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Original Article

Echinacea Glycoside Inhibits the Malignant Biological Behavior of Breast Cancer Cells by Regulating the LEF1CD44clinD1-Related Pathway

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ABSTRACT

Objectives: To investigate the inhibitory effect of Echinacoside on the malignant biological behavior of breast cancer cells through the regulation of the Lymphatic Enhancement Factor 1 (LEF1)-Cell Surface Adhesion molecule (CD44)-cyclin D1 pathway. **Materials and Methods:** A blank control group (MCF-10A without any treatment), BrCa group (MDA-MB-231 cells) and ECG group (MDA-MB-231 cells cultured in an incubator for 48 hr after the addition of 10 μg/mL ECG) were established. The proliferation ability of the cells was detected via the CCK8 assay, the protein and mRNA expression levels were detected via Western blot and qPCR and cell migration and invasion were detected via Transwell assays. **Results:** Compared with those in the control group, the cell proliferation and migration abilities in the BrCa group were greater, whereas those in the ECG group were lower. Compared with that in the control group, the cell invasion ability in the BrCa group was greater. Compared with that in the BrCa group, the overall apoptosis rate in the ECG group was lower. Compared with those in the control group, LEF1, CD44 and Cyclin D1 mRNA levels in the BrCa group were increased, whereas those in the ECG group were decreased compared with those in the BrCa group. The LEF1, CD44 and Cyclin D1 protein levels in the BrCa group were greater than those in the control group, whereas those in the ECG group were lower than those in the BrCa group. The protein levels of MMP-2, MMP-9 and VEGFA in the BrCa group were lower than those in the BrCa group. **Conclusion:** ECG can inhibit BrCa malignant behavior by regulating the LEF1-CD44-Cyclin D1 signaling pathway.

Keywords: Breast cancer, CD44, Cyclin D1, Echinoside, LEF1.

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INTRODUCTION

The disease is associated with inappropriate lifestyles, such as smoking, poor diet, lack of physical activity and changes in childbearing ages.[1] World-wide, breastage1. Breast Cancer (BrCa) is a common cancer among women worldwide. In less developed countries and among Chinese women, it is the leading cause of cancer death.[2] There is evidence that conventional radiotherapy and chemotherapy are effective at controlling the progression of cancer, but they are often associated with severe side effects.[3] The development of new therapeutic drugs is therefore especially important. Many studies have shown that natural compounds can prevent and treat cancer effectively and that their side effects are lower than those of conventional radiotherapy and chemotherapy.[4] Echinoside is a small-molecule substance extracted from the natural herbs *Cistanche deserticola* and *Echinacea*. It has antiaging, antifatigue and anticancer effects.[5] It has been reported that *Echinacea* Glycoside (ECG) has an anti-colorectal cancer effect by inducing tumor cell apoptosis;[6] however, its effect on BrCa cells and its specific mechanism are not clear. This study aimed to explore the inhibitory effect of ECG on the malignant biological behavior of BrCa cells by regulating the LEF1-CD44-Cyclin D1-related pathway.

MATERIALS AND METHODS

Materials and reagents

The American ATCC cell bank provided the immortalized human normal breast epithelial cell line MCF-10A and the human BrCa cell line MDA-MB-231 for the study. American Thermo Fisher Scientific provided 0.25% EDTA-grade trypsin, penicillin-streptomycin double antibodies, Fetal Bovine Serum (FBS) and L15 medium. The ECG was synthesized and analyzed

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as described in a previous study.[6] LEF1, CD44 and Cyclin D1 mRNA primers and β-actin primers (Sigma, USA); a CCK-8 detection kit, an Annexin VFITC/PI apoptosis kit and a QPCR detection kit (Shanghai Biyuntian Co., Ltd.); a Transwell chamber (Corning Company, USA); and artificial reconstructed basement membrane glue (Matrigel) purchased from BD Company of the United States were used. LEF1, CD44, clinD1, MMP-2, MMP-9, VEGFA and β-actin antibodies (Abcam Biotechnology Co., Ltd.) were used.

Methods

Cell culture and treatment

The cells were inoculated into L15 medium containing 10% FBS in a T25 culture bottle and cultured in a constant-temperature incubator (37°C, 5% CO_2). Cell growth was determined via digestion, passage and cryopreservation of trypsin. The rats were divided into a control group, a BrCa group and an ECG group. The cells in the control group were the immortalized human normal breast epithelial cell line MCF-10A without any treatment, the cells in the BrCa group were the human BrCa cells MDA-MB-231 without any treatment and were cultured routinely and the MDA-MB-231 cells in the pine ECG group were cultured in the incubator for 48 hr after the addition of 10 μg/mL of ECG.

Western blot

We determined the protein concentration via the BCA method, followed by gel preparation, electrophoresis for 90 min, gel separation, membrane separation for 90 min and milk sealing. The results were analyzed after the samples were cleaned, incubated with primary and secondary antibodies and developed via Bio-Rad image laboratory software.

