Int J Clin Exp Pathol 2017;10(10):10212-10222
www.ijcep.com /ISSN:1936-2625/IJCEP0060834

Original Article

Egr-1 suppresses breast cancer cells proliferation by arresting cell cycle progression via down-regulating CyclinDs

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Received July 5, 2017; Accepted September 13, 2017; Epub October 1, 2017; Published October 15, 2017

Abstract: Egr-1 is an important nuclear transcription factor in the early growth response gene family (Egr family). Egr-1 was reportedly involved in the tumorigenesis of diverse tumors. However, there was a paucity of data regarding the role of Egr-1 in the breast cancer. Herein, we investigated the expression of Egr-1 in breast tissues and breast cancer cell lines BT549 and Bcap37. Immunohistochemistry showed that Egr-1 was down-regulated in breast cancer tissues versus the normal paracancerous tissues. Overexpression of Egr-1 could arrest the progression of cell cycle in breast cancer cells. Luciferase reporter assay revealed Egr-1 could bind to the promoters of CyclinD1, CyclinD2 and CyclinD3. Together, these results suggested that Egr-1 could affect the cell cycle of breast cancer cells and defined the mechanism for the cells by inhibiting the process of G0/G1 phase. Our findings provide new insight into Egr-1 in breast cancer.

Keywords: Egr-1, breast cancer, cell cycle, CyclinD

Introduction

Breast cancer (BC) is the most common malignancy in women and the second leading cause of cancer-related mortality, which threatens women's health [1]. The incidence of new cases of BC in China accounts for 12.2% of the newly diagnosed cases in the world, with the morbidity and mortality increased [2]. The cause of BC is quite complex and related with genetic factors, hormones, immunity and a variety of environmental factors (physical and chemical, biological factors and lifestyle, etc.). With the advance of gene analysis, BC was reportedly driven by multi-gene mutation. The molecular-targeted therapy has altered the therapeutic approach for multiple tumors. It is critical to prevent the tumorigenesis for improving the understanding of the related gene's aberrant expression in BC [3]. Therefore, it is significant to identify the specific oncogene and the underlying mechanism for a new therapeutic target [4].

The cell cycle, as a basic process of cell activity, refers to the process from the end of one division to the end of the next division, two major events of which are DNA synthesis and cell division [5]. Cells in the evolution of the process have established a series of sound regulatory mechanisms to ensure that the cell cycle in a strict and orderly manner at all times [6]. Cell cycle regulatory proteins play an important role in the regulation of cell proliferation and directly affect cell proliferation [7]. The motility of cell cycle is mainly from cyclin-dependent kinase (CDK) which is controlled by cyclin and CDK inhibitors (CKI). Egr-1 was first discovered in 1987 and was induced by mitogen and differentiation factors [8]. Egr-1 contains a highly conserved DNA binding domain consisting of three zinc finger constructs that bind to GC-rich sequence GCGC (G/T) GGGCG [9]. In addition, Egr-1 includes one nuclear localization signal, two activation domains and one inhibitory domain, which is beneficial to the regulation of the expression of the target genes [10].

