more and more attention has been paid to the antitumor effect of Traditional Chinese Medicine (TCM) worldwide. *Trametes robiniophila* Murr. (Huaier) has been applied as a type of TCM drug for ~1,600 years. Huaier exhibits excellent clinical efficacy in the treatment of cancer, including prostate cancer. However, the mechanisms underlying the anti-prostate cancer effect of Huaier remain largely unclear. In the present study, we revealed that Huaier aqueous extract inhibited the proliferative and metastatic capabilities of human prostate cancer PC3 cells through CCK-8 assay, *in vitro* scratch assay and Transwell assay. Moreover, decreased Lamin B1 was implicated in Huaier-induced suppression of proliferative and metastatic potential of PC3 cells. Intriguingly, we demonstrated that Huaier treatment induced autophagic cell death in PC3 cells. This study sheds new light on the mechanisms underlying the activity of Huaier against prostate cancer and

provides a new theoretical basis for the clinical application of Huaier in prostate cancer.

Introduction

Prostate cancer is one of the leading causes of cancer-related death in men worldwide. At present, treatment approaches for prostate cancer mainly include surgery, chemotherapy and hormonal therapy (1,2). The selection of the treatment method for prostate cancer generally depends on the clinical stage, Gleason score, age of patients, and other factors. To date, the chemotherapeutic effect for prostate cancer is limited due to drug resistance and cytotoxicity (3-5), which makes it urgent to develop new therapeutic drugs to further improve the clinical outcomes of patients with prostate cancer.

Recently, increased attention has been paid to the anti-cancer effects of Traditional Chinese Medicine (TCM) largely due to the advantages of low toxicity and multi-targets (6-8). *Trametes robiniophila* Murr. (Huaier) has a long history of disease treatment for more than 1,600 years in China. Numerous studies have revealed that Huaier exhibits superior effects for the treatment of several types of cancers, such as hepatocellular carcinoma, breast, ovarian, lung and prostate cancer (9-14). The underlying mechanisms of the anticancer effects of Huaier include the inhibition of tumor cell proliferation, metastasis and angiogenesis, as well as induction of apoptosis (11,13,15-17). However, the mechanisms underlying the anti-prostate cancer effect of Huaier remain to be elucidated.

In the present study, we demonstrated that Huaier significantly inhibited the proliferative and metastatic potential of human prostate cancer PC3 cells. Moreover, downregulation of Lamin B1 was responsible for the inhibition of the proliferative and metastatic capacity of PC3 cells exposed to Huaier aqueous extract. More importantly, Huaier treatment activated autophagy in PC3 cells, and suppression of autophagy attenuated Huaier-induced cell death in PC3 cells. Thus, the present study provides a theoretical and experimental basis for the clinical application of Huaier for the treatment of prostate cancer.

Correspondence to: Dr Zhongdong Hu or Professor Pengfei Tu, Modern Research Center for Traditional Chinese Medicine, School of Chinese Materia Medica, Beijing University of Chinese Medicine, 11 North Third Ring Road, Chaoyang, Beijing 100029, P.R. China
E-mail: zdhu@bucm.edu.cn
E-mail: pengfeitu@163.com

Abbreviations: TCM, Traditional Chinese Medicine; FBS, fetal bovine serum; OD, optical density; CCK-8, Cell Counting Kit-8; TEM, transmission electron microscopy

Key words: Traditional Chinese Medicine, Huaier, prostate cancer, Lamin B1, autophagy

Materials and methods

Reagents and antibodies. Ham's F-12K (Kaighn's) medium, fetal bovine serum (FBS), penicillin-streptomycin solution, 0.25% trypsin, Matrigel and Transwell chambers were purchased from Corning Life Sciences (Corning, NY, USA). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). 3-Methyladenine (3-MA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bafilomycin A1 was obtained from Aladdin Shanghai Biochemical Technology Co., Ltd. (Shanghai, China). Bis-Tris Nu-PAGE gels (4-12%) were obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Super ECL Plus was from GE Healthcare (Pittsburgh, PA, USA). β -actin antibody was purchased from Abgent (cat. no. AM1829b; San Diego, CA, USA). Atg3 (cat. no. 3415), Beclin-1 (cat. no. 3495) and Atg5 (cat. no. 12994) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). LC3 antibody was from MBL (cat. no. PM036; Tokyo, Japan). Lamin B1 (cat. no. sc-20682), HRP-labeled goat anti-mouse (cat. no. sc-2005) and goat anti-rabbit (cat. no. sc-2004) antibodies were from Santa Cruz Biotechnology.

Preparation of Huaier aqueous extract. The electuary ointment of Huaier was obtained from Gaitianli Medicine Co., Ltd. (Jiangsu, China). It was dissolved in F-12K complete medium to obtain a 10 mg/ml stock solution and was stored at 4°C after sterilization by filtration.

Cell culture. Human prostate cancer PC3 cells were obtained from the Cell Culture Center of the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences (Beijing, China). PC3 cells were maintained in Ham's F-12K (Kaighn's) medium containing 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO₂.