Real-time quantitative reverse transcription (qPCR)

An RNA extraction kit was used to extract RNA, a Prime Script miRNA cDNA synthesis kit was used to reverse transcribe miRNA into cDNA and a miRNA fluorescence quantitative PCR detection kit was used to detect miRNA quantitatively in real time. After the reaction was complete, the relative expression of mRNA was calculated via the software.

Cell Counting Kit-8 (CCK8)

The proliferation ability of the cells in each group was determined by enzyme labeling at 450 nm after culture in a constant-temperature incubator for 4 hr. Each group of apoptotic cells was detected by flow cytometry after the corresponding reagent was added according to the instructions of the apoptosis kit.

Transwell

In the Transwell system, complete medium supplemented with 10% FBS was incubated for 4 hr after 105 cells were adjusted per well in the upper chamber and the cell migration ability was calculated by counting the number of transmembrane purple-stained cells under a microscope. The ultraclean worktable was first covered with Matrigel to detect cell invasion ability and the subsequent steps were the same as those for detecting cell migration.

Statistical methods

Statistical methods: The statistical software SPSS 22.0 was used for data processing and analysis. The Kolmogorov-Smirnov test was used for normality analysis of the measurement data. The Levene test was used for the variance homogeneity test. Measurement data conforming to a normal distribution are expressed as (image1). F variance analysis was used for multigroup comparisons. The LSD test was used for pairwise comparisons among multiple groups. $p<0.05$ was considered statistically significant.

RESULTS

Effect of ECG on the proliferation and migration of BrCa cells

The cell proliferative activity and migration in the BrCa group were greater than those in the control group, whereas those in the ECG group were lower than those in the BrCa group (Table 1).

Effects of ECG on the invasion and apoptosis of BrCa cells

The cell invasion ability in the BrCa group was greater than that in the control group, that in the ECG group was lower than that in the BrCa group and the overall apoptosis rate was greater than that in the BrCa group (Table 2).

Effect of ECG on LEF1, CD44 and Cyclin D1 mRNA in BrCa cells

The LEF1, CD44 and Cyclin D1 mRNA levels in the BrCa group were greater than those in the control group, whereas those in the ECG group were lower than those in the BrCa group (Table 3).

Effect of ECG on LEF1, CD44 and Cyclin D1 protein expression in BrCa cells

The LEF1, CD44 and Cyclin D1 protein levels in the BrCa group were greater than those in the control group, whereas those in the ECG group were lower than those in the BrCa group (Table 4).

Effect of ECG on MMP-2, MMP-9 and VEGFA protein expression in BrCa cells

Compared with those in the BrCa group, the protein levels of MMP-2, MMP-9 and VEGFA in the BrCa group were lower (Table 5).

DISCUSSION

Human health and quality of life are seriously threatened by cancer, which is one of the leading causes of death worldwide. Cancer cells exhibit uncontrolled growth and proliferation, apoptosis inhibition and metastasis.[7] Although progress has been made in various tumor treatment methods for BrCa patients, the 5-year survival rate of some BrCa patients is still very low.[8] Therefore, it is still necessary to explore new treatment methods or effective anticancer substances to improve the therapeutic effect in BrCa patients. Natural plants or resources have received increasing attention because of their rich and potential antitumor properties.[9] Natural plants or resources can achieve this goal by reducing toxicity and side effects, improving the efficacy of chemotherapeutic drugs and providing safer and more comprehensive treatments. In addition, active components in natural plants or resources usually have multitarget effects, which mean that they can simultaneously affect a variety of biological pathways and molecules associated with cancer.[10] This multitarget effect allows natural plants or resources to comprehensively interfere with tumor development and inhibit the growth and spread of tumor cells, thus enhancing the diversity and comprehensiveness of treatment.[11]

ECG is a small-molecule substance extracted from *Cistanche deserticola* and ECG that has a variety of biological functions.[12] In 5-FU-induced myelosuppressive mice, ECG can improve hematopoietic function by inhibiting MutT homolog 1 (MTH1)-induced apoptosis of cancer cells.[13] Recently, it has been reported that ECG can induce apoptosis through the Mitogen-Activated Protein Kinase (MAPK) pathway, thus inhibiting the growth of pancreatic adenocarcinoma cells.[14] However, research on the anti-BrCa ability of ECG is still lacking and its potential mechanism is not clear. This study aimed to explore the inhibitory effect of ECG on the malignant biological behavior of BrCa cells by regulating the LEF1-CD44-Cyclin D1-related pathway. Compared with those in the control group, the cell proliferative activity and migration ability in the BrCa group were greater, whereas those in the ECG group were lower than those in the BrCa group. The cell invasion ability in the BrCa group was greater than that in the control group, that in the ECG group was lower than that in the BrCa group and the overall apoptosis rate was greater than that in the BrCa group. These findings suggest that ECG can inhibit the malignant biological behavior of BrCa cells.

