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Original Research Article

Involvement of Wnt/β-catenin pathway in the inhibition of invasion and epithelial-mesenchymal transition in ovarian cancer cells

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Abstract

Purpose: *To investigate the effects of zerumbone on cell invasion, epithelial-mesenchymal transition (EMT) and the potential signaling pathway involved in ovarian cancer cells.*
Methods: *Caov-3 cell proliferation was assessed using 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay. Wound healing assay was used to determine Caov-3 cell migration while cell invasion was evaluated using Transwell assay. Protein expression was determined by western blot.*
Results: *Cell viability was reduced by 5, 10, 20, and 50 μM zerumbone ($p < 0.05$) in a concentration-dependent manner while cell migration and invasion were inhibited by 10 and 20 μM zerumbone ($p < 0.05$). Protein expression levels of E-cadherin and cytoplasm β-catenin were upregulated by zerumbone ($p < 0.05$) in a concentration-dependent manner. On the other hand, protein expression levels of N-cadherin, vimentin, ZEB1, nuclear β-catenin, and c-Myc were suppressed by zerumbone ($p < 0.05$) also in a concentration-dependent manner.*
Conclusion: *The results demonstrate that zerumbone inhibits cell proliferation, migration and invasion, but represses the EMT process via inactivation of Wnt/β-catenin signaling pathway.*

Keywords: *Zerumbone, Ovarian cancer, Wnt/β-catenin pathway, Epithelial-mesenchymal transition*

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INTRODUCTION

Ovarian cancer is the most aggressive cancer and the fifth leading cause of death from gynecological malignancies among women [1]. Approximately 70% of ovarian cancer cases are diagnosed at the advanced stage due to the lack of specific symptoms during the early stage [2]. Over the past 20 years, chemotherapy for ovarian cancer has consisted mainly of a combination of a platinum agent and a taxane [3]. There is a pressing need for new therapeutic regimens in order to improve the prognosis of ovarian cancer patients.

The epithelial-mesenchymal transition (EMT) is a cell biological process in which epithelial cells are transformed into quasi-mesenchymal cells [4],

resulting in cells migrating more aggressively to the surrounding tissues [5]. The EMT plays a key role in cancer development and metastasis [6]. Inhibition of EMT is a therapeutic approach in anticancer treatment [7].

The Wnt/β-catenin signaling pathway contributes to the regulation of cell proliferation, survival, and stem cell fate [8]. This pathway is strictly controlled in normal cells, and the dysregulation of this pathway is linked to disease progression, including cancer [9]. Increasing evidence reveals that the Wnt/β-catenin signaling pathway is involved in cancer development, metastasis, and cancer cell survival [10]. In ovarian cancer, Wnt/β-catenin signaling pathway promotes ovarian cancer cell proliferation and is involved in the EMT, leading to cancer initiation and progression [9]. Targeting the Wnt/β-catenin pathway may be effective in the treatment of ovarian cancer.

Zerumbone (2,6,9,9-tetramethyl-(2*E*,6*E*,10*E*)-cycloundeca-2,6,10-trien-1-one) is a monocyclic sesquiterpene compound (Figure 1A) extracted from the traditional plant *Zingiber zerumbet* [11]. Zerumbone has many biological effects, including antioxidant, anti-inflammatory, antibacterial, and anti-tumor effects [12,13]. In human colorectal cancer, zerumbone has been shown to prevent cancer invasion and metastasis through inhibition of the FAK/PI3K/NF-κB-uPA signaling pathway [14]. By targeting the TGF-β1 signaling pathway, zerumbone was shown to repress the tumorigenicity and mobility of triple-negative breast cancer cells [15]. The goal of this investigation was to investigate the effects of zerumbone on cell invasion and the EMT and to elucidate the potential signaling pathway involved in Caov-3 cells, a human ovarian cancer cell line.

