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 cytometer.

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;3 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 \textit{D. candidum} on breast cancer. The key molecules involved in cell proliferation of breast cancer cells, including ER\alpha, PGR, GATA3, p53, Ki67, and ELF5, were examined to inhibit the proliferation of tumor cells.

The materials and methods section details the experimental procedures and materials used in the study. The cells were cultured in appropriate media and treated with \textit{D. candidum} for various time periods. Western blot analysis was performed to confirm the expression of key molecules.

The materials and chemicals section lists the specific materials and chemicals used in the study. The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco), and DMEM with 2.5 mM 1-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.

The MTT assay was used to evaluate the cell viability after treatment. Cells were seeded in a 96-well plate and treated with \textit{D. candidum} at different concentrations. The MTT assay was performed after 12, 24, 48, and 72 hours of incubation. The optical density at 490 nm was measured.

Western blot analysis was performed to confirm the expression of key molecules. Cells were lysed and the protein expression was determined by Western blotting. The key molecules involved in cell proliferation were examined and their expression levels were quantified.

The research was conducted in accordance with the ethical guidelines and institutional review board for the use of these cell lines.
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™ (TaKaRa, Dalian, People’s Republic of China). Primers are listed in Table 1.

<table>
<thead>
<tr>
<th>RT-PCR Primers</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>β-actin</td>
<td>GCCGCCAGCTCACCACACT</td>
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<td>CDK1</td>
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<td>CTCACGACATTTCTCGAGTGG</td>
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<tr>
<td>p21 (CDKN1A)</td>
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<tr>
<td>p18 (CDKN2C)</td>
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<td>ERα</td>
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<tr>
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<td>ELF5</td>
<td>CTCCTTTGGACATGAGCACC</td>
<td>AGCAGTTGGAATCACGACTCCTG</td>
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</table>

| Abbreviations: ERα, estrogen receptor alpha; RT-PCR, quantitative reverse transcription polymerase chain reaction. |

Statistical analysis

The quantitative analysis of the Western blot images was performed by using the computer-assisted software Image Quant SoftTech (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* 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, the percentage of viable cells decreased.
D. candidum (4 and 8 mg/mL), the cell viability was increased slightly compared to the concentration of 2 mg/mL (data not shown). Therefore, in the following experiments, 2 mg/mL was used as the concentration of D. candidum.

Meanwhile, Figure 1D also showed that the cell viability significantly increased, followed by the increased treatment time of D. candidum, compared to the control group at 12 hours (from 24 to 72 hours) (Figure 1D, P<0.05). This result suggests that D. candidum illustrated the optimized antiproliferation effect at 48 hours treatment at all of the D. candidum concentrations.

**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 ERα, IGBP2, IGFBP4, and GATA3...
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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 (\( P > 0.05 \)).
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, IGBP2, IGFBP4, and GATA3 and significantly increased mRNA levels of ELF5, p53, 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 al21 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 al22 proved that the extracts of D. candidum have cytotoxic effects on human breast carcinoma cell line MCF-7 cells. Zhao et al17 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 al23 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 al24 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, IGBPBP2, IGFBP4, GATA3, and the mRNA levels of the apoptosis-associated biomarkers, including ELF5, p53, p21, p18, CDH1, CDH2, and p12. The results showed that all of the tumor growth biomarkers (ERα, IGBPBP2, 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. reported that p53 mutation could represent the prognosis and harbor potential utility as a clinical tool. Si et al. found that the expression of GATA3 is concurrently downregulated during breast cancer progression. Vernet-Tomas et al. 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. 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, 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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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.