Effect of cucurbitacin on malignant biological behavior of breast cancer cells, and its possible underlying mechanism

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Abstract

Purpose: To study the influence of cucurbitacin on malignant biological behavior of mammary carcinoma cells, and the likely mechanism involved.

Methods: Human mammary carcinoma cell line MDA-MB-436 was selected for cell culture and treated with different concentrations of cucurbitacin. The effect of cucurbitacin on cell activity, cell colony-formation capacity, cell invasion, migration potential, matrix metalloproteinase-9 (MMP-9) activity, and levels of vascular endothelial growth factor A (VEGFA), epithelial calcium adhesion (E-cadherin), and neurogenic calcium adhesion (N-cadherin) were measured. Moreover, levels of wave protein (vimentin), phosphorylated epidermal growth factor receptor (p-EGFR), phosphorylated signaling transduction, and transcription activation factor 3 (p-STAT3) and phosphorylated protein kinase B (p-Akt) were determined.

Results: With increase in cucurbitacin dose, there was significant decrease in cell viability, cell colony ratio, cell invasion and migration capacity, and expression levels of MMP-9, VEGFA, e-cadherin, n-cadherin, vimentin, P-EGFR, P-STAT3 and p-Akt (p < 0.05).

Conclusion: Cucurbitacin inhibits the proliferation, invasion, and migration of breast cancer cells by down-regulating the expressions of EGFR/STAT3/Akt signaling-related proteins, and inhibiting epithelial-mesenchymal transition transformation.

Keywords: Cucurbitacin, Breast cancer, Malignant biological behavior, Risk factors

INTRODUCTION

Carcinoma of the breast is a frequently diagnosed malignancy in women. Statistics indicate that over 1.65 million new breast cancer cases are diagnosed annually around the world, resulting in more than 510,000 deaths. Breast cancer is the most frequent cause of death from female malignant tumors, and it seriously affects the life and health of patients. With changes in living standards and increasing social pressure, cases of breast carcinoma are on the increase,
and it is gradually becoming rampant in the younger population of women.

Triple Negative Breast Carcinoma accounts for about 30% of breast cancer cases [1]. Studies have found that triple-negative breast cancer is one of the breast cancers with the highest migration and invasion potential. It easily metastasizes and invades the lungs, brain and other important organs, and it is associated with high degree of recurrence. There are no extant and efficacious targeted treatments for triple-negative breast cancer [2]. Thus, there is an important need to investigate the etiopathology of triple negative breast carcinoma so as to discover effective treatment options and improve the prognosis of patients.

Cucurbitacin is a class of tetracyclic triterpenoid compounds that are cytotoxic to cancer cells. In TCM, it is used to clear away heat, dampness and yellowing [3]. It has been reported that cucurbitacin inhibited the activation of signal transduction and transcription activation factor 3 (STAT3) signal pathway, thereby inhibiting the invasion and metastasis of cancer cells [4]. Moreover, it has been reported that cucurbitacin markedly suppressed the proliferative and invasion potential of non-small lung cancer [5]. However, the impact of cucurbitacin on mammary carcinoma cells, and the underlying mechanism remain unclear. In this research, the influence of cucurbitacin on biological behaviors of mammary cancer cells, and the related mechanisms were investigated.

EXPERIMENTAL

Materials

Human breast cancer cell line MDA-MB-436 was obtained from Shanghai Binho Biological Technology Co. Ltd.

Main instruments and reagents

The following instruments and reagents were used: electronic balance (Tianjin Deante Sensor Technology Co. Ltd, model: ES-E); thermostatic water bath (Shanghai Yike Instrument Co. Ltd, model: HH-20); cell incubator (Shanghai Muni Experimental Equipment Co. Ltd, model: GW-100); -80 °C ultra-low temperature refrigerator (Beijing Iris Biological Technology Co. Ltd, model DW-86L626); low-temperature, high-speed centrifuge (Shanghai Luxiang Instrument Centrifuge Instrument Co. Ltd, model: BH1200R); biological microscope (Shanghai Yuguang Instrument Co. Ltd, model: WMS-1033); penicillin-streptomycin double anti-

