Original Research Article

Zerumbone decreases BACH1 levels by upregulating miR-708 to inhibit breast cancer cell proliferation and invasion

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Abstract

Purpose: To investigate the potential mechanism by which zerumbone suppresses breast cancer (BC) cells.

Methods: Cell viability and Transwell assays were performed to assess the effect of zerumbone on BC cell growth. The downstream target of zerumbone was determined using quantitative polymerase chain reaction assays and immunoblotting. Cell viability assays and immunoblotting were conducted to detect if zerumbone had any effect on BACH1 (BTB domain and CNC homolog 1) expression.

Results: Zerumbone suppressed the proliferation, migration, and invasion of BC cells. It also upregulated the expression of microRNA (miR)-708 and, hence, suppressed BACH1 expression. Furthermore, zerumbone suppressed the proliferation and invasion of BC cells by promoting miR-708 expression and suppressing BACH1.

Conclusion: The findings help clarify the anti-tumor mechanism of zerumbone and provide theoretical and therapeutic bases for the anti-tumor effects of Chinese herbal medicine.

Keywords: Breast cancer, Zerumbone, Cell invasion, MiR-708, BACH1

INTRODUCTION

Breast cancer (BC) is the most common gynecological cancer, with the highest incidence among female malignant tumors [1]. Advanced BC has a poor prognosis due to its high metastasis rate and heterogeneity [2]. The standard treatments for advanced BC include surgery and chemoradiotherapy [3]. Surgical resection often results in BC recurrence, while chemoradiotherapy is associated with significant side effects, and the low concentration of chemoradiotherapy drugs at the tumor site limits its effects on tumor tissues [4]. Common anti-tumor drugs for BC have poor penetration and can induce drug resistance, limiting their efficacy [5]. Therefore, it is necessary to develop new treatments to improve the prognosis of patients with advanced BC.

Zerumbone is a type of double half mushroom compound extracted from ginger rhizomes demonstrated to have a variety of biological effects such as anti-inflammation and

ameliorating behavioral impairments [6]. Importantly, anti-tumor effects of zerumbone have been described in recent decades [7]. A previous study reported that zerumbone affected cell proliferation and induced cell cycle arrest in human laryngeal carcinoma HEP-2 cells [8]. It was shown to inhibit invasion by human non-small cell lung cancer (NSCLC) cells via the FAK/AKT/ROCK pathway [9]. Zerumbone also enhanced apoptosis in human colon cancer cells by inducing the death receptor, and it suppressed angiogenesis in pancreatic cancer by blocking nuclear factor (NF)-κB [10]. In BC, zerumbone was found to suppress BC cell invasion and induce apoptosis [11]. However, the potential mechanism remains unclear.

Zerumbone exerts biological activity by affecting microRNAs (miRNAs) [12]. For example, it attenuated obesity induced by a high-fat diet by regulating miR-146B levels and sirtuin 1-mediated fat production [13]. One recent study demonstrated decreased miR-708 expression in BC, and up-regulation of miR-708 suppressed tumor growth and drug resistance [14]. Here, we performed experiments to better understand the regulatory effects of zerumbone on BC progression.

EXPERIMENTAL

Cell culture

The BC cell line MDA-MB-231 was obtained from the American Type Culture Collection (ATCC, USA) and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (Invitrogen, USA) at 37 °C with 5% CO_2.

Cell viability assay

Cells were incubated in 96-well plates at a density of 2×10^3 cells in 100 μL medium per well for 24 h before use in experiments to allow total adherence. Cell viability was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays according to the manufacturer's instructions.

Colony formation assay

BC cells (1×10^3) were seeded into 6-well culture plates and incubated for 2 weeks. For visualization, cells were fixed with paraformaldehyde (PFA) for 10 min, and then stained with crystal violet staining solution and photographed. The numbers of colonies were counted manually.

Cell migration and invasion assays

Wound healing assays were used to determine cell migration. The cell layer was wounded by scraping with a 200-μL tip, followed by washing. Subsequently, serum-free culture medium was added to stimulate wound healing. Photographs were taken at 0 and 24 h to evaluate cell migration.