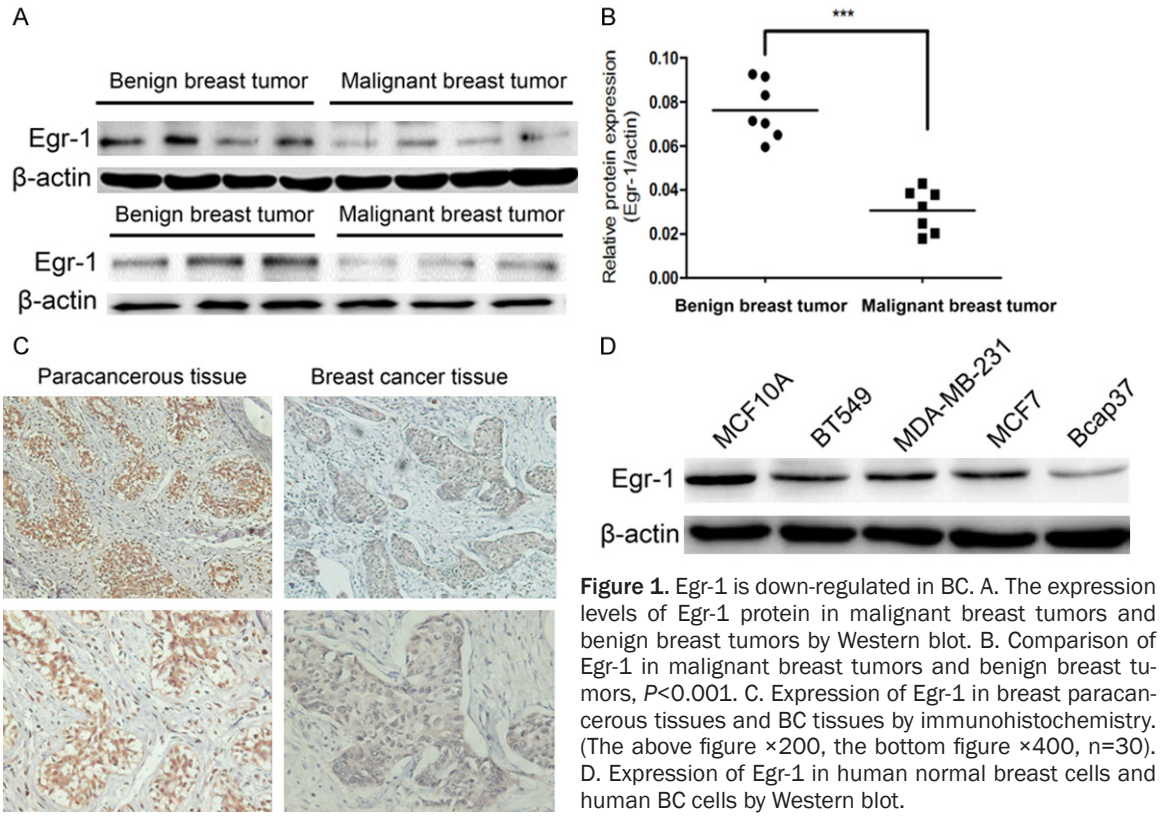


Figure 1. Egr-1 is down-regulated in BC. A. The expression levels of Egr-1 protein in malignant breast tumors and benign breast tumors by Western blot. B. Comparison of Egr-1 in malignant breast tumors and benign breast tumors, $P<0.001$. C. Expression of Egr-1 in breast paracancerous tissues and BC tissues by immunohistochemistry. (The above figure ×200, the bottom figure ×400, n=30). D. Expression of Egr-1 in human normal breast cells and human BC cells by Western blot.

Table 1. Patients' characteristics and Egr-1 expression

	Egr-1 staining			
Variables	Negative (%)	Positive (%)	Total	*P**
Tissues				0.000
Cancerous	40 (66.7)	20 (33.3)	60	
Paracancerous	5 (13.3)	52 (86.7)	60	
Age				0.439
≤49 years	18 (60)	12 (40)	30	
>49 years	22 (63.7)	8 (36.3)	30	
Tumor size				0.04
≤2 cm	6 (27.3)	16 (72.7)	22	
>2 cm	32 (84.2)	6 (15.8)	38	
pT status				0.04
pT_1	6 (27.3)	16 (72.7)	22	
pT_2	32 (84.2)	6 (15.8)	38	
pN status				0.238
pN_0	14 (53.9)	12 (46.1)	26	
pN_1	26 (76.5)	8 (23.5)	34	
TNM stage				0.04
I	6 (27.3)	16 (72.7)	22	
II	32 (84.2)	6 (15.8)	38	

*P values are obtained from χ^2 test.

Extracellular signals are evidenced to activate the expression of Egr-1 synthesis and regulate the transcription of the relevant target intranulear genes, which result in the cells producing adaptive changes through the cytoplasm of the signal transduction pathway. Thus, diverse biological functionalities of cells can be attributed to the activation of Egr-1. Our study was aimed to investigate the effect of Egr-1 on the cell cycle in BC cells and to explore its specific molecular mechanism, which might provide a new diagnostic and therapeutic target for BC.