Cell viability assay. PC3 cells were seeded into 96-well plates at a density of 3,500 cells/well. On the following day, the cells were treated with Huaier aqueous extract at the indicated concentrations for different times. Afterwards, 10 μ l of CCK-8 was added into each well and incubated at 37°C for 2 h. The optical density (OD) was then measured at 450 nm using a microplate reader (Perkin-Elmer, Waltham, MA, USA).

In vitro scratch assay. The scratch assay was performed as described previously (10,18). In brief, PC3 cells were seeded into 12-well plates with complete medium. Cells in a subconfluent state were starved with serum-free medium for 12 h, and then a straight cell-free wound was created using a 10- μ l pipette tip. Next, the cells were maintained in serum-free medium containing 4 mg/ml of Huaier extract. The scratch width was measured at 0 and 24 h. The cell migration distances were analyzed quantitatively.

Transwell assay. The Transwell system was established as previously described (19,20). PC3 cells (1 \times 10⁵) were resuspended in 200 μ l serum-free medium containing Huaier extract (4 mg/ml) and then added to the upper chamber of the Transwell system. The lower chamber was filled with 750 μ l complete medium containing 10% FBS. After incubation for

36 h, the cells were removed from the upper surface of the membrane with a cotton swab. Next, the cells on the bottom surface of the membrane were fixed and then stained with crystal violet. The Transwell chamber was washed twice with phosphate-buffered saline (PBS) to remove the dye. The invasive cells were observed and counted on 5 random fields under an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Quantitative real-time PCR. Total RNA was extracted from PC3 cells using E.Z.N.A.[®] Total RNA Kit I (Omega Bio-tek, Inc., Norcross, GA, USA) and reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara Biotechnology, Inc., Dalian, China) according to the manufacturer's protocols. cDNA was used as a template for the quantitative PCR using the TransStart Top Green qPCR SuperMix (Beijing TransGen Biotech Co., Ltd., Beijing, China). The primer sequences were: human *Lamin B1* forward, 5'-TTCTCGAAGCTTGATCTGGG-3' and human *Lamin B1* reverse, 5'-GATCGAGCTGGGCAAGTG-3'; human β -actin forward, 5'-GTTGTTCGACGACGAGCG-3' and human β -actin reverse, 5'-GCACAGAGCCTCGCCTT-3'.

Immunoblotting. Cells were washed twice with PBS and harvested with lysis buffer [10 mM Tris (pH 6.8), 2% SDS, 10% glycerol and 100 mM DTT], and then cell lysates were boiled for 10 min at 98°C. The levels of indicated proteins were detected by immunoblot analysis as previously described (21).

RNA interference. All siRNAs were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). PC3 cells were seeded in 6-well plates and then transfected with siRNAs targeting Lamin B1, Atg5 or Beclin-1 using Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. The siRNA target sequences were: *Lamin B1* (human), 5'-CGCGCTTGGTAGAGGTGGA-3'; *Atg5* (human), 5'-GGACGAATTCCAACCTTGTT-3'; *Beclin-1* (human), 5'-CAGTTTGGCACAATCAATA-3'; negative control (NC), 5'-TTCTCCGAACGTGTCACGT-3'.

Electron microscopy. Cells were collected with 2.5% glutaraldehyde, and then centrifuged (1,000 revolutions, 10 min) and washed twice with PBS. The samples were post-fixed with 1% osmium tetroxide at 4°C for 2 h in the dark and then washed three times with 0.1MPB. After dehydration, the permeation, paraffin embedding and section staining were performed as previously described (22). The ultrastructure of cells was observed under a JEM-1230 transmission electron microscope (JEOL, Ltd., Tokyo, Japan).

Acridine orange staining. PC3 cells treated with 8 mg/ml Huaier exact for indicated times were washed twice with PBS and stained with 10 μ g/ml acridine orange for 20 min at 37°C in the dark. The cells were observed under an inverted fluorescence microscope.

Immunofluorescence assay. Cells seeded on confocal dishes were washed with PBS and fixed in 4% paraformaldehyde for 10 min, and then permeabilized with PBS containing 0.5% Triton X-100 for 20 min. Next, the cells were washed three

