

Original Article

GANT 61 up-regulates E-cadherin expression and inhibits EMT and invasion of breast cancer cells

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Abstract: With the improvement of breast cancer diagnosis and treatment technology, the mortality rate of breast cancer is reduced to a certain extent, but the recurrence and metastasis rates are still high. The pathogenic factors are related to epithelial-mesenchymal transition (EMT) and breast cancer cell invasion. The Gli inhibitor GANT61 is used for treating several tumors. However, its role in breast cancer EMT and invasion, and related mechanisms have not been reported. The breast cancer cell line MCF-7 was cultured *in vitro* and randomly divided into control group and GANT61 treatment groups (20, 40, 60 μ mol/L), followed by analysis of cell proliferation using tetrazolium salt colorimetric (MTT) method, cell cycle by flow cytometry, cell apoptosis using Capase-3 activity assay, MMP-9 expression by real-time PCR, and cell invasion by transwell chamber. GANT61's effect on the expression of E-cadherin was detected by Western blot. Compared with control group, GANT61 significantly decreased the proliferation of MCF-7 cells, cell cycle arrest, increased Capase-3 activity, inhibited cell invasion, reduced MMP-9 expression and elevated E-cadherin expression (all $P < 0.05$) in a dose-dependent manner. GANT61 promotes apoptosis, regulates cell cycle, and inhibits cell proliferation and invasion of breast cancer cells by promoting E-cadherin expression.

Keywords: Gli, GANT61, E-cadherin, breast cancer, EMT

Introduction

Breast cancer (BC) mainly occurs in breast epithelial tissues, 99% of breast cancer patients are female. The incidence rate is increasing year by year, with younger ages being predominant [1, 2]. With the current pace of life, work pressure and environmental changes, the global incidence of breast cancer has further increased, and in China the incidence of breast cancer has it gradually becoming one of the countries with the highest incidence rates [3, 4]. According to statistics from global WHO incidences of cancer, more than one million people in the world are diagnosed with breast cancer every year. Breast cancer ranks first in women's malignant tumors. Although the current diagnosis and treatment of breast cancer has been improved, the mortality rate of breast cancer is still high [5, 6]. To date, the global medical community has given breast cancer a great deal of attention with treatments, surgery, radiotherapy and chemotherapy, and even individualized targeted therapies, but satisfactory treatment

effects have not been obtained regarding reducing the morbidity and mortality of breast cancer [7, 8]. The occurrence and development of breast cancer is a complex pathological process involving several endogenous or exogenous factors. Exogenous factors such as immunity, endocrine levels, genetic background and other external factors can lead to the occurrence of breast cancer [9]. Tumor invasion and EMT are the key to breast cancer recurrence and metastasis [10].

The Hedgehog (Hh) signaling pathway is involved in embryonic development, morphogenesis, cell division, organ formation, etc. [11]. Multiple signal pathways such as Hh signaling pathway are involved in tumor invasion and metastasis [12]. The zinc finger protein Gli is a key gene in the Hh signaling pathway, which activates transcription of downstream target genes and regulates the activation of Hh signaling pathways [13, 14]. GANT61 is Gli's most effective small molecule inhibitor and has been shown to play an important role in the invasion

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
MMP-9	TCCTTGCTACTACCGCTCT	TGTTGGTGTGTTGATGGGTATA

and metastasis of various tumors including prostate cancer and ovarian cancer. It can inhibit the proliferation and metastasis of tumor cells to varying degrees [15, 16]. However, GANT61's role in breast cancer EMT and invasion and related mechanisms have not been reported.

Materials and methods

Reagents and instruments

GANT61 was purchased from Sigma (USA). Breast cancer MCF-7 (cell number: CRL-103-17TM) cell line was purchased from the US ATCC cell bank. DMEM medium, cyan and chain double antibody were from Hyclone (USA). Dimethyl sulfoxide (DMSO), MTT powder was from Gibco; trypsin-EDTA digest was purchased from Sigma (USA). The Transwell chamber was bought from Corning (USA). PVDF membrane was purchased from Pall Life Sciences, Western blot related chemical reagents were purchased from Shanghai Biyuntian Biotechnology Co., Ltd., ECL reagent was purchased from Amersham Biosciences, rabbit anti-human E-cadherin monoclonal antibody, goat anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody was purchased from Cell signaling Corporation of the United States. The RNA extraction kit and the reverse transcription kit were purchased from Axygen (USA). The cell cycle assay kit was purchased from BD Corporation of the United States. The Capase-3 activity assay kit was purchased from R&D (USA). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad Corporation of the United States. The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI (USA). The SW-DJ ultra-clean workbench was purchased from Suzhou Sutai Purification Equipment Engineering Co., Ltd. The Melody C6 flow cytometer was purchased from BD Corporation of the United States.