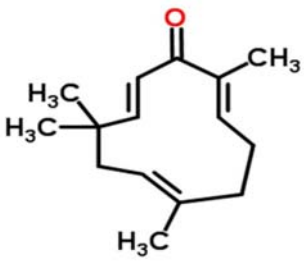

Figure 1: Chemical structure of zerumbone

EXPERIMENTAL

Cell culture and treatment

The Caov3 human ovarian cancer cell line was purchased from Wuhan Procell Life Science & Technology Co. Ltd, China. Caov-3 cells were cultured with DMEM which contained fetal bovine serum (FBS; 10%, v/v; Beyotime, China), penicillin-streptomycin (1%, Sigma-Aldrich, USA) with 95% and 5% CO_2 at 37°C. Caov-3 cells were seeded into sterile 6-well plates. At a cell confluence of 80–90%, cells were treated with vehicle 5, 10, 20, and 50 μM of zerumbone (Shyuanye, China) for 24 h.

3-(4,5)-Dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay

The MTT assay was conducted using a commercial MTT assay kit (Abcam, UK) according to the manufacturer's instructions. Briefly, Caov-3 cells were seeded into sterile 96-well plates. Following treatment with zerumbone for 24 h, the culture medium was removed, and 20 μL MTT reagent was added and incubated for 3 h at 37ºC. Next, the MTT solvent was added and the 96-well plate was incubated on an orbital shaker for 15 min. The optical density (OD) values at 590 nm were read in a spectrophotometer.

Wound healing and Transwell assay

Cell migration was examined using a wound healing assay. Caov-3 cells were cultured in 6-well plates. At a cell confluence of 80–90%, a straight line was scratched using a pipette tip on the bottom of the culture plate. Cells were then treated with different concentrations of zerumbone for 24 h as described above. The scratch width was photographed and measured under the microscope (Nikon, Japan).

Cell invasion was determined using the TranswellT assay. Caov-3 cells were cultured in the upper chamber of a 24-well insert (8-μm pore size; Corning, USA) that was precoated with Matrigel. The upper chamber was filled with FBS-free medium while the lower chamber was filled with FBS-containing medium. After treatment with zerumbone for 24 h, cells on the upper side were removed, and the membrane was fixed with methanol and stained with crystal violet. Invading cells were imaged under the microscope, and their numbers were recorded.

Western blot analysis

After treatment with zerumbone for 24 h, RIPA cell lysis buffer (Beyotime, China) and BCA Protein Assay Kit (Abcam, UK) were used to extract proteins and measure the protein concentration, respectively. Five micrograms of total protein were electrophoresed on a 10% polyacrylamide gel (SDS-PAGE) and then

transferred to PVDF membranes (Merck KGaA, Germany). The membranes were then incubated with blocking buffer (Abcam, UK) for 1 h and incubated with the following primary antibodies: E-cadherin (sc-21791, 1:1,000), N-cadherin (sc-8424, 1:2,000), Vimentin (sc-6260, 1:1,500), ZEB1 (sc-81428, 1:800), β-catenin (sc-7963, 1:1,000), c-Myc (sc-40, 1:1,000), and GAPDH (sc-47724, 1:10,000). After incubation overnight at 4ºC, the membranes were probed with secondary antibodies (Santa Cruz, USA). The strength of the protein signal was detected with SignalFire ECL reagent (Cell Signaling, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.1.0 and the data are expressed as mean ± SEM. Comparison between two groups was performed using one-way ANOVA followed by Bonferroni *post hoc* test. $P < 0.05$ was considered significant.

RESULTS

Zerumbone inhibited cell viability in Caov-3 cells

Results from the MTT assay revealed that cell viability was inhibited by 5, 10, 20, and 50 µM zerumbone when compared with the control group, and this inhibition was concentration-dependent (Figure 2).

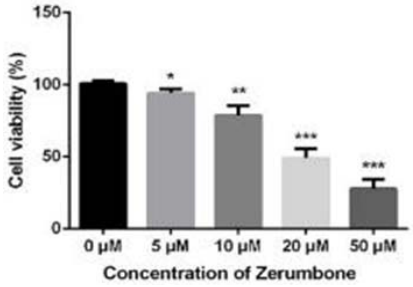

Figure 2: Effect of zerumbone on Caov-3 cell viability. *$p < 0.05$ vs. **$p < 0.01$ vs. 0 µM; ***$p < 0.005$ vs. 0 µM

Zerumbone reduced cell migration and invasion in Caov-3 cells

Following treatment with zerumbone for 24 h, the scratch width was larger in the cells treated with 10 and 20 µM zerumbone than in those treated with vehicle alone. Zerumbone inhibited Caov-3 cell migration in a concentration-dependent manner (Figure 2 A). The number of invading cells was reduced in cells treated with 10 and 20 µM of zerumbone, compared to vehicle-treated cells. Zerumbone inhibited Caov-3 cell invasion in a concentration-dependent manner (Figure 2 B).