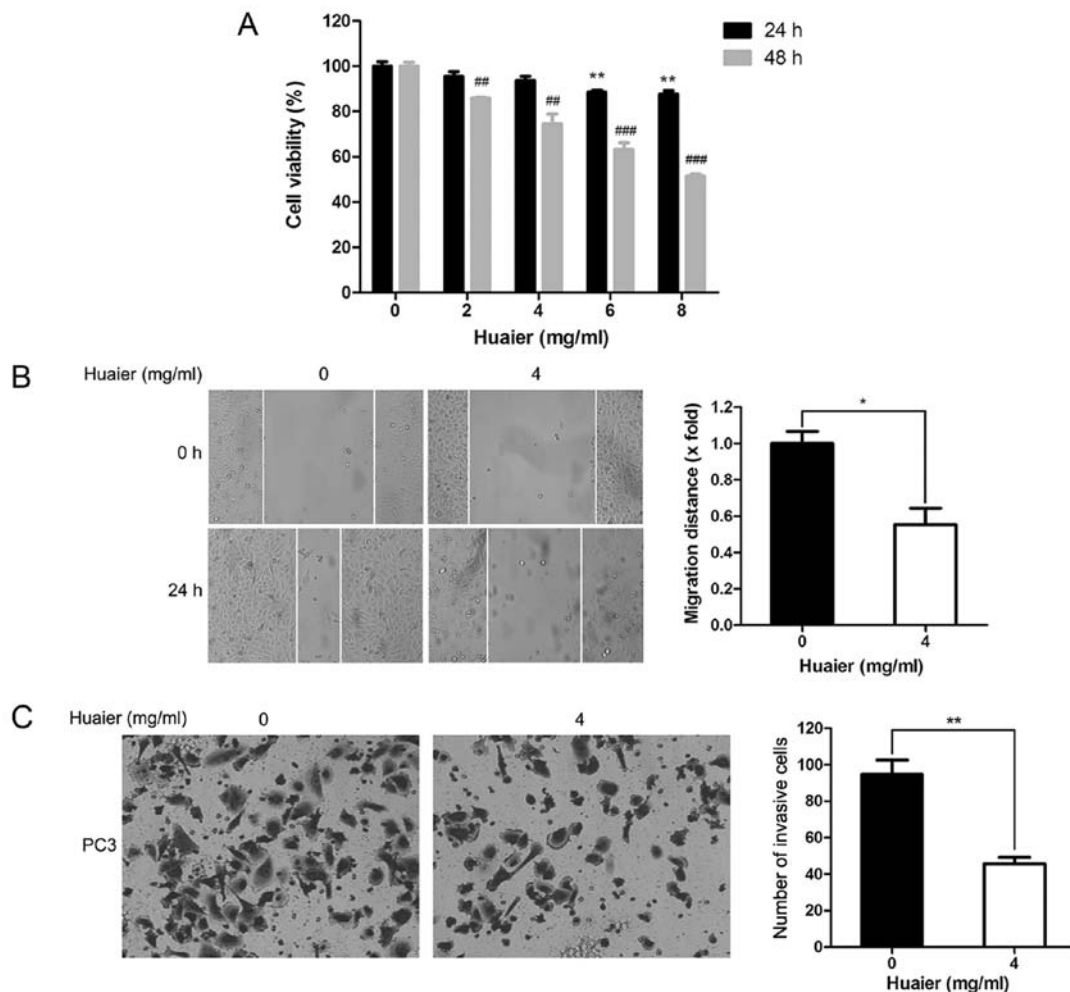


Figure 1. Huaier suppresses the proliferative and metastatic potential of human prostate cancer PC3 cells. (A) PC3 cells were treated with Huaier extract (0-8 mg/ml) for 24 and 48 h. Cell viability was measured by the CCK-8 assay. ^{**} $P < 0.01$, ^{##} $P < 0.01$, ^{###} $P < 0.001$. (B) PC3 cells treated with Huaier extract (4 mg/ml) were subjected to the scratch assay and observed at 0 and 24 h. Left panel, representative images are presented (magnification, $\times 100$); right panel, quantitative data. ^{*} $P < 0.05$. (C) PC3 cells treated with Huaier extract (4 mg/ml) for 36 h were subjected to Transwell assay. Left panel, representative images are presented (magnification, $\times 200$); right panel, quantitative data. ^{**} $P < 0.01$.

times with PBS and blocked with 1% BSA for 1 h at 37°C, and then incubated with the primary antibody (1:100) overnight at 4°C. Subsequently, the cells were washed with PBS and then incubated with anti-rabbit FITC-conjugated secondary antibody (1:100) for 1 h at 37°C. Nucleus was counterstained with DAPI in the dark after being washed with PBS. The cells were observed and photographed with a laser scanning confocal microscope (Olympus FV1000; Olympus Corp., Tokyo, Japan).

Statistical analysis. The data are presented as mean \pm SD of triplicate samples. The statistical analysis in this study was evaluated by the Student's t-test and ANOVA using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant result.

Results

Huaier inhibits the proliferative and metastatic potential of human prostate cancer PC3 cells. CCK-8 assay indicated that Huaier aqueous extract significantly inhibited proliferation of the PC3 cells in a time- and dose-dependent manner (Fig. 1A).

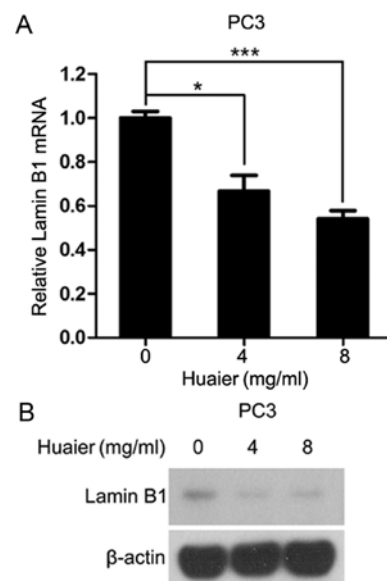


Figure 2. Huaier downregulates Lamin B1 expression in human prostate cancer PC3 cells. Total RNA (A) or cell lysates (B) collected from PC3 cells treated with Huaier extract (0-8 mg/ml) for 48 h were subjected to quantitative real-time PCR and immunoblotting, respectively. ^{*} $P < 0.05$, ^{***} $P < 0.001$.

