

Dendrobium candidum inhibits MCF-7 cells proliferation by inducing cell cycle arrest at G2/M phase and regulating key biomarkers

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Background: Breast cancer is one of the most frequently occurring cancers in women. In recent years, Dendrobium candidum has played a part in antihyperthyroidism and anticancer drugs. This study aims to examine the antitumor effect of D. candidum on breast cancer.

Methods: Human breast cancer cell line MCF-7 and normal breast epithelial cell line MCF10A were used to observe the effects of D. candidum treatment on human breast cancer. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to examine the cell proliferation of the MCF-7 and MCF10A cells. Western blot analysis and reverse transcription polymerase chain reaction were used to detect the key molecules and biomarkers in breast cancer pathology. Cell cycle was analyzed by using Becton Dickinson FACScan cytofluorometer.

Results: The results indicated that D. candidum significantly decreased cell viability at different concentrations compared to the control group (P<0.05). D. candidum-treated MCF-7 cells in the G2/M phase was significantly increased compared to the control group (P<0.05). The messenger RNA levels of estrogen receptor alpha, IGFBP2, IGFBP4, and GATA3 were significantly decreased, and the messenger RNA and protein levels of ELF5, p53, p21, p18, CDH1, CDH2, and p12 were significantly increased, compared to the control group (P<0.05). The protein levels of estrogen receptor alpha, PGR, GATA3, and Ki67 were significantly decreased and the protein levels of p53 and ELF5 were significantly increased compared to the control group (P<0.05). The general apoptosis biomarker, Bcl-2, was significantly decreased and the Bax was significantly increased compared to the control group (P<0.05). In contrast to that in MCF-7, D. candidum does not affect cell proliferation at any concentration and any time points in normal breast epithelial cells, MCF10A cells.

Conclusion: D. candidum could decrease the cell viability of MCF-7 cells by inducing cell cycle arrest at the G2/M phase and regulating the key biomarkers in breast cancer cells.

Keywords: breast cancer, D. candidum, proliferation, biomarker, inhibition

Introduction

Dendrobium candidum is one of the most medicine-valuable orchids, mainly distributes in Southeast and South Asia, such as the People’s Republic of China, Japan, etc.¹,² There are many functions for D. candidum, including 1) the dried stems of D. candidum can strengthen the stomach activity in traditional Chinese medicine;² 2) D. candidum also plays an important role in preventing cataract development, relieving throat inflammation, and fatigue;³ could reduce peripheral vascular obstruction and improve immunity; and 4) in recent years, D. candidum has played a part in antihyperthyroidism and anticancer drugs.⁴ Therefore, as an important traditional Chinese medical herb, it has been with a higher clinical value and potential application.⁴
Breast cancer is one of the most frequently occurring cancer in women worldwide. Approximately 14.2% (in the People’s Republic of China) and 26.4% malignant tumor patients (in the USA) are diagnosed with breast cancer annually. Recently, scientists have explored many differential gene expressions to advance individualized treatment or to assist in the cancer prognosis, such as the estrogen receptor (ER), progesterone receptor, and human epidermal growth factor-2. Meanwhile, many drugs that target these molecules have been designed.

Until now, many evolutionarily conserved key molecules have been discovered, which are involved in a variety of cellular processes including tumor suppression. These molecules could induce tumor suppression or inhibition via enhancing cell cycle arrest, repairing damaged DNA, and causing apoptosis by regulating key gene expression. Previous reports show that the therapeutic drugs interfere with DNA replication and in a further step can affect the development of tumor cells via the cell cycle. The G0, G1, and sub-G1 phase of cell cycle arrest could always induce the cell apoptosis and inhibit the proliferation of tumor cells.

The present study evaluated the antitumor effect of *D. candidum* on breast cancer. The key molecules involved in cell proliferation of breast cancer cells, including ERα, PGR, GATA3, p53, Ki67, and ELF5, were examined to evaluate and analyze the antitumor effects of *D. candidum* on the MCF-7 breast cancer cell line.

Materials and methods

Materials and chemicals

*D. candidum* were generously donated by Prof Jun Sheng (Yunnan Research Centre for Advance Tea Processing, Yunnan Agricultural University, Kunming, People’s Republic of China). Polyvinylidene fluoride membranes were purchased from EMD Millipore (Billerica, MA, USA). The necessary apparatus for sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot were bought from Bio-Rad.

