

Dietary Compound Diosmetin Chemosensitizes Breast Cancer Stem Cells by Suppressing HMGB1-mediated Autophagy

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Abstract: Breast cancer stem cells (CSCs) are a small cluster of highly tumorigenic cell subsets with self-renewal and multi-directional differentiation potential. Accumulating studies have shown that CSCs lead to chemotherapy resistance and recurrence of breast cancer, which is the main reason for the failure of chemotherapy in breast cancer. Autophagy is one of the key mechanisms to protect tumor cells from chemotherapeutic stimulation and other stresses, which contributes to the ultimate survival of CSCs in refractory or recurrent tumors. This study was carried out in terms of multiple strategies such as flow cytometry, stem-like detection, zebrafish tumor xenograft model construction, autophagy lentivirus construction, and western blotting analysis to prove that a natural flavonoid compound diosmetin safely and effectively promoted the tumor-killing effect of chemotherapeutic drug for breast cancer. The involved mechanism was closely related to its ability to reduce HMGB1 expression and subsequently block the autophagy pathway of breast CSCs. This study will provide new ideas and tools for the development of breast cancer drug resistance mechanism and targeted drugs.

1 Background

Breast cancer is one of the most common cancer diseases in women. According to the statistics, breast cancer accounts for 30% of all types of cancer among female populations. Since 2004, the incidence of breast cancer has been increasing at a rate of about 0.3% per year, becoming the second leading cause of death and the leading cause of cancer death in women aged 20-59 [1]. Although various treatments including surgery, radiotherapy, chemotherapy, endocrine and targeted therapy have significantly reduced the mortality rate of breast cancer, it still remains a clinically intractable problem due to its chemotherapy-resistance and recurrence [2].

Accumulating studies have shown that CSCs lead to chemotherapy resistance and recurrence of breast cancer, which is the ultimate reason for the failure of radiotherapy and chemotherapy for breast cancer. Breast CSCs are a small cluster of highly tumorigenic cell subsets with self-renewal and multi-directional differentiation potential. Tumor multidrug resistance (MDR) includes primary and acquired categories of drug resistance. CSCs are inherently resistant to chemotherapies, and even simultaneously become multi-chemoresistant to several chemodrugs that are structurally unrelated and have no common mechanisms of action. The autophagy activation caused by anti-cancer treatment has been described as an adaptive mechanism for CSCs to survive from tumor microenvironment [3]. Autophagy has been described as a

lysosomal degradation process. In addition to being critical for the adaptive response to stress and the maintenance of cellular and tissue homeostasis in all eukaryotes, it also plays a critical role in the origin, maintenance, and invasiveness of CSC [4]. In our pilot study, it was found that the autophagic flux of CSCs was significantly higher than that of normal tissue cells, suggesting that they might be more prone to chemo-resistant and self-renewal. What's more, we applied a high-throughput autophagy chip to screen out autophagy-related genes closely related to breast cancer chemosensitivity, and the expression of autophagy-related high mobility group box 1 (HMGB1) increased most significantly.

At present, natural small molecule compounds have become the research focus of anti-tumor drugs due to their obscure side-effects and potential clinical value. In our pilot study, diosmetin was screened out and proved to be harboring strong anti-breast tumor activity. Diosmetin is a natural flavonoid compound, which is widely distributed in chrysanthemum, bergamot and other plants. It has anti-inflammatory, antibacterial, antioxidant and anti-tumor effects. Other study reported that diosmetin not only inhibited the proliferation and migration of tumor cells, but also suppressed tumor growth, angiogenesis and metastasis in mice, consequently serving as a potential adjuvant of chemotherapy drugs [5]. This study will further clarify its chemosensitizing effect and molecular mechanism on breast CSCs, and the research results will provide new

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ideas and tools for the development of breast cancer drug resistance mechanism and targeted drugs.