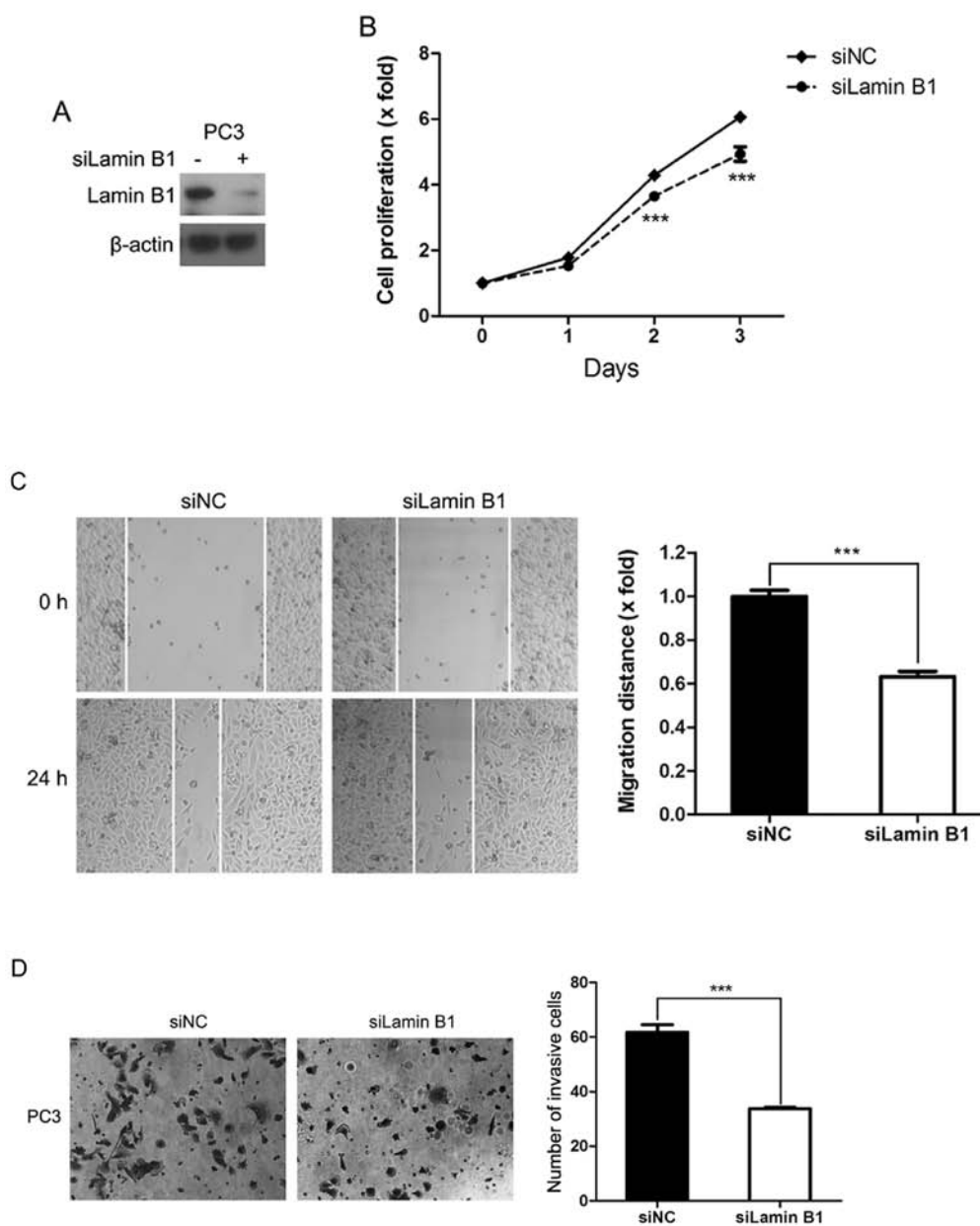


Figure 3. Lamin B1 is required for the proliferation and metastatic potential of PC3 cells. (A) PC3 cells were transfected with siRNAs against Lamin B1 for 48 h and total protein lysates were harvested for immunoblotting. (B) The proliferation of PC3 cells transfected with Lamin B1 siRNAs or negative control siRNAs was assessed by CCK-8 assay. (C) PC3 cells transfected with Lamin B1 siRNAs or negative control siRNAs were subjected to the scratch assay and observed at 0 and 24 h. Left panel, representative images are presented (magnification, x100); right panel, quantitative data. (D) PC3 cells transfected with Lamin B1 siRNAs or negative control siRNAs were subjected to Transwell assay. Left panel, representative images are presented (magnification, x200); right panel, quantitative data. ***P<0.001.

The IC₅₀ value of PC3 cells exposed to Huaier for 48 h was 8.18 mg/ml. The wound healing assay is widely used to evaluate the migratory ability of cells *in vitro*. As shown in Fig. 1B, the migration capacity of PC3 cells was decreased after treatment with Huaier, and the migration inhibition rate of PC3 cells treated with Huaier was 44.68±16.39%. Moreover, the invasion capacity of cells *in vitro* is frequently examined by using Transwell assay. As shown in Fig. 1C, after treatment of PC3 cells with 4 mg/ml Huaier aqueous extract for 36 h, the number of cells that had successfully passed through the Matrigel-coated membrane was markedly reduced compared with that of the control group cells. Taken together, Huaier markedly suppressed the proliferative and metastatic capability of the human prostate cancer cells.