Cells culture

The human breast cancer cell line MCF-7 and the human breast epithelial cell line MCF10A were generously donated by Prof Xin Hu (Jilin University, Changchun, People’s Republic of China) and cultured as described in a previous article. No ethics statement was required from the institutional review board for the use of these cell lines. Briefly, MCF-7 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin/streptomycin. MCF10A cells were cultured in Ham’s F12 medium and DMEM with 2.5 mM l-glutamine (DMEM:F12, Gibco), 5% horse serum (Gibco), 20 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ, USA), 100 ng/mL cholera toxin (Sigma-Aldrich Co., St Louis, MO, USA), 10 μg/mL insulin (Sigma-Aldrich), 0.5 mg/mL hydrocortisone (Sigma-Aldrich), and 100 IU/mL penicillin/streptomycin. Cells were maintained at 37°C and 5% CO₂ in a humidified incubator.

MTT assay

Cells were seeded in a 96-well plate to a final concentration of 5,000 cells/well and incubated in growth media with varying concentrations of *D. candidum* for 12, 24, 48, and 72 hours. Medium was removed and fresh medium was added to each well along with 10 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL). After 4 hours of incubation at 37°C, the medium was drained and replaced by 150 mL of dimethyl sulfoxide. The plates were read at wavelength of 490 nm using a microplate reader (BioTek, Winooski, VT, USA). Six reduplicate wells were used for each treatment, and experiments were repeated three times.

Western blot analysis

Cells were treated with 2 mg/mL *D. candidum* for 48 and 72 hours. Western blot was performed as previously described. The cells were collected in ice-cold phosphate-buffered saline and lysed in ice-cold whole cell extraction buffer containing 25 mM β-glycerophosphate (pH 7.3), 5 mM ethylenediaminetetraacetic acid, 2 mM ethylene glycol tetraacetic acid, 5 mM β-mercaptoethanol, 1% Triton X-100, 0.1 M NaCl, and a protease inhibitor mixture (Roche Applied Science, Penzberg, Germany). The protein concentration cell lysates were determined by using the Bradford method and boiled in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris, pH 6.8; 100 mM dithiothreitol; 2% SDS; 0.1% bromophenol blue; and 10% glycerol). The proteins were separated on 8%–10% SDS polyacrylamide gel and electrotransferred to polyvinylidene fluoride membrane. After blocking with 3% bovine serum albumin-Tris-HCl buffer saline Tween, primary antibodies (PGR, MILLIPORE 2437345; ELF5, Abcam ab136119; GATA3, Abcam ab11693; ERα, Santa Cruz Biotechnology sc-542; p53, Abcam ab26; Ki67, Abcam ab15580) were detected using horseradish peroxidase-conjugated antirabbit antibodies and visualized on Tanon-5200 Chemiluminescent Imaging System (Tanon Science & Technology, Shanghai, People’s Republic of China). β-Actin (ZSGB-BIO TA-09, Beijing, People’s Republic of China) was also tested to confirm equal loading.
Table 1 RT-PCR primer sequences (all sequences from 5’ to 3’)

<table>
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<tr>
<th>RT-PCR Primers</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>β-actin</td>
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<td>TTTGCTGAGTGTGACTG</td>
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<td>TGAGGGGCTTGTCTAGTTC</td>
<td>CAAAGGGACCTTACCAGGCC</td>
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<td>AGGTCACCCATTCTTTACAATTCTCA</td>
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<td>GAGACATTGGCCACCCGGTT</td>
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<td>TCGATGGTGATGCAGTCAGAGC</td>
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<tr>
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<td>p18 (CDK2C2)</td>
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<tr>
<td>CDH2 (N-cadherin)</td>
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<td>EFL5</td>
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Abbreviations: ERα, estrogen receptor alpha; RT-PCR, quantitative reverse transcription polymerase chain reaction.