2 Materials and methods

2.1. Cell growth analysis

The cell proliferation was firstly detected by CCK8 kit according to the manufacturer's instructions. For cell number analysis, 3×10^5 cells were planted into each well of a 6-well plate and counted following the indicated treatment. For colony formation assay, treated cells were fixed with 4% PFA, stained with 0.5% crystal purple and observed with a microscope.

2.2. The assessment of CSC function

CSC population was determined by flow cytometry analysis under BD LSRFortessa. In addition, CSC self-renewal potency was evaluated by sphere-forming assay, while its differentiation ability was further confirmed by CSC differentiation experiment.

2.3. Establishment of zebrafish xenotransplantation model

The procedure was conducted following the previous study^[6].

2.4. LC3-mRFP-GFP Lentiviral Transfection

The autophagic activity was evaluated by using HBLV-mRFP-GFP-LC3-puro lentiviral vectors following the manufacturer's instructions.

2.5. Western Blotting Analysis

The procedure was followed with the previous study^[4]. The primary antibodies against LC3, P62, and HMGB1 were derived from Proteintech (Rosemont, United States). In addition, β -actin from CST (Boston, MA, USA) was served as a loading control. A chemiluminescent detection reagent (Tanon, Shanghai, China) was prepared to visualize the protein bands.

2.6. Statistical analysis

Either Student's t-test or ANOVA analysis based on SPSS results was used to determine the significance of the data between groups, while a significant difference was considered to be $p < 0.05$.