Huaier downregulates Lamin B1 expression in human prostate cancer PC3 cells. Lamin B1 is a member of the lamin family making up the nuclear matrix and is abnormally over-expressed in multiple types of cancers including prostate, hepatocellular carcinoma and pancreatic cancer (19,23-25). Moreover, Lamin B1 positively regulates the proliferation and invasion of pancreatic cancer cells (25). Our previous study revealed that as a pro-oncogenic gene, Lamin B1 was dramatically decreased in human hepatoma SKHEP-1 cells exposed to Huaier (10). Intriguingly, qRT-PCR and western blot analysis demonstrated that Huaier aqueous extract significantly inhibited the mRNA and protein levels of Lamin B1 in PC3 cells in a dose-dependent manner (Fig. 2A and B).

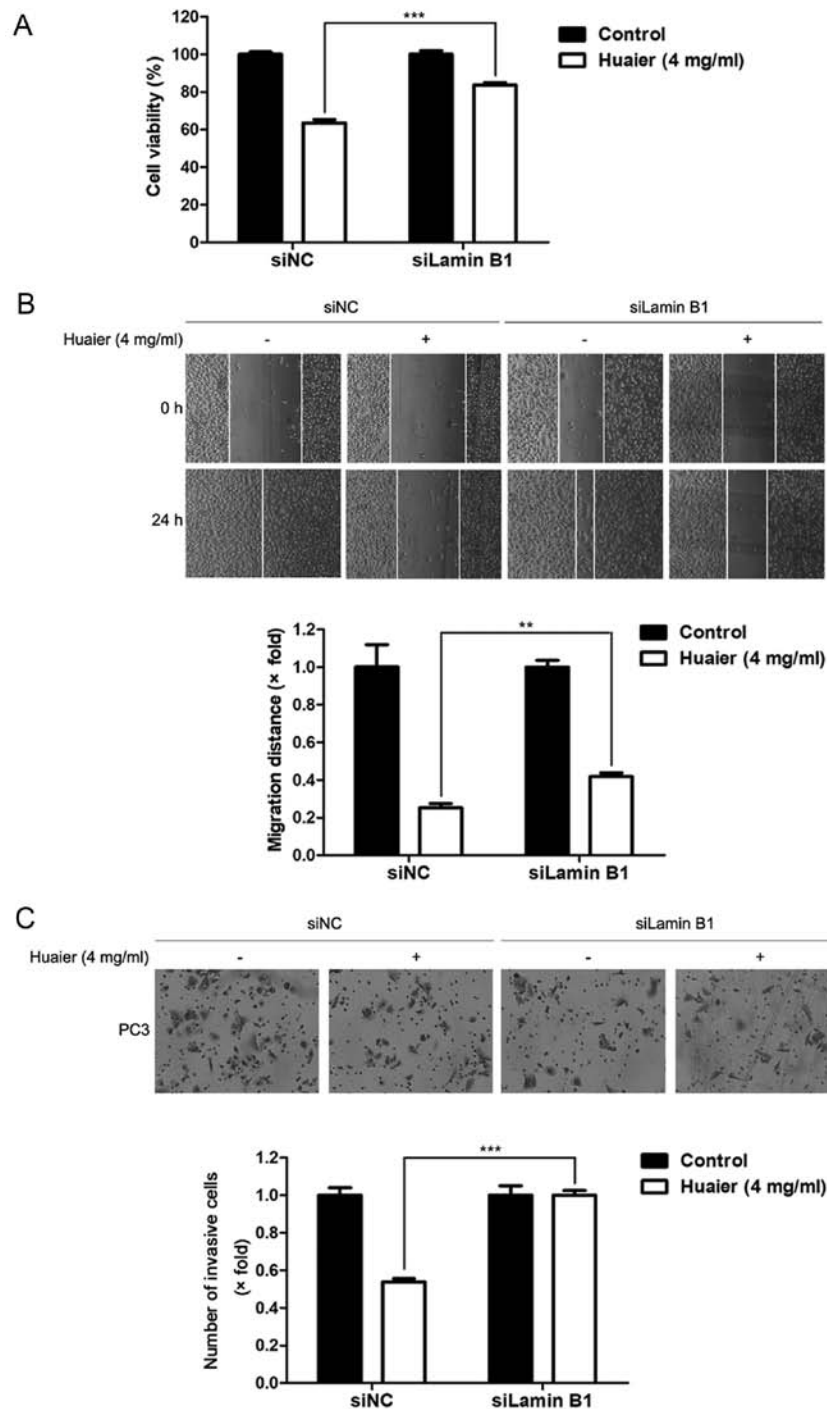


Figure 4. Depletion of Lamin B1 compromises the inhibitory effect of Huaier on the proliferative and metastatic potential of PC3 cells. (A-C) PC3 cells transfected with Lamin B1 siRNAs or negative control siRNAs were treated with or without Huaier extract. (A) Cell viability of the indicated cells was measured by CCK-8 assay. (B) The indicated cells were subjected to the scratch assay and observed at 0 and 24 h. Left panel, representative images are presented (magnification, x100); right panel, quantitative data. (C) The indicated cells were subjected to Transwell assay. Left panel, representative images are presented (magnification, x200); right panel, quantitative data. ** $P < 0.01$, *** $P < 0.001$.