Quantitative reverse transcription polymerase chain reaction

Cells were treated with 2 mg/mL D. candidum for 48 hours. RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays were performed as described in previous articles. Total RNA from cells was extracted with TRIzol reagent (Invitrogen, Waltham, MA, USA) following the manufacturer’s instruction, and complementary DNA was synthesized using Moloney murine leukemia virus reverse transcriptase with random primers. Complementary DNA was generated with BioTeke super RT kit (BioTek) according to the manufacturer’s protocol. qRT-PCR was performed using SYBR Premix Ex Taq™ (TaqRa, Dalian, People’s Republic of China). Primers are listed in Table 1.

Cell cycle analysis

Cells were treated with graded concentrations of D. candidum (0–2 mg/mL) for 48 hours. Cell cycle distribution was evaluated using cell cycle detection kit (BestBio, People’s Republic of China) following the manufacturer’s instruction. Briefly, the cells were harvested, washed twice with phosphate-buffered saline, and fixed at 4°C for 1 hour with 70% ethanol, and then stained with a propidium iodide solution (containing RNase) at 4°C for 30 minutes. At least 20,000 cells were analyzed by Becton Dickinson FACScan cytometer (Mansfield, MA, USA). Cell cycle distribution was calculated using ModFIT cell cycle analysis software (version 2.01.2; Becton Dickinson).

Statistical analysis

The quantitative analysis of the Western blot images was performed by using the computer-assisted software Image Total Tech (2.0 version). In brief, the images of Western blot were scanned with the Typhoon and digitalized, and saved in JPEG format. The values of the Western blot bands were calculated. Data were presented as mean ± SD of at least three independent experiments. Statistical analysis of data was performed by Student’s t-test for two-group comparison. P<0.05 represents the statistically significant difference.

Results

D. candidum decreases the cell viability of MCF-7 cells

In order to observe the antitumor proliferation effects of D. candidum, MTT assay was used to examine the cell viability of the MCF-7 cells. First, the median lethal dose value of D. candidum was calculated; the preliminary study showed that the median lethal dose of D. candidum is 2 mg/mL (data not shown). Second, the appropriate concentration of D. candidum I was investigated. The MTT assay results indicated that the treatment of D. candidum could significantly decrease the cell viability at different concentrations (0.25, 0.5, 1, and 2 mg/mL) compared to the control group at 24, 48, and 72 hours, respectively (Figure 1A–C, P<0.05). The cell viability was also decreased, followed by the increased concentration (range from 0.25 to 2 mg/mL) of D. candidum on different time points. However, when the concentration of D. candidum was higher than 2 mg/mL.
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D. candidum triggers MCF-7 cell cycle arrest at G2/M phase

The outcomes of cell cycle assay showed that the percentage of the D. candidum-treated MCF-7 cells in the G2/M phase was significantly increased compared to the control group (Figure 2A and B, \( P < 0.05 \)). Also, the percentage of the MCF-7 cells in G2/G0 phase in D. candidum-treated group increased, and S phase decreased compared to the control group (Figure 2B).

Furthermore, the cell cycle arrest-related CDK family proteins transcription has also been examined by using the qRT-PCR assay. The results indicated that the treatment of D. candidum significantly decreased the transcription of CDK family members compared to the control group (Figure 2C, \( P < 0.05 \)).

D. candidum regulates the key molecules involving in the MCF-7 proliferation

To clarify the specific mechanism of the antitumor proliferation effects of D. candidum, the key molecules involved in MCF-7 proliferation were examined. First, we examined the apoptosis- or proliferation-related molecules by using the qRT-PCR assay. The results showed that the messenger RNA (mRNA) levels of ER\( \alpha \), IGBP2, IGFBP4, and GATA3
were significantly reduced, and the mRNA levels of ELF5, p53, p21, p18, CDH1, CDH2, and p12 were significantly increased compared to the control group (Figure 3A, $P < 0.05$). From the mRNA detection results, we found that apoptosis biomarkers (ELF5, p21, CDH1, CDH2, and p12) were increased; therefore, we also examined the protein levels of these biomarkers. The result also confirmed the similar changes of the ELF5, p21, CDH1, CDH2, and p12 protein as the mRNA levels (Figure S1).

Furthermore, the general biomarkers for apoptosis, such as Bcl-2 and Bax, were also detected. The results indicated that Bcl-2 was significantly decreased and Bax was significantly increased compared to the control group (Figure S2).