Cell culture and grouping

The liquid nitrogen-preserved breast cancer cell line MCF-7 cells were resuscitated, cultured, and passaged, and randomly divided into 4 groups; control group, low concentration of

GANT61 (20 μ mol/L) group, medium concentration of GANT61 (40 μ mol/L) group and high concentration of GANT61 (60 μ mol/L) group.

Real-time PCR detection of MMP-9 mRNA expression in MCF-7 cells

Total RNA was extracted using Trizol reagent, and reverse transcribed into cDNA according to the kit instructions. The primers were designed according to each gene sequence by PrimerPremier 6.0 and synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Table 1). Real-time PCR was performed using the conditions as follows: 56°C 1 min, 92°C 30 S, 58°C 45 S, 72°C 35 S, a total of 35 cycles. GAPDH was selected as a reference. According to the fluorescence quantification, the starting cycle number (CT) of all samples and standards were calculated. Based on the standard CT value, a standard curve was drawn, and then the semi-quantitative analysis was carried out using the $2^{-\Delta Ct}$ method.

Western blot

The total protein of breast cancer MCF-7 cells was extracted separately: RIPA lysate containing protease inhibitor was added, and the cells were lysed on ice for 15-30 min, sonicated for 5 s \times 4 times, and centrifuged at 4°C, 10,000 \times g for 15 min to obtain the proteins, whose concentration was quantified using BCA assay and stored at -20°C. The isolated protein was separated on a 10% SDS-PAGE, transferred to a PVDF membrane, and blocked with 5% skim milk powder for 2 h. Then, 1:1,000 dilution of primary antibody E-cadherin monoclonal antibody, was incubated with the membrane at 4°C overnight. After PBST wash, 1:2,000 diluted goat anti-rabbit secondary antibody was incubated with the membrane under dark conditions for 30 min. After washing with PBST, the chemiluminescence was added and developed for 1 min, followed by X-ray exposure. X-film and strip density measurements were separately scanned using protein image processing system software and Quantity one software. The experiment was repeated four times (n = 4).

MTT analysis of proliferation of cells in each group

The logarithmic growth phase MCF-7 cells were inoculated into the 96-well culture plate with

GANT61 inhibits invasion of breast cancer cells

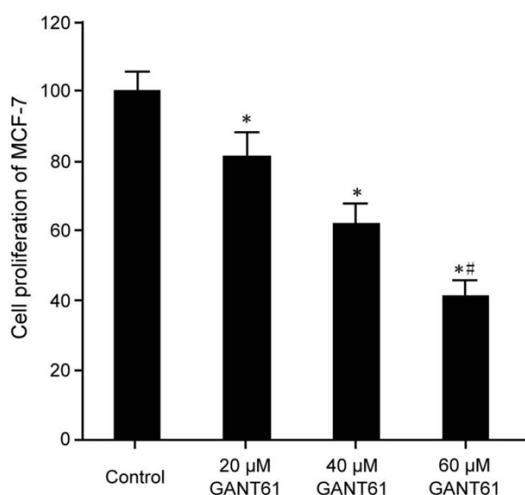


Figure 1. Analysis of the proliferation of breast cancer cells by GANT61. Compared with the control group, * $P < 0.05$; compared with the 20 $\mu\text{mol/L}$ GANT61 group, # $P < 0.05$.

DMO medium containing 10% fetal bovine serum at a cell number of 5×10^3 , the supernatant was discarded after 24 hours of culture, and cells were randomly divided into three treatment groups. After 48 h culture, 20 μl of sterile MTT was added and 3 replicate wells were set in each treatment group. After 4 hours of continuous culture, the supernatant was completely removed, 150 $\mu\text{l/well}$ of DMSO was added, and shaken for 10 min. After the purple crystals were fully dissolved, the absorbance (Absorbance, A) value was measured at a wavelength of 570 nm, and the proliferation rate of each group was calculated. This assay was repeated three times.