Lamin B1 is involved in the inhibition of proliferation and metastatic potential of PC3 cells exposed to Huaier. Next, we investigated whether downregulation of Lamin B1 contributes to Huaier-mediated inhibition of proliferative and metastatic capacity of PC3 cells by using RNA interference. Transfection with siRNAs targeting Lamin B1 markedly reduced Lamin B1 expression (Fig. 3A). Moreover, depletion of Lamin B1 significantly blunted the proliferation of PC3 cells (Fig. 3B). In addition, reduction of Lamin B1 substantially suppressed

the migration and invasion of PC3 cells (Fig. 3C and D). Thus, Lamin B1 positively regulates the proliferation and metastatic potential of human prostate cancer PC3 cells. To further investigate the role of Lamin B1 in the inhibition of proliferative and metastatic potential of PC3 cells in the presence of Huaier, we treated PC3 cells transfected with Lamin B1 siRNAs or negative control siRNAs with Huaier aqueous extract. As depicted in Fig. 4A, depletion of Lamin B1 attenuated the inhibitory effect of Huaier on the proliferation of PC3 cells.

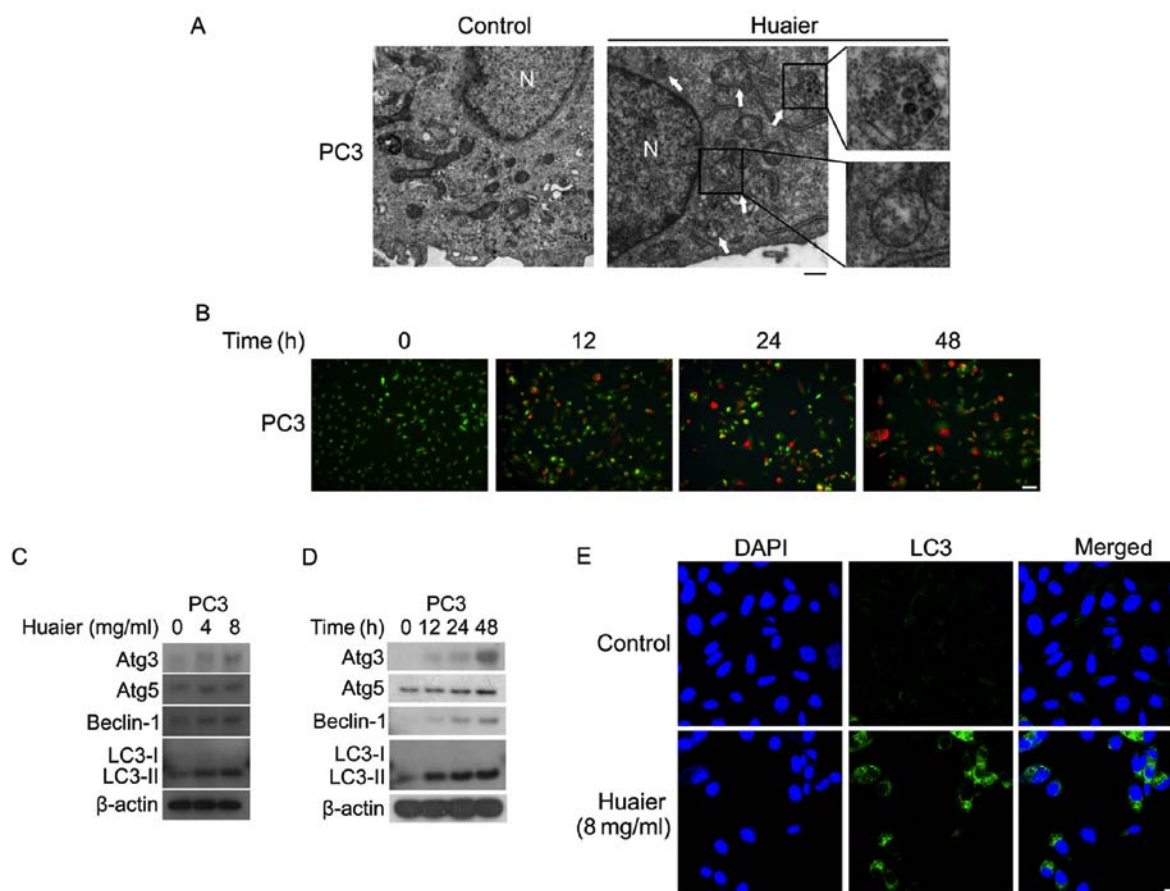


Figure 5. Huaier induces autophagy in PC3 cells. (A) Transmission electron microscopy (TEM) analysis of PC3 cells treated with Huaier extract (8 mg/ml) for 48 h. White arrows indicated autolysosomes/autophagosomes. Scale bar, 500 nm. N, nucleus. (B) PC3 cells treated with Huaier extract (8 mg/ml) for 0, 12, 24 and 48 h were stained with acridine orange (10 μ g/ml), and observed under a fluorescence microscopy. Scale bar, 100 μ m. (C and D) Total cell lysates harvested from PC3 cells treated with Huaier extract at the concentrations of 0, 4 and 8 mg/ml for 48 h (C) or treated with 8 mg/ml Huaier extract for the indicated times (0, 12, 24 and 48 h) (D) were subjected to immunoblotting for detection of Atg3, Atg5, Beclin-1 and LC3. (E) PC3 cells treated with 8 mg/ml Huaier extract for 48 h were stained with anti-LC3 antibody. The LC3 puncta (green) were observed through immunofluorescence. Nuclei were stained with DAPI (blue). Representative images are presented. Scale bar, 20 μ m.