Transwell chambers (8-μm pore size; Corning, USA) with 20 % Matrigel in the upper chamber were filled with BC cells (Becton Dickinson, USA). Complete medium was added to the bottom chamber. After 48 h of incubation, cells in the upper chamber were induced to migrate toward the bottom chamber. Then the upper chamber medium and the lower chamber cells were fixed in PFA, stained with 0.1 % crystal violet, and counted under a microscope.

Quantitative polymerase chain reaction (PCR)

Total RNA was extracted from cells by TRIzol reagent (Invitrogen, USA) and reverse-transcribed with a Transcriptor First Strand cDNA Synthesis Kit (Takara, Japan). A SYBR Ex Taq kit (Takara) was used to amplify indicated genes.

The expression levels of related genes were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). For miR-708 quantitative PCR (qPCR), we used a TaqMan microRNA RT kit (Applied Biosystems, USA). The primers used in this assay were listed as follows:

BACH1 (BTB domain and CNC homolog 1): forward: 5'- ATTCATGCTTCTGTTCAGCCAA-3', Reverse: 5'- GGCACTGAGAAGCAGGATCTTT-3'; GAPDH: forward: 5'-CTCTGCTCCTCCTG TTCGAC-3', Reverse: 5'-ACCAAATCCGTTGA CTCCGA-3'.

Luciferase reporter assay

BACH1 primers (Forward: 5'-CAGACTAGTA AGCCAATGGAACCCTTGATT-3' and Reverse: 5'-CAGAAGCTTGCCTTGAAACATTTTCTTAGA A-3') were used for amplification. BC cells were seeded in 12-well plates 1 day before transfection with pGL3-wt-Bach1-UTR, pGL3-mu-Bach1-UTR, miR-708 mimic, or negative control mimics using Lipofectamine 2000 (Invitrogen). At 48 h after transfection, the cells were collected for relative luciferase activity measurement using Dual Luciferase Assays (Cat#: E1910, Promega, USA).

Immunoblotting

Cells were harvested and lysed for protein extraction. Denatured protein samples were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride membranes (Millipore Corporation, USA). Antibodies used for immunoblot analysis were against BACH1 (1:200 dilution, sc-14700, Santa Cruz Biotechnology, USA), matrix metalloproteinase (MMP)2 and -9 (1:1500 dilution, Santa Cruz Biotechnology), and GAPDH antibody (1:3000 dilution, sc-32233, Santa Cruz Biotechnology) was used as loading control. All protein bands were detected using an enhanced chemiluminescent substrate (Cell Signaling Technology, USA).

Statistics

GraphPad 5.0 software was used for all statistical analyses. Data are presented as mean ± SEM. Student' t-tests was performed to compare groups, and $p < 0.05$ was considered significant.

RESULTS

Zerumbone inhibits BC cell proliferation, migration, and invasion

Viability assay was performed using MDA-MB-231 cells treated with different doses of zerumbone to investigate its effects on BC cell proliferation. Cell viability was reduced after exposure to higher concentrations of zerumbone (Figure 1 A). Next, Transwell assays were performed. Zerumbone significantly reduced both BC cell migration and invasion at the dose of 50 µM (Figure 1 B). These results show that zerumbone suppressed BC cell proliferation, migration, and invasion *in vitro*.
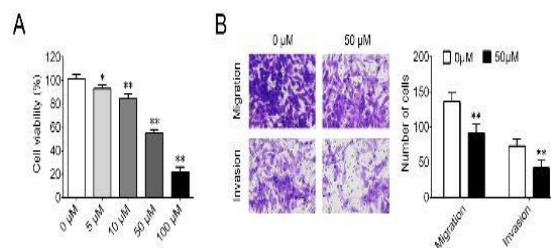

Figure 1: Zerumbone inhibits BC cell proliferation, migration, and invasion. (A) Cell viability assays showing reduced proliferation in zerumbone-treated cells. (B) Transwell assays showing that zerumbone inhibits MDA-MB-231 cell migration and invasion; *$p < 0.05$, **$p < 0.01$ versus control