Cell cycle detection

The cells were collected after digestion, suspended and slowly added to the pre-cooled 90% ethanol, and the cells were washed with PBS. One hundred μl of the cells were added to 500 μl of RNase and propidium iodide staining solution (PI), followed by gentle mixing, and incubation at room temperature for 20 min in the dark. After that, 300 μl of $1 \times$ Binding buffer was added, mixed and cell cycle was measured by flow cytometry.

Caspase3 activity detection

The changes in Caspase3 activity in each group of cells were examined according to the kit

instructions. Trypsin-digested cells were centrifuged at $600 \times g$ at 4°C for 5 min, discarded the supernatant, added cell lysate, lysed on ice for 15 min, 20,000 g, centrifugation at 5°C for 5 min, added 2 mM Ac-DEVD-pNA, and measured the optical density (OD) at 405 nm wavelength. Change in value was calculated with Caspase3 activity.

Transwell chamber for detecting cell invasion

After 48 h of transfection, cells were cultured in DMEM medium containing 10% fetal bovine serum, and then cultured for 24 h in serum-free DMEM. The bottom of the Transwell chamber and the upper chamber of the membrane were coated with 50 mg/L Matrigel (1:5 dilution) and air dried at 4°C . The residual liquid in the plate was aspirated, and 50 μl of serum-free DMEM containing 10 g/L of BSA was added to each well at 37°C for 30 min. The Transwell chamber was placed in a 24-well culture plate, added 500 μl of 10% fetal bovine serum to DMEM medium in a small chamber, and add 100 μl of tumor cell suspension in a small chamber. The culture medium was serum-free DMEM medium, and each group was repeated 3 times.

Statistical analysis

All data are presented as mean \pm standard deviation (SD). The mean values of the two groups were compared by student t-test, analyzed by SPSS 11.5 software. The differences between groups were analyzed by analysis of variance (ANOVA). $P < 0.05$ indicated a significant difference.

Results

GANT61 inhibits the proliferation of breast cancer cells

MTT assay showed that GANT61 significantly decreased the proliferation of breast cancer MCF-7 cells compared with the control group ($P < 0.05$); and the inhibition was enhanced with increased concentration (**Figure 1**).

GANT61 inhibits cell cycle of breast cancer cells

Flow cytometry analysis showed that GANT61 significantly inhibited the cell cycle of breast cancer MCF-7 cells, mainly blocked in G2/M

GANT61 inhibits invasion of breast cancer cells

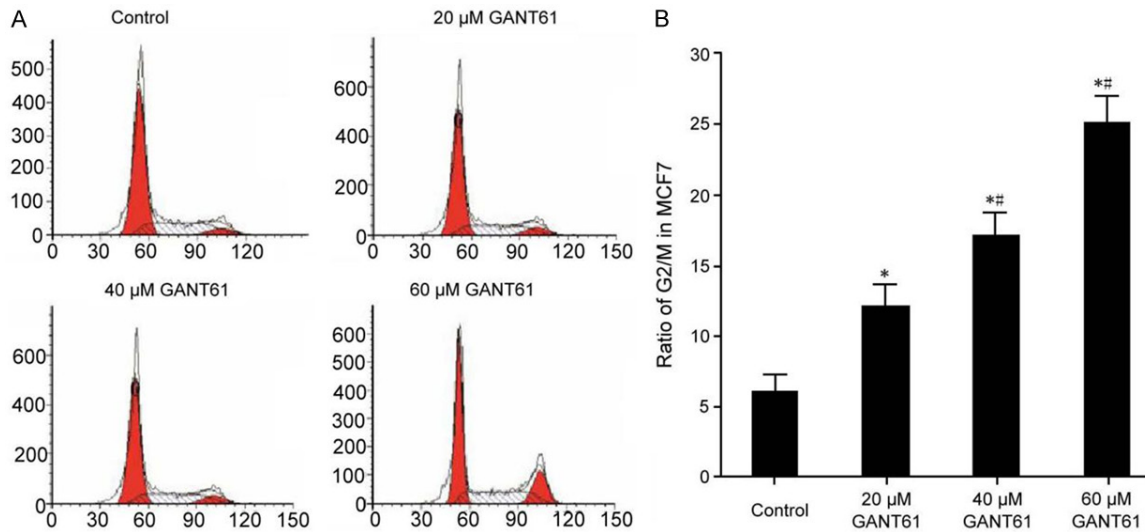


Figure 2. Analysis of the effect of GANT61 on cell cycle of breast cancer; A. flow cytometry analysis of the effect of GANT61 on cell cycle of breast cancer; B. Cell cycle statistical analysis. Compared with the control group, * $P < 0.05$; compared with 20 μmol/L GANT61 group, # $P < 0.05$.