solution (Shanghai Mingbo Biological Technology Co. Ltd); fetal bovine serum (Shanghai Yubo Biological Technology Co. Ltd); mouse anti-human EGFR polyclonal antibody (Hangzhou Haoxin Biotechnology Co. Ltd); mouse anti-human STAT3 polyclonal antibody (Shanghai Hengfei Biotechnology Co. Ltd); rabbit anti-human Akt monoclonal antibody (Shanghai Ziqi Biotechnology Co. Ltd), and cucurbitacin (Chengdu Pfield Biotechnology Co. Ltd., purity > 98%).

Study design

Cell culture

The MDA-MB-436 cells were cultured with 10% fetal bovine serum in a cell incubator at 37 °C and 5% CO2 atmosphere. The culture medium contained penicillin-streptomycin (100 U/mL). When the cell density reached about 75 – 85%, subculture was carried out. The culture medium was replaced timely during the culture process. A cell suspension was prepared using digestion with 0.25% trypsin, and an appropriate amount of the cell suspension was placed in the incubator for further culture.

Evaluation of cell viability/proliferation

The MDA-MB-436 cells were exposed to different cucurbitacin concentrations (10, 20 and 30 μM) in different wells for 24 h. Then, 10 μL MTT solution (5mg/mL) was added to each well and culturing was continued for 2 h. Thereafter, the medium was discarded, and the formazan formed was solubilized in dimethyl sulfoxide. Absorbance of the formazan solution was read at 520 nm. Each group of cells had 4 wells.

Clone formation studies

Appropriate amount of cell suspension was cultured for 24 h, after which 2 mL of different concentrations of cucurbitacin (10, 20 and 30 μM) were added to different wells. Following 2 weeks of culture, cell fixation in HCHO solution was done, followed by staining using crystal purple solution. The stained cells were washed with distilled water, examined under a microscope, and photographed.

Measurement of cell invasion

Cells (200 μL) were inoculated in top Transwell compartment (10,000 cells/mL), while 200 μL of zero-serum medium and matrix gel were evenly placed at the bottom of the chamber, until the matrix gel was solidified. Then, 400 μL of complete medium and 10% FBS were put in the
lower compartment. The cells were cultured for 12 h at 37 °C, followed by fixing, cleaning, drying, staining with crystal violet for 20 min, and rinsing with distilled water. Four fields of view were randomly selected and observed under a microscope.

**Cell migration assay**

The procedure was similar to that used in invasion assay, except that no matrix glue was added to the Transwell chamber.

**Western blotting**

The protein levels of MMP-9, VEGF, E-cadherin, N-cadherin, vimentin, p-EGFR, p-STAT3 and, p-Akt were determined with immunoblot assay. Cells in good growth state and in logarithmic growth phase were subjected to protein extraction using lysing with RIPA buffer, and after centrifugation, protein concentration of the lysate was determined with BCA procedure. Then, the proteins were separated with SDS-PAGE, followed by transfer to PVDF membranes which, following sealing, were incubated overnight at 4 °C with primary antibodies for MMP-9, VEGF, E-cadherin, N-cadherin, vimentin, p-EGFR, p-STAT3, P-Akt and GAPDH, followed by washing with TBST, and incubation with HRP-conjugated secondary antibody at room temperature for 2 h. Then, the membranes were washed with TBST. Finally, ECL was used for color development, and Image J Image analysis system was used for analysis of protein expression levels, relative to GAPDH which was the internal control.

**Statistical analysis**

Results are expressed as mean ± SD. Measurement data on cell activity, cell clone formation, cell invasion and migration, and expression levels of MMP-9, VEGF, E-cadherin, n-cadherin, vimentin, P-EGFR, P-Stat3 and p-Akt were determined with normal distribution. The SNK-q test was used for two-group comparison, while single-factor multivariate mean comparison was used for multiple-group comparison. The SPSS22.0 software was used for all statistical analyses, and p < 0.05 was taken as indicative of statistically significant difference.

