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Research on the mechanisms of zerumbone in inhibition of proliferation and induction of apoptosis in human esophageal cancer cell line Eca-109.

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Abstract

Objective: To investigate the mechanisms of zerumbone in proliferation and inducing effect on apoptosis of human esophageal cancer cell line Eca-109.

Methods: Eca-109 cell lines were divided into two zerumbone treatment groups (10 μmol/L and 20 μmol/L) and one blank control group (0 μmol/L). Thereafter, MTT assay was performed to observe the inhibitory effect of zerumbone on the *in-vitro* growth of Eca-109 cells; flow cytometry was performed for detecting the distribution of cell cycles; inverted phase contrast microscope was used to observe the morphology of Eca-109 cells; RT-PCR and Western blotting assay were carried out for detecting the changes in mRNA and protein expressions of CXCR4.

Results: Zerumbone had a significant inhibitory effect on the growth of Eca-109 cells, and the inhibitory effect was continuously elevated with an increase in concentration ($p<0.05$). Besides, zerumbone could induce the apoptosis of Eca-109 cells, and the apoptotic rate was increased in a concentration-dependent manner ($p<0.05$). After 48 h and 72 h of treatment with zerumbone in different concentrations, we found that the incidence of poor adhesive growth and cell death was increased against the augmentation in concentration of zerumbone; with the time extension, the quantity of Eca-109 cells would be decreased gradually after treatment with zerumbone in a certain concentration. The results of RT-PCR and Western blotting assay also showed that compared with the control group, the mRNA and protein expressions of CXCR4 in 10 μmol/L and 20 μmol/L groups were significantly decreased ($p<0.05$), in which the decrease in 20 μmol/L group was more significant.

Conclusion: Zerumbone can inhibit the growth of Eca-109 cells with a promoting effect on apoptosis, and the mechanism might be correlated with the downregulation of CXCR4.

Keywords: Zerumbone, Human esophageal cancer, Eca-109, CXCR4.

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Introduction

Zerumbone, a kind of sesquiterpenes extracted from herbal, is characterized with the anti-inflammatory effect and anti-tumor effect in some studies [1]. Some other studies [2] showed that zerumbone can inhibit the invasion of breast cancer cells mediated by CXCR4. Currently, there remain few studies reporting the inhibitory and inducing effects of zerumbone on proliferation and apoptosis of Eca-109 cells, respectively, and the intrinsic mechanism of zerumbone in inducing cell apoptosis is still unclear. CXCR4, a highly conservative G-protein-coupled receptor, has been confirmed to be involved in a variety of biological behaviors in many tumors, such as cell proliferation, metastasis, invasion and apoptosis [3,4]. In this study, human esophageal cancer Eca-109 cell lines were treated with zerumbone in different concentrations, thereby investigating the mechanism of zerumbone in inhibiting the proliferation and inducing the apoptosis in human esophageal cancer cells, and its effect on the mRNA and protein expressions of CXCR4.

Materials and Methods

Cell culture

Eca-109 cells (Institute of Biochemistry and Cell Biology, SIBS, CAS) in logarithmic phase were enrolled in the control group and combined group, and cultured in the RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 0.1% penicillin and streptomycin. Cell culture was carried out in presence of HEPES/3% glutamine (pH 7.2-7.2) at 37°C and 5% CO_2 in an incubator.

Detecting the growth inhibition ratio

Eca-109 cells were inoculated onto a 96-well plate at density of 1×10^4/ml and cultured at 37°C for 24 h in an incubator containing 5% CO_2. Zerumbone in different concentrations (10 μmol/L and 20 μmol/L) was used for treatment of cells for 48 h and 72 h, while the cells in control group received the treatment of zerumbone in concentration of 0 μmol/L, in which 6 replicative wells were set in each group. In each well, 10 μL MTT reagent (5 mg/mL) was dropped followed by 4 h of culture at 37°C and 5% CO_2. Afterwards, 100 μL DMSO was added into each well followed by vibration for 10 min and detecting the optical density (OD) at wavelength of 570 nm to calculate the growth inhibition rate of Eca-109 cells according to the following formula: growth inhibition rate (%)=(1-OD drug treatment group/OD control group) × 100% [5].

Observing the cell morphology under the inverted phase-contrast microscope

Eca-109 cells were inoculated onto a 96-well plate at density of 1×10^4/mL. After 48 h and 72 h, cells were placed under the inverted phase-contrast microscope (200X) to observe the morphological changes in cells in blank control group and the drug-treatment groups.

Analysis of changes in cell cycle

Cells in logarithmic phase were collected and inoculated into the culture flask at density of 1×10^4/mL (10 mL/flask, 6 flasks for each group). After culture at 37°C overnight, 10 and 20 μmol/L zerumbone was respectively added into the drug-treatment groups, while the DMEM medium in same volume was added into the control group. Culture was terminated at 12 h. After 24 h, centrifugation was carried out to collect the Eca-109 cells in control group and drug-treatment groups. These cells were rinsed with phosphate-buffer saline (PBS) three times, and stained with propidium iodide in a dark place for detection *via* FCM.