3 Results

3.1. Diosmetin remarkably promoted chemosensitivity in breast cancer

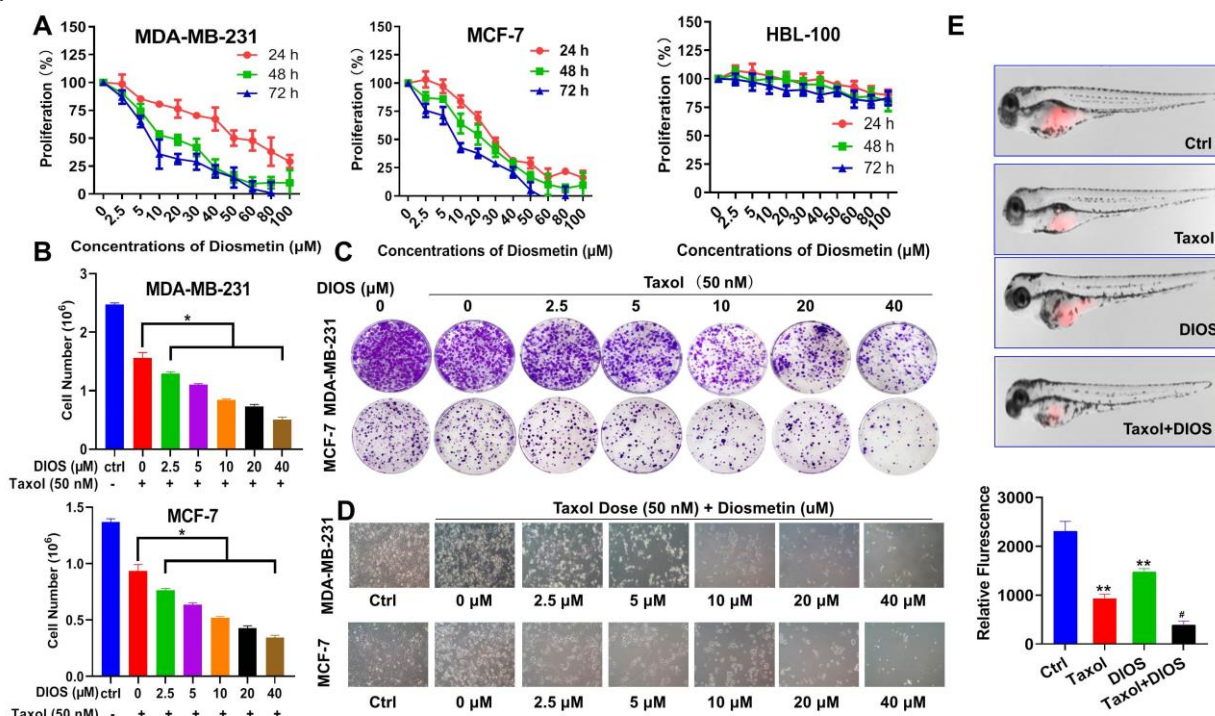


Fig. 1. Diosmetin remarkably promoted chemosensitivity in breast cancer. (A) CCK8 assay demonstrated that DIOS exerted an inhibitory effect on breast cancer cell lines MDA-MB-231 and MCF-7, while posing little cytotoxicity on a normal human breast epithelial cell line HBL-100; (B) Cell counting assay, (C) colony formation assay, and (D) morphological observation showed a synergistic effect of DIOS with 50 nM Taxol in MDA-MB-231 and MCF-7 cells; (E) DIOS significantly enhanced the inhibitory effects of Taxol on the zebrafish models bearing Dil-labelled MDA-MB-231 cells.

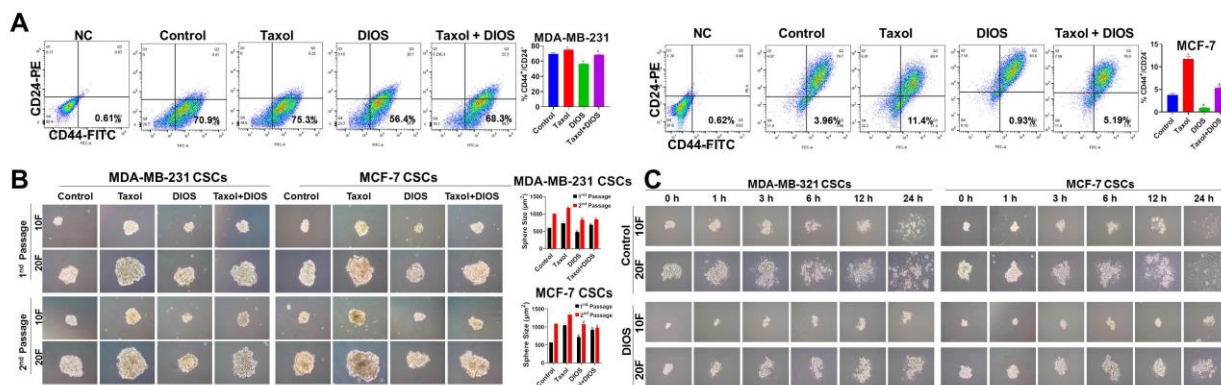


Fig. 2. The chemosensitivity of diosmetin was closely related to its anti-CSCs effect. (A) 48 h-treatment of DIOS led to a remarkable reduction of CD44⁺CD24⁻/low subsets in MDA-MB-231 cells and MCF-7 cells; (B) 20 μ M DIOS with or without 50 nM Taxol markedly limited number and sizes of the primary and secondary spheres; (C) DIOS administration inhibited the differentiation ability of breast CSCs in a time-dependant manner.

For the *in vitro* study, we firstly observed the effect of DIOS on the proliferation of breast cancer cell lines MDA-MB-231, MCF-7 and a normal human breast epithelial cell line HBL-100. The results of CCK-8 experiment were shown in Fig. 1A. DIOS significantly inhibited the proliferation of MDA-MB-231 and MCF-7 cells in a dose-and time-dependent manner, but posed no significant inhibitory effect on the normal human mammary epithelial cell HBL-100. The IC₅₀ of MDA-MB-231 cells at 24h, 48h, 72h was 50.8 μ M, 21.2 μ M, 7.684 μ M. The IC₅₀ of MCF-7 cells was 30.635 μ M, 22.542 μ M, and 8.625 μ M at 24h, 48h, and 72h, respectively, while different concentrations of DIOS had no significant inhibitory effect on HBL-100. Secondly, we detected the combinatory effect of DIOS with chemotherapeutic agent Taxol on the growth of breast cancer cells. MDA-MB-231 and MCF-7 cells were seeded in 6-well plates and treated with Taxol alone or in combination with different concentrations of DIOS (0 μ M, 2.5 μ M, 5 μ M, 10 μ M, 20 μ M, 40 μ M) for 48 h. As shown, DIOS increased the cytotoxicity of Taxol in breast cancer cells in a dose-dependent manner, presented as suppressed cell proliferation (Fig. 1B), decreased colony formation (Fig. 1C) and morphological changes under a microscope (Fig. 1D).