Additionally, the suppression of migration and invasion of PC3 cells caused by Huaier treatment were significantly impaired by knockdown of Lamin B1 (Fig. 4B and C). Collectively, decreased Lamin B1 is partially responsible for the inhibition of proliferation and the metastatic potential of PC3 cells in the presence of Huaier.

Huaier induces autophagy in PC3 cells. Autophagy is one of the underlying mechanisms for drug-induced cell death (26,27). In the present study we determined the effect of Huaier on autophagy in PC3 cells. Observation of autophagosomes and other related subcellular structures in the cytoplasm with transmission electron microscope is widely used for autophagy assessment (22,28,29). Ultrastructural changes of PC3 cells treated with Huaier were observed by transmission electron microscope. As shown in Fig. 5A, PC3 cells treated with Huaier exhibited typical characteristics of autophagy: autophagosomes and autolysosomes which contained cytoplasmic components. Moreover, acridine orange staining was used to observe the accumulation of autophagy vesicles. The number of acidic vesicles marked by orange fluorescence increased in response to Huaier treatment in a time-dependent manner (Fig. 5B). A series of autophagy-related proteins partic-

ipate in different stages of autophagosome formation, such as Atg3, Atg5 and Beclin-1 (30-33). LC3 is an autophagosomal marker protein (34). LC3-II, one form of LC3, accumulates on the membranes of autophagosomes and is widely used as a marker for autophagy evaluation (34,35). Western blot analysis revealed that Huaier treatment led to increase in the expression of Atg3, Atg5, Beclin-1 and LC3-II in a dose- and time-dependent manner in PC3 cells (Fig. 5C and D). Moreover, Huaier treatment markedly increased LC3 puncta in number and intensity in PC3 cells under a fluorescence microscope (Fig. 5E), indicating that the number of autophagosomes was increased in the presence of Huaier. Taken together, Huaier evidently promoted autophagy in PC3 cells.

Autophagy inhibition attenuates Huaier-induced inhibition of proliferation of PC3 cells. To investigate whether autophagy was involved in the inhibition of proliferation of PC3 cells by Huaier, we suppressed autophagy with two autophagy inhibitors and RNA interference. As depicted in Fig. 6A and B, inhibition of autophagy with 3-MA or bafilomycin A1 significantly impaired the sensitivity of PC3 cells to Huaier treatment. Moreover, Atg5 and Beclin-1, two essential components involved in autophagosome