Detecting the mRNA expression of CXCR4 via RT-PCR

After 48 h of treatment, RNA extraction kit (TaKaRa) was applied to extract the total RNA from Eca-109 cells in each group for reverse transcription, in which the primers were designed with Primer Premier 5.0 and synthesized by Sangon Biotech (Shanghai) Co., Ltd. CXCR4 primer sequences are shown as follows: forward 5'-AAAATCTTCCTGCCCACCAT-3', reverse 5'-TTGCTATCGTCTGCCTCACT-3', 440 bp. GAPDH primer sequences are shown as follows: forward 5'-AGAAGGCTGGGGCTCATTTG-3', reverse: 5'-AGGGGCCATCCACAGTCTTC-3', 258 bp. PCR was carried out in following conditions: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 s, annealing at 55°C (for CXCR4) and 59°C (for GAPDH) for 30 s and extension at 72°C for 1 min; extension at 72°C for 5 min. Products were separated *via* electrophoresis on 2% agarose, and the electrophoresis results were collected by gel imaging system for analysis of gray value with Quantity One and calculation of the ratios of CXCR4 band to the GAPDH band. This experiment was conducted in triplicate.

Detecting the protein expression of CXCR4 via Western blotting assay

After 48 h of treatment, RIPA agent was added into the cells for lysis to extract the total protein. Following the quantification analysis of CXCR4 with BCA kit, we took 50 μg protein for electrophoresis on SDS-PAGE, and then the protein was transferred onto the PVDF membrane. After the membrane was blocked with skimmed milk, the primary antibodies of CXCR4 (1:300) and GAPDH (1:500) were added onto the membrane for incubation at 4°C overnight followed by rinsing with TBST. Then, HRP-labeled goat anti-rabbit IgG (1:1000) was added for incubation for 1 h. Thereafter, color development was performed for proteins with ECL kit followed by gray value analysis in Quantity One, and this experiment was conducted in triplicate.

Statistical analysis

SPSS 21.0 was utilized to perform one-way ANOVA, and SNK test was adopted for analysis of the significant differences between groups, in which $p<0.05$ suggested that the difference had statistical significance.

Results

Results of MTT assay

Zerumbone exhibited significant inhibitory effect on the growth of Eca-109 cells, and the inhibitory effect was continuously elevated with an increase in concentration ($p<0.05$, Table 1).

Table 1. *The MTT assay results of several groups (n=6).*

Groups	Inhibition Rate (%)	
	48 h	72 h
Control	0	0
10 μmol/L Zerumbone	15.76 ± 1.38	29.95 ± 3.15
20 μmol/L Zerumbone	27.05 ± 3.12	49.37 ± 4.03
F	11.031	17.654
p	<0.05	<0.05

Variations in distribution of cell cycle in each group

Besides, zerumbone could induce the apoptosis of Eca-109 cells, and the apoptotic rate was increased in a concentration-dependent manner ($p<0.05$; Table 2).

Table 2. *Distribution of cell cycle in each group (n=6).*

Groups	Cell cycle			Apoptotic cells
	G0-G1	S	G2-M	
Control	53.16 ± 2.48	34.03 ± 3.05	9.16 ± 2.76	-
10 μmol/L zerumbone	17.05 ± 0.32	8.75 ± 0.52	63.43 ± 0.47	3.48 ± 0.12
20 μmol/L zerumbone	22.72 ± 1.22	24.58 ± 2.01	47.31 ± 1.05	10.22 ± 1.05
F	21.054	19.823	16.741	22.006
p	<0.05	<0.05	<0.05	<0.05

Morphological observation of Eca-109 cells

Cells in the control group grew well and presented in intensive adhesive growth pattern. After 48 h and 72 h of treatment with zerumbone in different concentrations, we found that the incidence of poor adhesive growth and cell death were increased against the augmentation in concentration of zerumbone; with the time extension, the quantity of Eca-109 cells would be decreased gradually after treatment with zerumbone in a certain concentration (Figure 1).

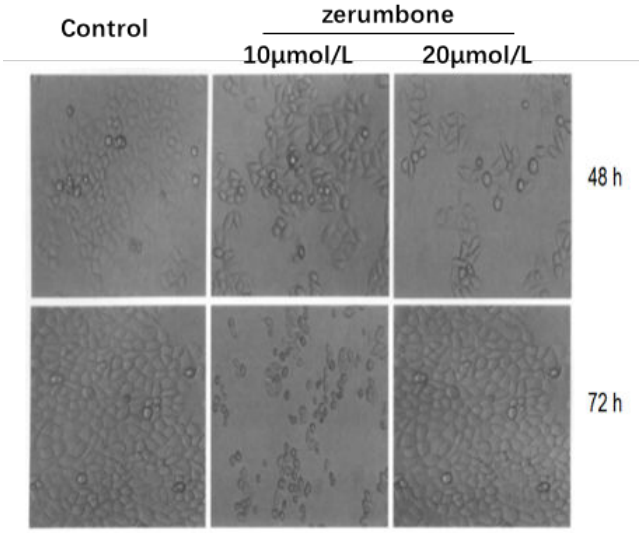

Figure 1. *Morphological observation of Eca-109 cells after 48 h and 72 h of treatment with zerumbone in different concentrations (200X).*

Effect of zerumbone on gene expression of CXCR4 in Eca-109 cells

The results of RT-PCR and Western blotting assay also showed that compared with the control group, the mRNA and protein expressions of CXCR4 in 10 μmol/L and 20 μmol/L groups were significantly decreased ($p<0.05$), in which the decrease in 20 μmol/L group was more significant (Figure 2).