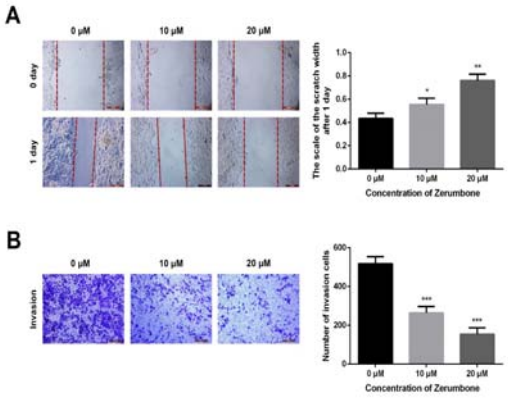

Figure 3: Caov-3 cell migration (A) and invasion (B) were inhibited by zerumbone; *$p < 0.05$ vs. 0 µM; **$p < 0.01$ vs. 0 µM; ***$p < 0.005$ vs. 0 µM

Zerumbone prevented epithelial-mesenchymal transition in Caov-3 cells

After treatment with zerumbone for 24 h, overexpression of E-cadherin was observed in cells treated with 5, 10, 20, and 50 µM zerumbone, and this upregulation of E-cadherin was enhanced in a concentration-dependent manner. The protein expression levels of N-cadherin, vimentin, and zinc-finger E-box-binding 1 (ZEB1) were reduced in cells treated with 5, 10, 20, and 50 µM of zerumbone compared to those treated with vehicle alone, in a concentration-dependent manner (Figure 3).

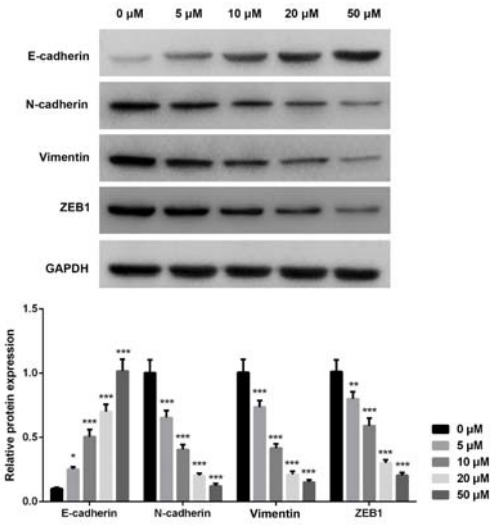

Figure 4: Epithelial-mesenchymal transition was suppressed by zerumbone; *$p < 0.05$ vs. 0 µM; ***$p < 0.005$ vs. 0 µM

Zerumbone repressed the activation of Wnt/β-catenin signaling pathway in Caov-3 cells

Following treatment with zerumbone for 24 h, the expression of β-catenin in the cytoplasm was upregulated in cells treated with 5, 10, 20, and 50 μM zerumbone in a concentration-dependent manner. Expression of β-catenin in the nucleus was downregulated in cells treated with 5, 10, 20, and 50 μM zerumbone in a concentration-dependent manner. Suppression of c-Myc was observed in cells treated with 5, 10, 20, and 50 μM of zerumbone in a concentration-dependent manner (Figure 4).

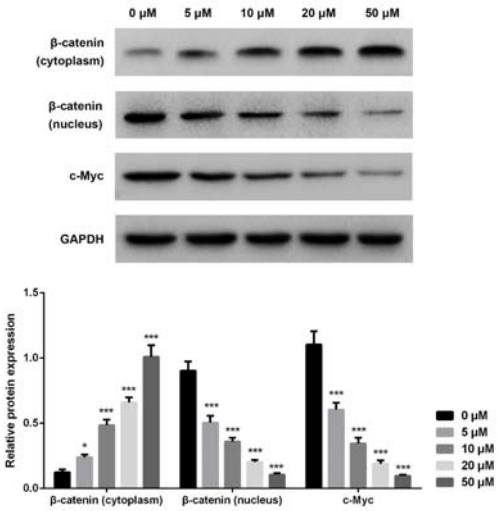

Figure 5: Activation of Wnt/β-catenin signaling pathway was repressed by zerumbone; *p < 0.05 vs. 0 μM; ***p < 0.005 vs. 0 μM