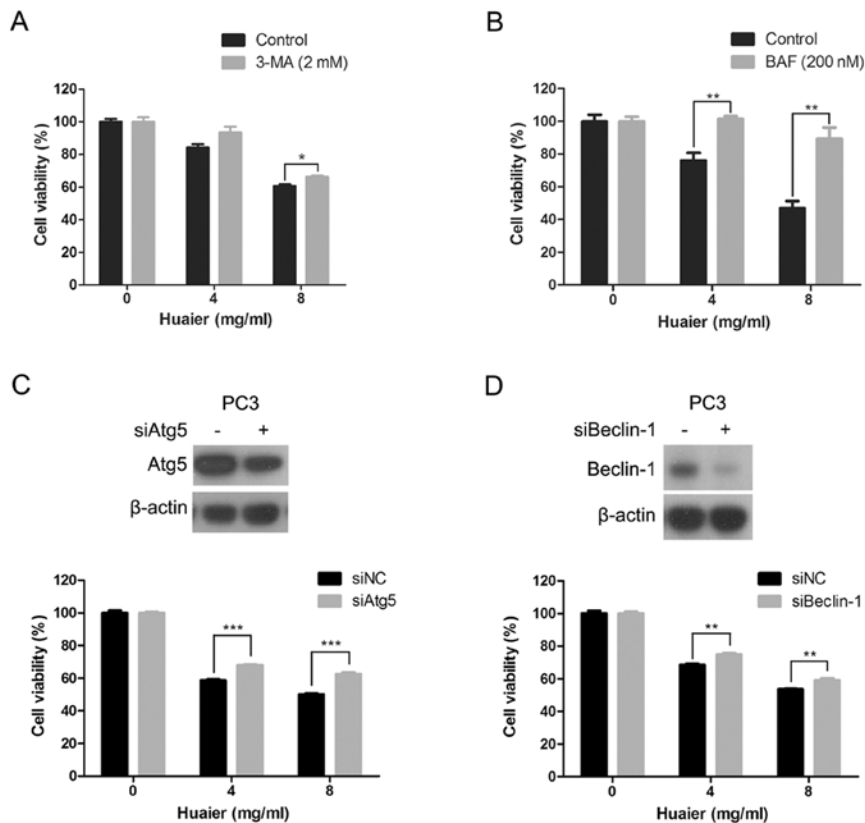


Figure 6. Inhibition of autophagy attenuates Huaier-induced cytotoxicity in PC3 cells. (A) PC3 cells were treated with Huaier extract (0-8 mg/ml) for 48 h in the absence or presence of 3-MA (2 mM). (B) PC3 cells were treated with Huaier extract (0-8 mg/ml) for 48 h in the absence or presence of bafilomycin A1 (200 nM). (C) PC3 cells transfected with Atg5 siRNAs or negative control siRNAs were treated with Huaier extract (0-8 mg/ml) for 48 h, and the protein level of Atg5 was examined by immunoblotting. (D) PC3 cells transfected with Beclin-1 siRNAs or negative control siRNAs were treated with Huaier extract (0-8 mg/ml) for 48 h, and the protein level of Beclin-1 was examined by immunoblotting. The cell viability was measured with the CCK-8 assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

formation (36), were efficiently knocked down in the PC3 cells transfected with siRNAs (Fig. 6C and D). Consistent with the results of autophagy inhibitors, suppression of autophagy with siRNAs against Atg5 or Beclin-1 remarkably blunted the inhibitory effect of Huaier on the proliferation of PC3 cells (Fig. 6C and D). Collectively, the inhibitory proliferation of PC3 cells exposed to Huaier is partially mediated by activation of autophagy.

Discussion

In recent years, the antitumor effects of Huaier have drawn the attention of cancer researchers. Many clinical applications have demonstrated that Huaier can be used for the treatment of multiple types of cancers including prostate cancer. However, the anti-prostate cancer effect of Huaier and its underlying mechanisms remain elusive. In the present study, we demonstrated that Huaier inhibited the proliferation and metastatic potential of human prostate cancer PC3 cells partially through downregulation of Lamin B1. In addition, we revealed that Huaier treatment induced autophagic cell death in PC3 cells.

Uncontrolled cell proliferation is one of the hallmarks of cancer (37). Inhibition of cancer cell proliferation can be used as a strategy to treat cancer. Metastasis is a complex multi-step process which plays a pivotal role in the progression of cancer (38). Cancer metastasis is responsible for ~90%

of human cancer-related deaths (39). Metastases to distant organs such as the bone, liver, lungs and brain frequently occur in the advanced stage of prostate cancer (40,41). Most prostate cancer-related deaths result from metastases. Herein we revealed that Huaier had excellent anti-proliferative and anti-metastatic effects in PC3 cells. Thus, Huaier can be used as a candidate drug for targeting the proliferation and metastasis of cancer cells in the treatment of human prostate cancer. Lamin B1 is an important member of the lamin protein family (23) and was reported to be a carcinogenic gene (25). The levels of Lamin B1 are elevated in tumors of patients with hepatocellular carcinoma (24). Moreover, Lamin B1 expression was found to be markedly increased in malignant prostate cancer (19). Knockdown of Lamin B1 expression by siRNAs induced apoptosis in HeLa cells (42). Our previous study demonstrated that Lamin B1 was remarkably downregulated in human hepatoma SKHEP-1 cells exposed to Huaier (10). In the present study, we revealed that Huaier treatment markedly reduced Lamin B1 expression in human prostate cancer PC3 cells. Furthermore, Lamin B1 was required for the inhibition of proliferation and metastatic potential of PC3 cells by Huaier. As an oncogenic protein, Lamin B1 is a novel target of Huaier for prostate cancer treatment.

Autophagy is a normal physiological process in which the cytoplasmic components including misfolded proteins and damaged organelles are surrounded to form autophagosomes

that are eventually transported to lysosomes for degradation (20,43). As one of the underlying mechanisms of cell death, autophagy frequently occurs in cancer cells in response to antitumor therapies (44-47). Huaier triggered autophagy in human breast cancer MDA-MB-231, MDA-MB-468 and MCF7 cells, and autophagy inhibition impaired Huaier-induced cell death in these cancer cells. Huaier-induced cytotoxicity in human breast cancer cells was partially due to activation of autophagy (48). In addition, Huaier augmented tamoxifen-induced autophagy in ER-positive breast cancer cells (49). We demonstrated that Huaier extract dramatically triggered autophagy in PC3 cells through electron microscopy observation, acridine orange staining, western blotting and immunofluorescence assay. Moreover, inhibition of autophagy via drugs or siRNAs significantly abrogated Huaier-induced cytotoxicity in PC3 cells. Therefore, autophagic cell death is involved in Huaier-induced cytotoxicity in human prostate cancer cells.

In this study, we demonstrated that Huaier-induced cytotoxicity and decreased cell mobility were at least partially mediated by downregulation of Lamin B1 and autophagic cell death in prostate cancer PC3 cells. The multiple mechanisms reported in our study contribute to the understanding of the complex anti-prostate cancer effects of Huaier. The present study provides a new theoretical basis for the clinical application of Huaier in prostate cancer.

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Availability of data and materials

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

Authors' contributions

AY designed and performed the experiments, analyzed data and wrote the manuscript. YaZ, YW, XZ and YuZ analyzed data and revised the manuscript. PT and ZH supervised the study, designed experiments and revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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