Second, the breast cancer biomarkers (ER$\alpha$, PGR, and GATA3) and oncogenes (p53, Ki67, and ELF5) were also examined by using the Western blot assay. The results indicated that the protein levels of ER$\alpha$, PGR, GATA3, and Ki67 were significantly decreased and the protein levels of p53 and ELF5 were significantly increased compared to the control group (Figure 3B and C, $P < 0.05$).

**D. candidum** did not affect the normal breast epithelial MCF10A cell growth

Does **D. candidum** affect the normal breast epithelial cell lines proliferation? MCF10A cells were used to evaluate the effects of **D. candidum** on normal breast cell lines. In contrast to the effects in cancer cells exposed to various concentrations, the MCF10A cells showed no differences in cell proliferation at the concentration of 0.5, 1, and 2 mg/mL (Figure 4A, $P > 0.05$). Meanwhile, **D. candidum** (at the concentration of 2 mg/mL) also did not affect cells proliferation in MCF10A cells at 24 hours (Figure 4B), 48 hours (Figure 4C), and 72 hours (Figure 4D) compared to that in MCF-7 cells ($P > 0.05$).
Figure 3 Dendrobium candidum affects the mRNA and protein levels of key molecules involved in breast cancer cell growth and survival.

Notes: (A) qRT-PCR analysis showing significantly reduced mRNA levels of ERα, IGFBP2, IGFBP4, and GATA3 and significantly increased mRNA levels of ELF5, p53, p21, p18, CDH1, CDH2, and p12 in D. candidum-treated cells. Cells were treated with 2 mg/mL D. candidum for 48 hours. Data represent the mean ± SD, (n=3), **P<0.01, ***P<0.001 versus control cells. (B) Effects of D. candidum on protein levels of breast cancer biomarker (ERα, PGR, and GATA3), oncogene (p53, Ki67, and ELF5) in MCF-7 cells. Cells were treated with 2 mg/mL D. candidum for 48 and 72 hours. Then total cellular extracts were prepared and subjected to Western blot assay using antibody against ERα, GATA3, PGR, Ki67, p53, and ELF5, respectively. β-Actin was also tested to confirm equal loading. (C) Quantities were calculated as fold changes of untreated control cells (represented as 100%). Data are presented as mean ± SEM, (n=3), *P<0.05, **P<0.01, ***P<0.001 versus control cells.

Abbreviations: ERα, estrogen receptor alpha; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SD, standard deviation; h, hours; Con, control group; SEM, standard error of the mean.
Discussion

Previous studies reported that *D. candidum* could be served as a kind of promising functional food or medicine, but little scientific findings about its therapeutic effects were explored.\(^{17,18}\) There are many important components in *D. candidum*, including benzenes and their derivatives (with high concentration), lignans, flavonoids, phenolic, and lactone; meanwhile, 18 kinds of new pigments were also discovered in *D. candidum*.\(^{19}\) Recently, many studies have reported that *D. candidum* has some potential therapeutic functions on various pathologic forms, including hyperglycemic, immunity, cancer, and inflammation.\(^{20}\) Bao et al\(^ {21}\) found that *D. candidum* could inhibit the HepG2 liver cancer cell proliferation in vitro and preliminarily proved the anticancer effects of *D. candidum*. Although the studies have showed the therapeutic effects of *D. candidum*, the specific mechanisms of the anticancer effects have not been fully clarified.

The cell proliferation is the most important characteristic of the tumor cells and could be indicated by the cell viability. Therefore, in this study, we observed the cell viability in the control and *D. candidum*-treated cells, and explored the antiproliferation effects of *D. candidum* in vitro. The results indicated that the treatment of *D. candidum* could significantly decrease the cell viability at different concentrations (0.25, 0.5, 1, and 2 mg/mL) compared to the control group at 24, 48, and 72 hours, respectively (Figure 1A–C, \(P<0.05\)). Tokgun et al\(^ {22}\) proved that the extracts of *D. candidum* have cytotoxic effects on human breast carcinoma cell line MCF-7 cells. Zhao et al\(^ {17}\) found that *D. candidum* could inhibit the proliferation of the HCT-116 cells. Our result is consistent with these two studies.