Results

Egr-1 is down-regulated in BC

Seven cases of malignant breast tumor tissues and seven cases of benign breast tumor tissues collected from the Affiliated Hospital of Xuzhou Medical University were employed to determine the expression of Egr-1. The Egr-1 was significantly down-regulated in malignant BC tissues versus the benign ones ($P<0.05$, **Figure 1A**, **1B**). In parallel, the immunohistochemistry (IHC) of 60 cases of BC tissues and paracancerous tissues showed that Egr-1 was mainly

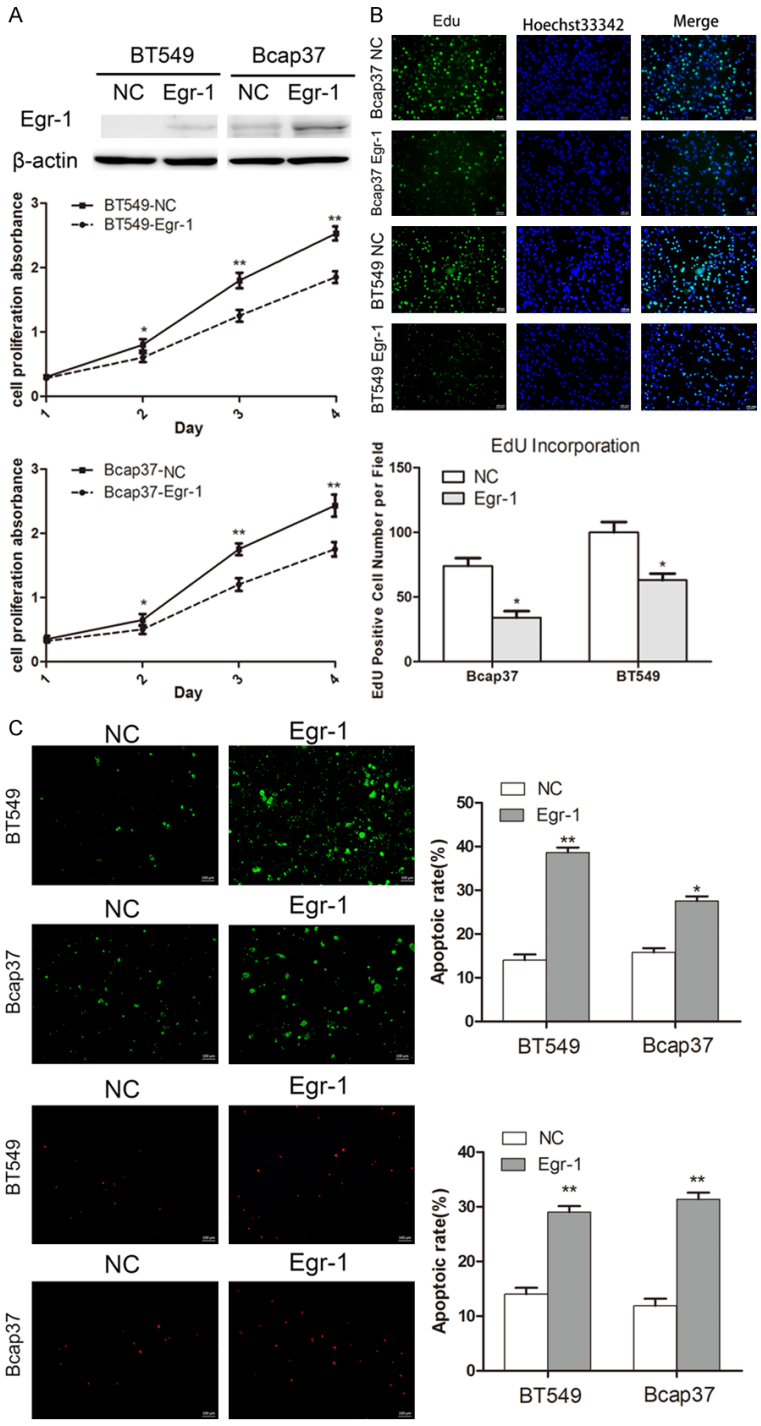


Figure 2. Egr-1 suppresses the proliferation of BC cells. A. Western blot analyzed the expression of Egr-1 protein in BC cell lines BT549 and Bcap37 transfected with pcDNA3.1-Egr-1 (Egr-1) and pcDNA3.1 (NC). CCK8 analysis was performed to examine the cell proliferation of BC cells transfected with pcDNA3.1-Egr-1 (Egr-1) and pcDNA3.1 (NC). The cell proliferation absorbance was detected in 24 h, 48 h, 72 h and 96 h. B. Egr-1 inhibited Edu incorporation. Bcap37 and BT549 cells were transfected with pcDNA3.1-Egr-1 (Egr-1) and pcDNA3.1 (NC). The cells were fixed for anti-Edu staining. The EdU-positive cells were measured and shown as a bar graph. C. Annexin V-FITC binding assay was used to observe apoptotic cells by fluorescence microscope in BC cells transfected with pcDNA3.1-Egr-1 (Egr-1) and pcDNA3.1 (NC). Prophase apoptotic cells were recognized by binding with FITC on the membrane (cell membrane displays green). Anaphase apoptotic cells were recognized by binding with FITC and PI on the nuclei (nuclei displays red). Data shown were from a typical experiment performed in triplicate.

located in the nucleus of BC paracancerous tissues. Egr-1 was significantly suppressed in BC specimens than those in paracancerous tissues (P< 0.05, **Figure 1C**; **Table 1**). It was indicated that decreased expression of Egr-1 in BC patients was correlated with tumor size, primary tumor and TNM stage (P<0.05, **Table 1**). However, the similar correlation trend was not observed in terms of age and lymph node involvement. These results indicate that Egr-1 was significantly decreased in BC tissues. Subsequently, we examined the expression level of Egr-1 protein in diverse human BC cell lines (BT549, MDA-MB-231, MCF7 and Bcap37) and the normal human breast cell line MCF10A (**Figure 1D**). The results of Western blot revealed that Egr-1 was statistically down-regulated in breast tumor cell lines as compared to the normal breast cell line MCF10A.

Egr-1 suppresses the proliferation and promotes the apoptosis of BC cells

To investigate the effect of Egr-1 in BC cell proliferation, we performed gain-of-function experiments. Cell counting Kit-8 (CCK8) assays showed that after being transfected with the pcDNA3.1-Egr-1 (Egr-1) BT549 cells and Bcap37 cells proliferation were inhibited as compared with the pcDNA3.1 (NC) group (**Figure 2A**). Similar effects are observed in Edu experiment (**Figure**