To further determine the anti-tumor effect of DIOS *in vivo*, MDA-MB-231 CSCs were stained with DiI (red fluorescent probe) and micro-injected into the abdomen of zebrafish larvae to construct a breast cancer xenograft model. They were divided into Ctrl group, DIOS (20 μ M) group, Taxol (50 nM) group as well as DIOS (20 μ M) + Taxol (50 nM) group. The chemosensitivity of zebrafish breast cancer cells to DIOS was observed under fluorescence microscope after 48 h administration. As shown in Fig. 1E, the fluorescence intensity (representing the tumor) in the DIOS group was significantly lower than that in the Ctrl group, indicating

that DIOS has a strong anti-tumor effect on tumors formed by CSCs. The inhibitory effect of DIOS combined with Taxol on tumor was significantly enhanced compared with DIOS or Taxol alone, indicating that DIOS could promote the chemosensitivity of breast CSCs.

3.2. The chemosensitivity of diosmetin was closely related to its anti-CSCs effect

CSCs are roots of chemoresistance in cancer. Herein, we confirmed that the chemosensitivity of DIOS was closely related to its ability to inhibit breast CSCs by flow cytometry, which showed that DIOS could significantly reduce the proportion of CSC populations in MDA-MB-231 and MCF-7 (Fig. 2A). Compared with the Ctrl group, the size of taxol-treated CSC spheres was larger in the sphere-formation assay, indicating that Taxol could enhance the ability of CSC sphere formation or self-renewal ability. When 20 μ M DIOS was used in combination with Taxol, the size of CSC spheres was significantly reduced, indicating that DIOS can effectively weaken the self-renewal ability of cancer stem cells induced by chemotherapeutic drugs (Fig. 2B). Furthermore, the CSC spheres which grew for about 7 days were selected and cultured in complete culture medium using adherent culture dishes, and their differentiation ability was compared at different time points. We found that the differentiation ability of CSC spheres was significantly reduced after DIOS treatment, indicating that DIOS could reduce the differentiation ability of breast CSCs (Fig. 2C).