Zerumbone upregulates miR-708 and downregulates BACH1

MDA-MB-231 cells were treated with varying doses of zerumbone, and miR-708 and BACH1 expression were measured via qPCR. The results demonstrated that miR-708 and BACH1 were up- and downregulated, respectively (Figure 2 A and B). Immunoblotting showed that BACH1 protein levels were decreased by zerumbone in a dose-dependent manner (Figure 2 C).
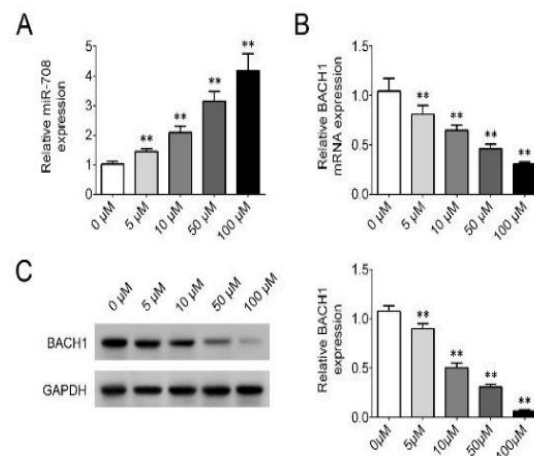

Figure 2: Zerumbone upregulates miR-708 and downregulates BACH1 expression. (A) Increased miR-708 was observed in qPCR assays of MDA-MB-231 cells following zerumbone treatment. (B) Decreased BACH1 expression was observed in qPCR assays following zerumbone treatment. (C) BACH1 levels were confirmed by immunoblot assays; **$p<0.01$ versus control

Zerumbone downregulates BACH1 by upregulating miR-708

To investigate the possible link between BACH1 and miR-708, a search was performed in the starBase website (http://starbase.sysu.edu.cn/index.php) to identify potential targets of miR-708. BACH1 was listed as a putative target of miR-708 (Figure 3 A). Luciferase assays were performed to validate the interaction between BACH1 and miR-708. Wild-type BACH1 luciferase activity was significantly decreased in miR-708 mimic-transfected cells (Figure 3 B). However, mutant BACH1 luciferase activity was not significantly different in BC cells transfected with miR-708 mimic, indicating that miR-708 interacted with BACH1. Moreover, zerumbone treatment decreased BACH1 levels, which were increased after addition of an miR-708 inhibitor (Figure 3 C).
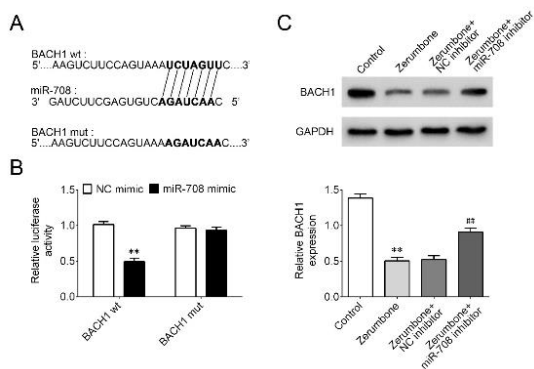
Figure 3: Zerumbone downregulates BACH1 via upregulating miR-708. (A) The predicted binding sites between BACH1 and miR-708 and the BACH1 mutant sequence. (B) Luciferase assays for BACH1 (Wt) or BACH1 (Mut) reporter activity following miR-708 mimic transfection in MDA-MB-231 cells. (C) Immunoblotting showing that the zerumbone-induced reduction of BACH1 could be reversed by miR-708 inhibitor; **$p<0.01$ and ##$p<0.01$ versus control

Zerumbone inhibits BC cell proliferation, migration, and invasion by suppressing BACH1 expression

The final set of experiments examined whether zerumbone affected BC cell proliferation, migration, and invasion through BACH1. Cell proliferation was assessed in zerumbone-treated BC cells with or without BACH1 overexpression. The results revealed that BACH1 transfection blocked the anti-proliferative effect of zerumbone (Figure 4 A). Consistent with this observation, BACH1 also reversed the inhibitory effects of zerumbone on BC cell migration and invasion as indicated by MMP2 and -9 levels (Figure 4 B). Taken together, these findings support the hypothesis that zerumbone inhibits BC cell proliferation, invasion, and migration by regulating BACH1.
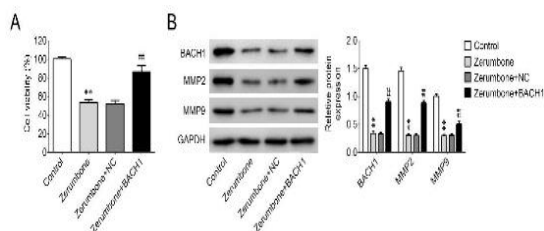