Effect of zerumbone on protein expression of CXCR4 in Eca-109 cells

The results of Western blotting assay showed that compared with the control group, the protein expressions of CXCR4 in 10 μmol/L and 20 μmol/L groups were significantly decreased ($p<0.05$), in which the decrease in 20 μmol/L group was more significant (Figure 3).

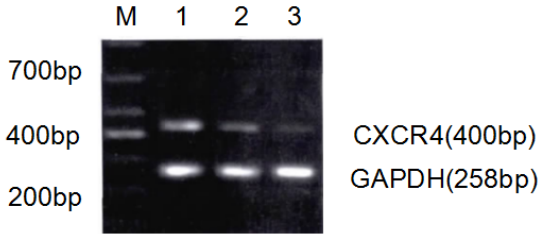

Figure 2. *mRNA expression of CXCR4 in cells of each group (M: standard DNA; 1: control group; 2: 10 μmol/L drug-treatment group; 3: 20 μmol/L drug-treatment group).*

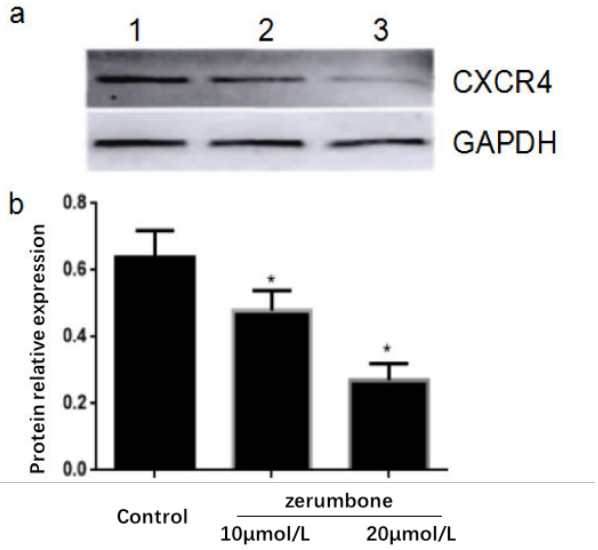

Figure 3. *Protein expression of CXCR4 in cells of each group. Note: *p<0.05 vs. control group. A) Protein expression of CXCR4 (1: control group; 2) 10 μmol/L drug-treatment group; 3) 20 μmol/L drug-treatment group); B) Analysis of relative protein expression of CXCR4.*

Discussion

Literatures [6,7] have indicated that zerumbone, a cytotoxic ingredient extracted from the root and stem of wild ginger, can significantly inhibit cell growth with the potential anti-tumor effect. Some other studies [8,9] also revealed that zerumbone is strongly cytotoxic to lymphocytes. Currently, inducing the apoptosis of tumor cells but without any effect on surrounding normal cells and tissues has been regarded as a key indicator in evaluating the efficacy of chemotherapeutics. The results of this study showed that after treatment with zerumbone for Eca-109 cells, the MTT assay showed that the growth of Eca-109 cells was significantly inhibited in a dosage-dependent manner, suggesting that zerumbone can suppress the growth of Eca-109 cells.

Activated CXCR4 will initiate the transduction of ERK/MAPK signal pathways, thereby upregulating the relative genes [10,11]. Existing studies [12,13] indicated that CXCR4 are expressed in tumor tissues like breast cancer and ovarian cancer. Some studies [14,15] reported that after the expression of CXCR4 was silenced by RNAi, the proliferation rate of breast cancer cell would be decreased, and the suppression on activity of CXCR4 could promote cell apoptosis. The results of this study showed that in Eca-109 cells that were treated with zerumbone, there were decreases in mRNA and protein expressions of CXCR4, and with an increase in concentration, the decreases would be more evident, suggesting that zerumbone can inhibit the growth of Eca-109 cells and promote the apoptosis, which might be associated with the downregulation of CXCR4.

In conclusion, we found that after Eca-109 cells were treated with zerumbone, the growth of esophageal cancer cells could be inhibited; besides, zerumbone could significantly inhibit the growth of Eca-109 cells and promote the apoptosis possibly through suppressing the mRNA and protein expressions of CXCR4. However, in-depth research is required to further elucidate the mechanism of zerumbone inhibiting the growth and promoting the apoptosis of cancer cells.

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