Multiple signaling pathways are activated in BrCa cells, including the Wnt/β-catenin, LEF1-CD44-Cyclin D1 and Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathways.[15] As one of these signals, the abnormally activated LEF1-CD44-Cyclin D1 signal plays an important role in BrCa development. Reducing LEF1-CD44-Cyclin D1 signaling can effectively inhibit BrCa development.[16] Abnormal activation of the LEF1-CD44-Cyclin D1 signaling pathway is often observed in human BrCa. This imbalance of signaling pathways can promote the progression of many types of cancers, including colorectal cancer and BrCa. Therefore, targeting the LEF1-CD44-Cyclin D1 signal is a promising method for cancer treatment.[17] Compared with those in the BrCa group, LEF1, CD44 and Cyclin D1 mRNA levels in the ECG group were lower. These findings suggest that ECG can inhibit BrCa malignant biological behavior by regulating the LEF1-CD44-Cyclin D1 signaling pathway. Cyclin D1, CD44 and LEF1, important target genes of the Wnt/β-catenin pathway, are involved in the proliferation, apoptosis and growth regulation of tumor cells. CyclinD1 is a classic onco-promoting gene and its high expression can promote mitosis, accelerate proliferation and is a specific factor for malignant tumors.[18]

Different biological processes are involved in cancer, including persistent proliferation signals, escape from growth inhibitors,

Table 1: Effect of ECG on the proliferation and migration of BrCa cells.

Group	N	Cell proliferative activity	Number of cell migration
control	6	0.10±0.01	73.25±15.70
BrCa	6	0.22±0.04[a]	142.74±24.34[a]
ECG	6	0.15±0.03[b]	106.48±17.44[b]
F		25.154	19.022
P		0.000	0.000

Table 2: Effects of ECG on the invasion and apoptosis of BrCa cells.

Group	N	Overall apoptosis rate (%)	Number of cell invasion
Control	6	9.97±1.23	51.72±10.31
BrCa	6	9.51±1.13	121.05±20.26[a]
ECG	6	31.34±4.25[b]	93.12±16.35[b]
F		134.293	26.157
P		0.000	0.000

Table 3: Effect of ECG on LEF1, CD44 and Cyclin D1 mRNA in BrCa cells.

Group	N	LEF1 mRNA	CD44 mRNA	Cyclin D1 mRNA
control	6	1.24±0.32	0.83±0.35	0.770.09
BrCa	6	2.47±0.54[a]	2.59±0.67[a]	2.05±0.46[a]
ECG	6	1.65±0.40[b]	1.76±0.43[b]	1.32±0.35[b]
F		12.744	18.451	21.687
P		0.000	0.000	0.000

Table 4: Effects of ECG on LEF1, CD44 and clinD1 protein expression in BrCa cells.

Group	N	LEF1	CD44	Cyclin D1
control	6	0.280.04	0.300.03	0.330.14
BrCa	6	0.860.28[a]	0.760.11[a]	0.780.12
ECG	6	0.570.08[b]	0.520.09[b]	0.440.14[b]
F		15.521	45.156	18.481
P		0.000	0.000	0.000

Table 5: Effects of ECG on MMP-2, MMP-9 and VEGFA protein expression in BrCa cells.

Group	N	MMP-2	MMP-9	VEGFA
control	6	0.330.17	0.370.09	0.310.03
BrCa	6	0.840.18	0.860.16	0.640.03
ECG	6	0.350.14[b]	0.430.14[b]	0.490.04[b]
F		15.521	45.156	144.529
P		0.000	0.000	0.000

replication immortality, invasion and metastasis, angiogenesis and resistance to cell death. Genomic instability and inflammatory processes also contribute to the development of this disease.[18] VEGFA is also involved in metastasis. MMP-2 and MMP-9 belong to the matrix metalloproteinase family and play important roles in wound healing and tumor invasion.[19] By secreting VEGFA and metalloproteinases, tumor cells promote the digestion of the extracellular matrix to facilitate tumor migration and invasion.[20] Several studies have shown that inhibiting MMP-2, MMP-9 and VEGFA expression can inhibit tumor growth, metastasis and angiogenesis.[21] Compared with the ECG group, the BrCa group presented lower levels of the MMP-2, MMP-9 and VEGFA proteins. Therefore, ECG may inhibit BrCa growth and metastasis by inhibiting MMP-2, MMP-9 and VEGFA expression. These proteins are related to the regulation of the LEF1-CD44-clinD1 signaling pathway.[22]

The *in vitro* experiment involved only one breast cancer cell line and did not investigate other breast cancer cells. Owing to the specificity of Estrogen (ER), Progesterone (PR), Human Epidermal Growth factor (HER)-2, Cytokeratin (CK)5/6, Epidermal Growth Factor Receptor (EGFR), Ki-67 and progesterone receptor (AR) expression in different cell lines, the results may differ. *In vivo* tumor-bearing experiments were not carried out in this study because whether the metabolism and accumulation of echinacoside in animals are affected by metabolism and changes in the metabolic rate and kinetic parameters *in vivo* need further tumor-bearing experiments for verification.

CONCLUSION

ECG can inhibit BrCa malignant biological behavior by regulating the LEF1-CD44-clinD1 signaling pathway.

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ABBREVIATIONS

ECG: *Echinacea* glycoside; **BrCa:** Breast cancer; **FBS:** Fetal bovine serum.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Research ethics approval was received from the First Affiliated Hospital, Jiangxi Medical College, Nanchang University.

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