Figure 4: Zerumbone downregulates BACH1 by upregulating miR-708. (A) Cell viability assays showing that zerumbone's inhibition of cell proliferation was abrogated by BACH1 overexpression. (B) Transwell assays showing that decreased invasion in the presence of zerumbone was reversed by BACH1 overexpression; **$p < 0.01$, ##$p < 0.01$, versus control group

DISCUSSION

In recent years, several subsets of BC have been described, and the associated morbidity and mortality are high [15]. Beyond surgical resection and chemoradiotherapy, a variety of new treatment methods have helped improve BC patient prognosis and the cure rate of advanced patients [16]. Endocrine and targeted therapies for BC have made progress, and traditional Chinese medicine also has unique advantages in the fight against advanced BC [17]. A variety of traditional Chinese medicine extracts, such as the active triterpenoid monomer compound KHF16, have significant anti-tumor effects and inhibit BC cell proliferation [18,19]. To further improve the prognosis of patients with advanced BC, it is necessary to identify effective anti-tumor Chinese medicine extracts and characterize their effects.

MTT viability and colony formation assay data revealed that zerumbone suppressed BC cell proliferation in a concentration-dependent manner. Wound closure and Transwell assays further demonstrated that zerumbone has inhibitory effects on BC cell migration and invasion. Immunoblotting, qPCR, and luciferase assays further confirmed the compound's anti-tumor effects on BC through the miR-708-BACH1 axis. This is in line with previous publications describing the anti-tumor effects of zerumbone [20].

One group reported that zerumbone inhibited colorectal cancer cell invasion via the FAk/PI3K/NFκB-uPA pathway [9]. Another found that zerumbone suppressed esophageal cancer cell migration by promoting Rac1 ubiquitination [21]. Zerumbone can affect the proliferation, migration, invasion, and apoptosis of multiple types of cancers, such as prostate, cervical, hepatoma, and oral cancers [22]. These studies, together with our findings, demonstrate that zerumbone exerts anti-tumor effects through different pathways.

Zerumbone affects multiple cellular processes by targeting miRNAs [12]. Zerumbone suppressed epithelial-mesenchymal transition by inhibiting beta-catenin signaling through regulation of miR-200c [12]. Another study demonstrated that zerumbone ameliorated high-fat diet-induced adiposity via targeting miR-146b [13]. Interestingly, miR-708 was down-regulated in BC tissues, and a previous study found that miR-708 upregulation was correlated with tumor metastasis [23]. In the present study, we observed that zerumbone regulated miR-708 expression and might suppress BC progression.

The results reveal a novel mechanism underlying the anti-tumor activity of zerumbone.

BACH1 is an oncogene that affects the progression and metastasis of multiple types of tumors, including pancreatic, lung , and colorectal cancers [24]. BACH1 serves as a prognostic biomarker and affects BC cell migration and apoptosis [25]. Collectively, our results show that zerumbone affected BC cell proliferation, migration, and invasion by targeting miR-708 and BACH1 and suggest that identifying inhibitors that target BACH1 could be an effective therapeutic strategy for BC.

CONCLUSION

Zerumbone suppresses the proliferation, migration, and invasion of BC cells *in vitro*. These effects are due to zerumbone's ability to upregulate miR-708 and suppress BACH1 expression. Thus, zerumbone is a promising drug for the management of BC.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done 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. Fuguang Zhao and Yong Cai designed the study, supervised the data collection, and analyzed the data; Bo Ma and Zhenye Lv interpreted the data and prepared the manuscript for publication; Jie Chen, Yuanjie Cai, and Chunjing Xu supervised the data collection, analyzed the data, and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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