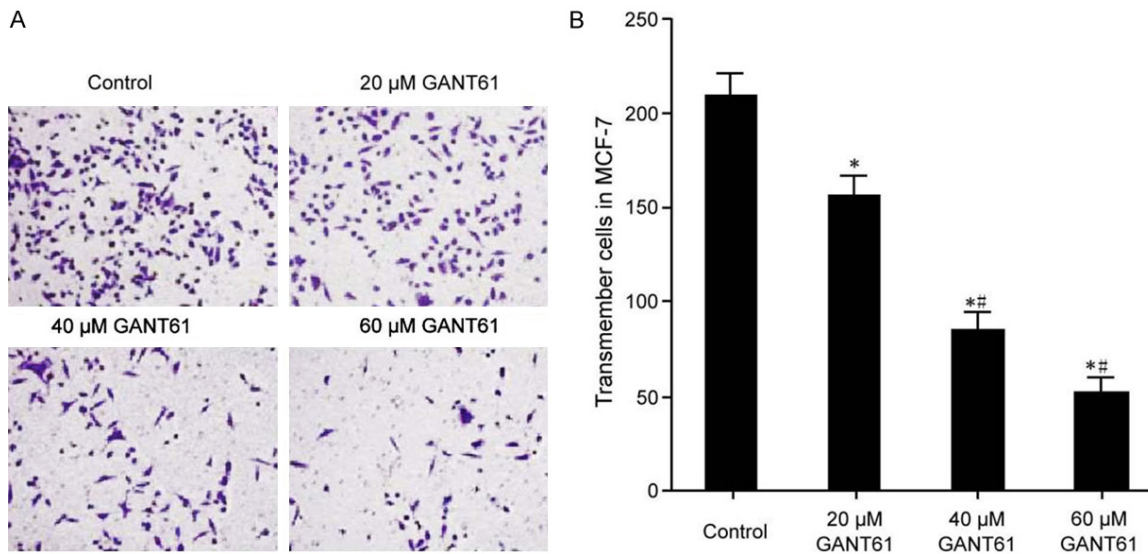


Figure 3. Analysis of the invasion of breast cancer cells by GANT61. A. Transwell chamber analyzes the invasive effect of GANT61 on breast cancer cells; B. Statistical analysis of invasive ability. Compared with the control group, * $P < 0.05$; compared with 20 μmol/L GANT61 group, # $P < 0.05$.

phase, compared with the control group ($P < 0.05$). With increased concentration, the proportion of G2/M cells was increased (Figure 2).

GANT61 decreased invasion of breast cancer cells

Transwell chamber assay showed that GANT61 significantly inhibited the invasion ability of breast cancer MCF-7 cells, compared with the con-

trol group ($P < 0.05$); and the inhibitory effect was enhanced with increase of the concentration (Figure 3).

GANT61 increases Caspase3 activity in breast cancer cells

GANT61 significantly promoted the increase of Caspase3 activity in breast cancer MCF-7 cells, compared with the control group ($P < 0.05$);

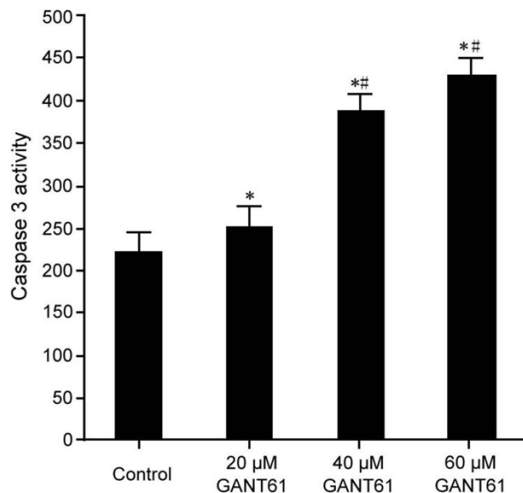


Figure 4. Analysis of Caspase3 activity in breast cancer cells by GANT61. Compared with the control group, *P < 0.05; compared with the 20 μmol/L GANT61 group, #P < 0.05.