3.3. Diosmetin inhibited autophagy activity of breast CSCs by down-regulating HMGB1 expression

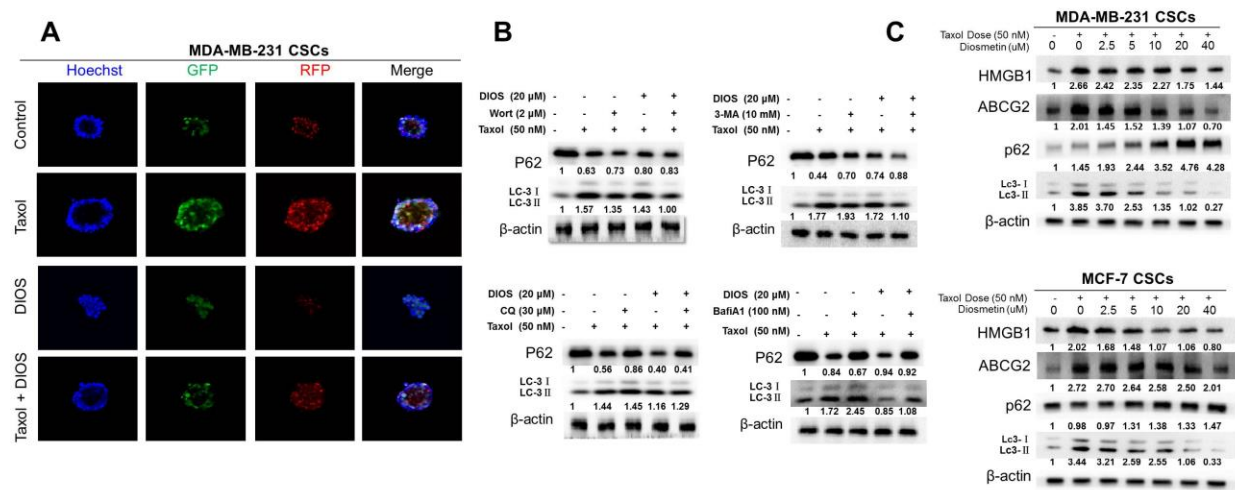


Fig. 3. Diosmetin inhibited autophagy activity of breast CSCs by down-regulating HMGB1 expression. (A) Fluorescence photographs of autophagic flux transfected with HBLV-mRFP-GFP-LC3-puro in CSC spheres after the indicated treatment; (B) Representative bands of LC3 II and P62 in MDA-MB-231 CSCs with or without the early-phase autophagy inhibitor (wortmannin, 3-MA) and the late-phase autophagy inhibitor (CQ, bafilomycin A1) after the indicated treatment; (C) Western blotting analysis showing the synergistic effects of DIOS with Taxol 50 nM.

MDA-MB-231-LC3 stable cells infected with LC3-mRFP-GFP autophagic lentivirus were used to detect changes in autophagic flow. MDA-MB-231-LC3 CSCs were cultured into spheres for the next experiments. Compared with the Ctrl group, the CSC spheres in Taxol group were larger and the autophagic flow was more obvious. It suggested that chemotherapeutic drugs could induce an increase in autophagy of CSC spheres, and such increase could be attenuated by DIOS (Fig. 3A). To further investigate the effect of DIOS on autophagy, we used different stages of autophagy inhibitors in conjunction with diosmetin. As shown in Fig. 3B, the effect of DIOS was consistent with that of the early-phase inhibitors wortmannin and 3-MA, that is, LC3II was reduced and P62 was increased by blocking the formation of autophagosomes, while LC3II and P62 were reduced by the late-phase inhibitors CQ and bafilomycin A1. Our result suggested that DIOS could reduce the early autophagy level of CSCs, subsequently reducing the self-renewal and differentiation ability of breast CSCs. In our pilot study, we used high-throughput autophagy chip to screen autophagy-related genes closely related to the sensitivity of breast cancer chemotherapy, and the expression of autophagy-related HMGB1 increased most significantly. Herein, Taxol administration resulted in increased HMGB1, ABCG2, and autophagic flow compared to Ctrl, while the addition of DIOS resulted in the decrease of HMGB1 expression and autophagy levels. Our finding indicated that DIOS might regulate the autophagy-related pathway to promote breast CSC chemosensitization by down-regulating HMGB1 expression. As yet, it is still unclear exactly how DIOS down-regulates HMGB1 expression to induce chemosensitization of breast CSCs *via* the autophagy pathway, which deserves further study (Fig. 3C).

4 Discussion and conclusion

In summary, this study demonstrated that diosmetin had significant anti-tumor activity and chemosensitizing effect, which was at least partly due to the weakened stem-like potency of breast CSCs. Furthermore, its inhibitory effect was closely related to its reduction of HMGB1 expression to block the autophagic pathway of breast CSCs. Our data also suggested that HMGB1 might increase the chemoresistance of CSCs by autophagic activation, and targeting HMGB1 to suppress autophagy might become one of the key therapeutic mechanisms targeting CSCs.

Acknowledgement

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