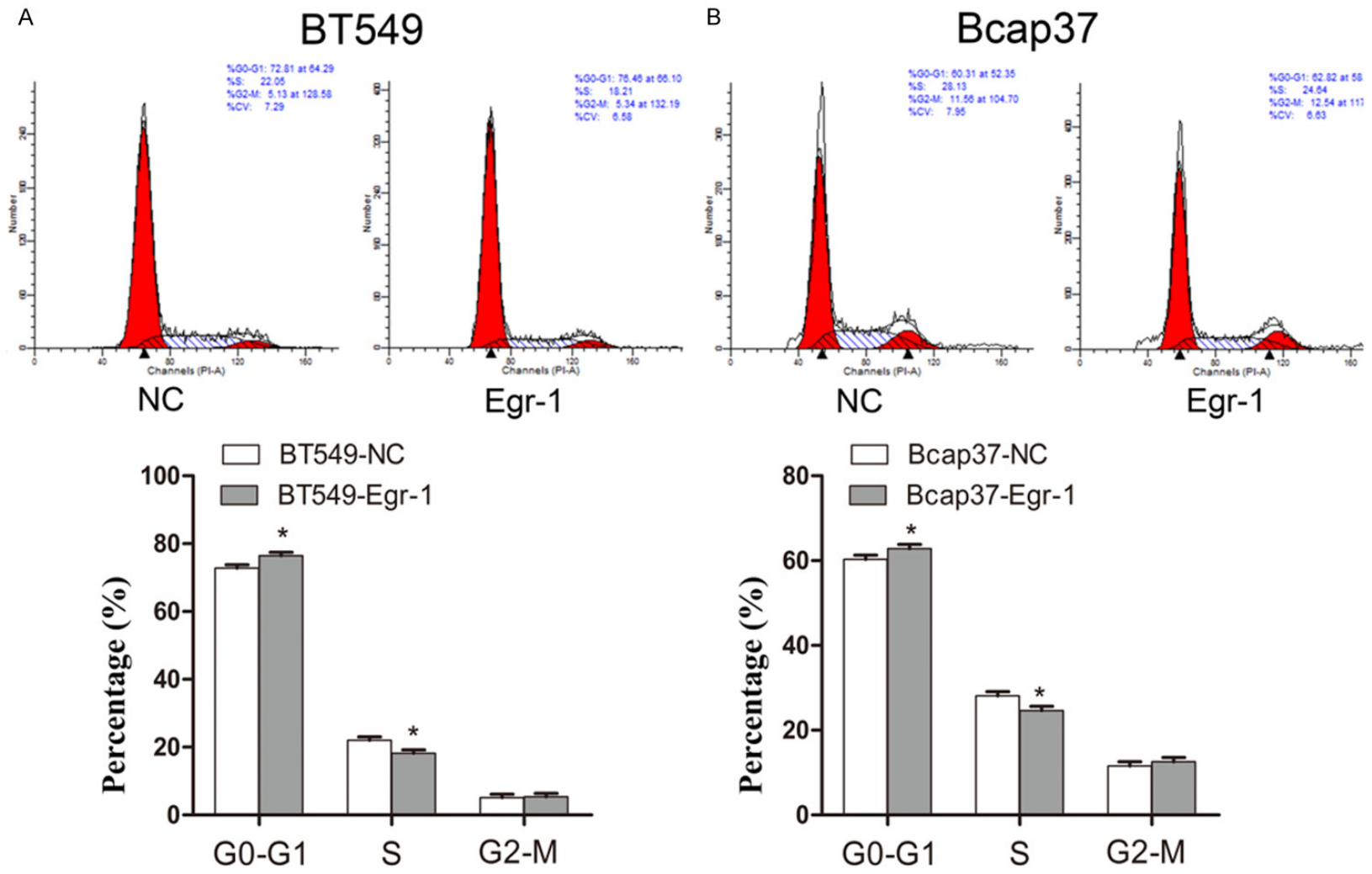