**RESULTS**

**Effect of cucurbitacin on cell activity**

Compared with untreated group (control), cell viability decreased significantly with increase in cucurbitacin dose (p < 0.05). These results are presented in Table 1.

**Table 1: Effect of cucurbitacin on cell viability (mean ± SD, n = 4)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.00±0.01</td>
</tr>
<tr>
<td>10 μM</td>
<td>83.26±3.58&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20 μM</td>
<td>71.85±5.26&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>30 μM</td>
<td>54.33±1.51&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b, c</sup> P < 0.05, vs control<sup>a</sup>; vs 10 μM cucurbitacin group<sup>b</sup>; vs 20 μM cucurbitacin group<sup>c</sup>

**Effect of cucurbitacin on clone formation potential of cells**

Table 2 and Figure 1 show that, relative to control, cell clone formation potential was markedly reduced with increase in cucurbitacin dose (p < 0.05).

**Figure 1:** Impact of cucurbitacin on clonogenic ability. A: control group; B: 10 μM group; C: 20 μM group; D: 30 μM group

**Table 2: Effect of cucurbitacin on the clonogenic ability of cells (mean ± SD, n = 4)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell clone formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>99.87±0.26</td>
</tr>
<tr>
<td>10 μM</td>
<td>81.26±2.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20 μM</td>
<td>58.52±3.57&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>30 μM</td>
<td>39.73±2.46&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b, c</sup> P < 0.05, vs control<sup>a</sup>; vs 10 μM cucurbitacin group<sup>b</sup>; vs 20 μM cucurbitacin group<sup>c</sup>

**Effect of cucurbitacin on cell invasion potential**

The invasion potential of the cells was reduced significantly with increase in cucurbitacin dose, relative to control (Table 3 & Figure 2).

**Effect of cucurbitacin on cell migration**

Compared with control, with increase of cucurbitacin dose, the migration ability of cells decreased significantly (p < 0.05). These results are shown in Table 4 and Figure 3.
Figure 2: Effect of cucurbitacin on cell invasion capacity. A: control group; B: 10 μM group; C: 20 μM group; D: 30 μM group

Table 3: Effect of cucurbitacin on cell invasion capacity (mean ± SD, n = 4)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell invasion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>99.64±0.43</td>
</tr>
<tr>
<td>10 μM</td>
<td>85.71±4.28</td>
</tr>
<tr>
<td>20 μM</td>
<td>63.16±5.86</td>
</tr>
<tr>
<td>30 μM</td>
<td>37.48±6.2</td>
</tr>
<tr>
<td>F</td>
<td>93.15</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*a, b, c P < 0.05, vs control*a; vs 10 μM cucurbitacin groupb, vs 20 μM cucurbitacin groupc

Figure 3: Effect of cucurbitacin on cell migration. A: control group; B: 10 μM group; C: 20 μM group; D: 30 μM group

Table 4: Effect of cucurbitacin on cell migration (mean ± SD, n = 4)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell migration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>99.57±0.36</td>
</tr>
<tr>
<td>10 μM</td>
<td>86.06±4.33</td>
</tr>
<tr>
<td>20 μM</td>
<td>68.47±3.56</td>
</tr>
<tr>
<td>30 μM</td>
<td>42.66±2.76</td>
</tr>
<tr>
<td>F</td>
<td>246.71</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*a, b, c P < 0.05, vs control*a; vs 10 μM cucurbitacin groupb, vs 20 μM cucurbitacin groupc

Protein levels of MMP-9, VEGFA, E-cadherin, N-cadherin, vimentin, P-EGFR, P-Stat3 and p-Akt

The expression levels of MMP-9, VEGFA, E-Cadherin, N-cadherin, vimentin, P-EGFR, P-Stat3 and p-Akt were significantly decreased with increase in dose of cucurbitacin, when compared to the control group (p < 0.05; Figure 4).

DISCUSSION

Triple negative breast cancer is basal-like, and it is negative for progesterone receptor, human epidermal growth factor receptor-2 and estrogen receptor. However, triple negative breast cancer has a high degree of invasion and recurrence, poor prognosis and low 5-year survival, which pose a great threat to the quality of lives, survival and health of patients.