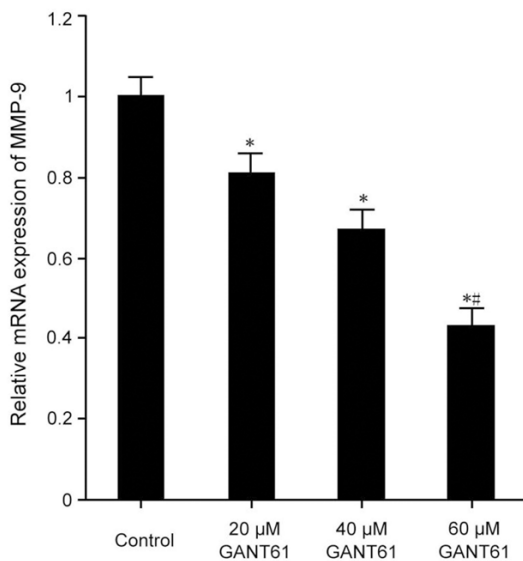


Figure 5. Effect of GANT61 on the expression of MMP-9 in breast cancer cells. Compared with the control group, *P < 0.05; compared with the 20 μmol/L GANT61 group, #P < 0.05.

and the increase of the concentration promoted the increase of apoptotic activity (**Figure 4**).

Effect of GANT61 on the expression of MMP-9 in breast cancer cells

Real-time PCR analysis found that GANT61 significantly reduced the expression of MMP-9 in breast cancer MCF-7 cells in a dose-dependent

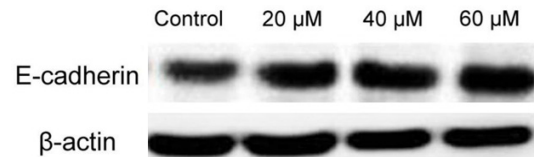


Figure 6. Effect of GANT61 on the expression of E-cadherin in breast cancer cells.

manner, compared with the control group (P < 0.05) (**Figure 5**).

Effect of GANT61 on E-cadherin expression in breast cancer cells

Western blot analysis showed that GANT61 promoted E-cadherin expression in breast cancer cells in a dose-dependent manner (**Figure 6**).

Discussion

Abnormal activation of Hh signaling pathway was found during tumorigenesis and development, including rhabdomyosarcoma, digestive system tumors, respiratory tumors, and gynecologic tumors [17]. In addition, studies have reported that the Hh signaling pathway is closely associated with the invasion and metastasis of various tumors including gastric cancer and esophageal cancer [18, 19]. The tumor cells that are detached from the primary site of the tumor tend to cause invasion and metastasis of the tumor, invasive growth of surrounding tissues or distant tissue metastasis. In the Hh signaling pathway, Gli can participate in the regulation of angiogenesis-related genes, promote angiogenesis, and regulate cell cycle progression [20, 21].

GANT61, developed by the National Cancer Institute, is a novel selective transcription factor target inhibitor of Gli, it can participate in the regulation of the development of several tumors [22] and is a research hotspot in recent years. However, the role of GANT61 in the development and invasion of EMT in breast cancer cells has not been reported. EMT is a process in which epithelial cells transform into mesenchymal phenotype cells, during which the epithelial cells lose their phenotype and connection to the basement membrane, which in turn leads to degradation of the extracellular matrix [23]. E-cadherin is a cell-marker adhesion molecule of epithelial cells, and its expression is reduced, which can lead to the dis-

appearance of the epithelial cytoskeleton, which in turn leads to the transformation into mesenchymal cells [24]. MMP-9, an important member of the matrix metalloproteinase family, can further promote the breakdown of the extracellular matrix, promote the occurrence and invasion of tumor cell EMT, and promote angiogenesis [25]. In this study, GANT61 inhibited MCF-7 cell proliferation, cell cycle arrest, increased Capase-3 activity, inhibited cell invasion, decreased MMP-9 expression, and increased E-cadherin expression; suggesting that GANT61 can up-regulate the expression of E-cadherin, inhibit the occurrence of EMT, down-regulate the expression of MMP-9, and prevent tumor invasion. At the same time, GANT61 blocks cell cycle, delays tumor cell proliferation and promotes tumor cell apoptosis. In further research, will be necessary to deeply analyze the related effects and mechanisms of GANT61 on tumor progression in breast cancer patients, and provide a research basis for clinical breast cancer treatment strategies.

Conclusion

GANT61 promotes cell apoptosis, regulates cell cycle, inhibits cell proliferation and invasion by promoting E-cadherin expression; indicating that GANT61 might be used as a novel approach for the prevention and treatment of breast cancer invasion and metastasis, as well as providing a new theoretical basis for the choice of clinical breast cancer treatment options.

Disclosure of conflict of interest

None.

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