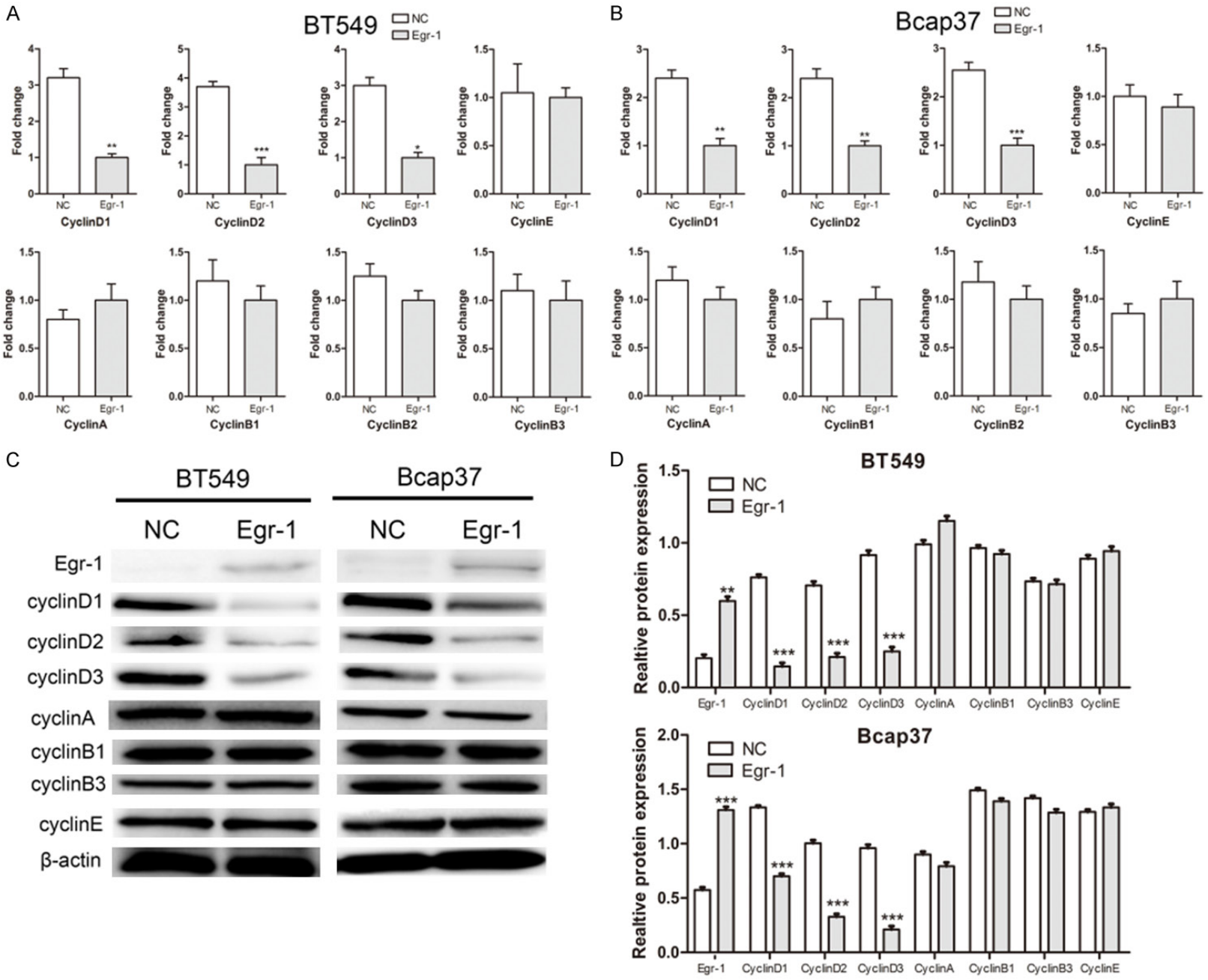