DISCUSSION

The present study demonstrated that zerumbone plays an anti-tumorigenic role in ovarian cancer. Cell proliferation, migration, and invasion were prevented by zerumbone, consistent with findings in previous studies that zerumbone induced cell cycle arrest in ovarian and cervical cancer cells [16]. Furthermore, the EMT process was also repressed by zerumbone, confirming that zerumbone exerted protective and therapeutic effects on ovarian cancer. These results demonstrate that zerumbone might inhibit ovarian cancer progression and metastasis through the suppression of cell proliferation, migration and invasion, and by inhibiting the EMT process.

Initiation of the EMT is accompanied by the overexpression of mesenchymal marker proteins, such as N-cadherin, vimentin, and ZEB1, and suppression of E-cadherin, a cell adhesion molecule [17,18]. During EMT, upregulation of N-cadherin contributes to cell mobility and invasion s[19]. In this study, a reduction in N-cadherin was observed after treatment with zerumbone, and cell migration and invasion were also inhibited by zerumbone, indicating that downregulation of N-cadherin is associated with decreased cell migration and invasion. E-cadherin is typically expressed in epithelial cells, and loss of E-cadherin occurs during EMT in many cancers [20].

In this study, the expression of E-cadherin was increased with zerumbone treatment, indicating that the EMT process was reversed by zerumbone treatment. Furthermore, the expression of vimentinv and ZEB1 is required for the EMT in cancer [21]. Taken together, these data indicate that expression levels of N-cadherin, vimentin, and ZEB1 were downregulated, and that of E-cadherin was upregulated, upon zerumbone treatment, suggesting that zerumbone prevents the EMT process and that all these four proteins involved in the EMT are potential therapeutic targets for ovarian cancer treatment.

Activation of the Wnt/β-catenin signaling pathway has been observed in almost all types of ovarian cancers [22]. By binding to Wnt receptors, Wnt proteins induce the deactivation of GSK-3β through Ser9 phosphorylation, and release β-catenin, resulting in the translocation of activated β-catenin from cytoplasm to the nucleus, where β-catenin interacts with transcription factors to activate cell proliferation and survival [23]. In this study, β-catenin expression in both the cytoplasm and nucleus was increased in a concentration-dependent manner following treatment with zerumbone, demonstrating that translocation of β-catenin is inhibited by zerumbone.

c-Myc is one of the target genes of the Wnt/β-catenin signaling pathway [24]; overexpression of c-Myc has been observed in ovarian cancer, and contributes to platinum resistance in ovarian cancer [25]. The results of this study indicate that zerumbone causes a reduction in c-Myc expression in a concentration-dependent manner, with the consequent inactivation of the Wnt/β-catenin signaling pathway and suppression of ovarian cancer progression. Because the Wnt/β-catenin signaling pathway is involved in the regulation of EMT [9], the data from this study also indicate that suppression of the EMT by zerumbone is mediated via inhibition of the Wnt/β-catenin signaling pathway. In summary, cell viability, migration, and invasion are repressed by zerumbone, indicating that its

protective effects are mediated through the inactivation of the Wnt/β-catenin signaling pathway.

CONCLUSION

The findings of this study indicate that zerumbone inhibits cell proliferation, migration, and invasion in ovarian cancer cells and also suppresses the EMT mediated by the activation of the Wnt/β-catenin signaling pathway. Thus, zerumbone can potentially serve as a food supplement for the prevention of ovarian cancer as well as a potential therapeutic drug for the management of ovarian cancer.

DECLARATIONS

Acknowledgement

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Competing interest

The authors state that there are no conflicts of interest to disclose with regard to this work.

Contribution of authors

We declare that this work was carried out by the researchers listed in this article. All liabilities related to the content of this article will be borne by the authors. QG designed all the experiments and revised the paper. BE, EG and LH performed the experiments. XW, NL and LW wrote the paper.

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