The current clinical therapy of mammary cancer involves extensive surgical resection in combination with radiation and chemotherapy. Although this strategy has resulted in some successes, the morbidity and mortality in patients (with poor prognosis) remain high. In addition, the susceptibility of triple negative breast cancer to endocrine therapy or molecular targeted therapy is low, and resistance to chemotherapy drugs occurs easily [6]. These problems increase difficulty in the treatment of patients. Trichosanthes kirilowii Maxim is one of the important herbs used in Traditional Chinese medicine (TCM). It is effective in relieving microvascular circulation disorders and reducing serum cholesterol levels [7].

Cucurbitacin is one of the bioactive components of Trichosanthes kirilowii. In a study, Ueno et al. found that cucurbitacin significantly inhibited the growth of lymphoma cells [8]. Moreover, cucurbitacin significantly altered the cell cycle and led to apoptotic changes colon carcinoma [9]. The purpose of this research was to determine the effect of cucurbitacin on biological behavior of mammary carcinoma cells, and the mechanism involved.
Cell proliferation, invasion and migration are important characteristics of malignant tumors. Lymph node metastasis is the most important and common mode of metastasis in breast cancer patients. Cell migration and invasion are complex processes which are important for tumor progression. Cell-cell adhesion, adhesion between cells and stroma, and cytoskeleton remodeling are important processes in cell migration and invasion [10]. Matrix metalloproteinase-9 (MMP-9) is a member of the MMPs family. Studies have found that when breast cancer occurs, the secretions of tissue inhibitors of MMPs are suppressed, and MMP activity is significantly increased, resulting in increased breakdown of extracellular matrix and enhancement of the invasion and migration of cancer cells [11]. It is known that vimentin and E- and N-cadherins are important indexes of epithelial-mesenchymal transformation, and they are involved in regulation of tumor cell invasion and migration [12].

In this study, the effects of cucurbitacin on the proliferation, invasion and migratory potential of MDA-MB-436 cells were determined. It was shown that cucurbitacin significantly inhibited the proliferation, invasion and migration of cells, and suppressed the angiogenesis of tumor cells. These data are in cinch with extant findings which showed that cucurbitacin significantly enhanced the activity of calcium protease by promoting MAPK-extracellular signaling and controlling the kinase reaction cascade; it stimulated the cleavage of plaque protein, affected cytoskeletal proteins, and altered cell morphology, thereby enhancing the invasion and migration of tumor cells [13,14].

The pathogenesis of tumor is a multi-gene and multi-stage biological phenomenon involving a variety of cell events in which abnormal cell signal transduction pathways play an important role [15]. The EGFR is a transmembrane glycoprotein that is clearly expressed in a variety of malignant tumors. It has been reported that when tumors occur due to the mutation of some genes, there are marked up-regulations in the number of proteins involved in the translation process, resulting in the proliferation and metastasis of tumors [16]. In a mouse study, Kumar et al. found that tumor growth was significantly slowed down when EGFR expression was inhibited, thereby suppressing the EGFR/JNK/Akt signaling pathway. This suggests that EGFR may regulate the tumor proliferative potential by regulating the expressions of the relevant proteins, or the activation of related pathways. In this study, it was found that cucurbitacin significantly suppressed the activation of the EGFR/STAT3/Akt pathway.

CONCLUSION

Cucurbitacin inhibits the proliferation, invasion and migration of breast cancer cells and suppresses epithelial-mesenchymal transformation by down-regulating the expressions of EGFR/STAT3/Akt signaling pathway-related proteins. Thus, cucurbitacin may be useful for clinical treatment of breast cancer.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Shengya Wang designed the study, supervised the data collection, and analyzed the data. Yun Ren interpreted the data and prepared the manuscript for publication. Yun Ren, Guangyuan Huang, Qiang Xue, Qinjing Lv, Yayun Wu, Qianfei Wu, Wei Yao, Bowen Wang and Shengya Wang supervised the data collection, analyzed the data, and reviewed the draft of the manuscript.

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REFERENCES