In this study, we demonstrated that *D. candidum* inhibits the MCF-7 cell viability by enhancing the cell cycle arrest in the G2/M phase. Ben Sahra et al\(^ {23}\) proved that the metformin could inhibit the proliferation of prostate cancer cells by blocking the cell cycle in the G2/M phase and decreasing the cell growth. Achari et al\(^ {24}\) also reported that microRNA-34c affects the cell cycle mainly by inducing an arrest in the G2/M phase and frequently triggers the inhibitory effects of the breast cancer cell proliferation. Together with the above two studies, our study also showed that the induction of the G2/M cell cycle arrest in cancer cells could inhibit the cancer cells’ proliferation. Therefore, we explored the key molecules that regulate the processes of the breast cancer cell cycle arrest.
We examined the mRNA levels of the tumor growth-associated biomarkers, including ERα, IGBP2, IGFBP4, GATA3, and the mRNA levels of the apoptosis-associated biomarkers, including ELFS, p53, p21, p18, CDH1, CDH2, and p12. The results showed that all of the tumor growth biomarkers (ERα, IGBP2, IGFBP4, and GATA3) were significantly decreased or inhibited when treated with D. candidum. Also, all of the apoptosis biomarkers (ELF5, p53, p21, p18, CDH1, CDH2, and p12) were significantly increased. We also detected the protein levels and found that the breast cancer markers (ERα, PGR, and GATA3) were significantly decreased. While the oncogene Ki67 was significantly decreased, the oncogenes p53 and ELF5 were significantly increased. Grawenda et al\textsuperscript{25} reported that p53 mutation could represent the prognosis and harbor potential utility as a clinical tool. Si et al\textsuperscript{26} found that the expression of GATA3 is concurrently downregulated during breast cancer progression. Vernet-Tomas et al\textsuperscript{27} proved that the expression of p53 in breast cancer patients could predict a tumor with low probability of non-sentinel nodes infiltration. Fernandes et al\textsuperscript{28} also reported that PGR, ESR1, human epidermal growth factor-2, and Ki67 could act as the prognostic biomarker in different molecular subtypes of breast cancer. Comparing the above studies,\textsuperscript{25–28} we systematically analyzed the key molecules or biomarker, which could provide the targeting pathway for the therapy in clinical.

In addition, the normal breast epithelial cell line MCF10A was also used as a control to examine the cancer specificity of D. candidum. Treatment with D. candidum at any concentration and any time points caused no inhibitory effect on cell proliferation, suggesting that D. candidum selectivity inhibits MCF-7 breast cancer cell proliferation.

In conclusion, D. candidum could selectivity inhibit the breast cancer cell (MCF-7) proliferation by inducing cell cycle arrest at the G2/M phase and regulating the key biomarkers in breast cancer cells. Meanwhile, D. candidum does not affect the normal breast epithelial cells (MCF10A) growth.

Acknowledgments
Jun Sheng and Xin Hu contributed equally to this work.

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Disclosure
The authors report no conflicts of interest in this work.

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5. Alghamdi IG, Hussain II, Alghamdi MS, El-Sheemy MA. The power of the age standardized incidence rate to detect the gene link between breast cancer diseases: development of a new epidemiological method to save money, time and effort for genetic scientists. Onco Target Ther. 2015;8:677–687.
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Supplementary materials

**Figure S1** *Dendrobium candidum* affects the protein levels of the apoptosis biomarkers in breast cancer cells.

**Notes:** Total cellular extracts were prepared and subjected to Western blot assay using antibody against CDH2, CDH1, ELF5, p21, and p12, respectively. Actin was also tested to confirm equal loading. Quantities were calculated as fold changes of untreated control cells (represented as 100%). Data are presented as mean ± SEM, (n=3). *P<0.05, **P<0.01, ***P<0.001 versus control cells.

**Abbreviations:** h, hours; Con, control group; SEM, standard error of the mean.

**Figure S2** *Dendrobium candidum* decrease the Bcl-2 and increased the Bax level in breast cancer cells.

**Notes:** Total cellular extracts were prepared and subjected to Western blot assay using antibody against Bcl-2 and Bax, respectively. Actin was also tested to confirm equal loading. Quantities were calculated as fold changes of untreated control cells (represented as 100%). Data are presented as mean ± SEM, (n=3). *P<0.05, **P<0.01, ***P<0.001 versus control cells.

**Abbreviations:** h, hours; Con, control group; SEM, standard error of the mean.