Figure 3. Egr-1 arrests cell cycle progression in BC cells. A. Flow cytometry was used to detect the effect of pcDNA3.1-Egr-1 (Egr-1) and pcDNA3.1 (NC) on the cell cycle progression of BT549 cells. The percentage of cells in the G0/G1 phase was increased in Egr-1 group compared with the NC group. The percentage of cells in the S phase was decreased in Egr-1 group compared with the NC group, **P*<0.05, n=3. B. Flow cytometry was used to detect the effect of pcDNA3.1-Egr-1 (Egr-1) and pcDNA3.1 (NC) on the cell cycle progression of Bcap37 cells. The percentage of cells in the G0/G1 phase was increased in Egr-1 group compared with the NC group. The percentage of cells in the S phase was decreased in Egr-1 group compared with the NC group, **P*<0.05, n=3.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BT549
NC
Egr-1
Fold change
CyclinD1
CyclinD2
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CyclinB1
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B
Bcap37
C
BT549
Bcap37
NC
Egr-1
cyclinD1
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cyclinD3
cyclinA
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β-actin
D
Realtive protein expression

Figure 4. Effects of Egr-1 on cell cycle-related proteins in BC cells. A. qPCR was used to detect the effect of overexpression Egr-1 on cell cycle-related protein mRNA levels in BT549 cells, **P*<0.05, ***P*<0.01, ****P*<0.001, n=3. B. qPCR was used to detect the effect of overexpression Egr-1 on cell cycle-related protein mRNA levels in Bcap37 cells, ***P*<0.01, ****P*<0.001, n=3. C. Effects of overexpression Egr-1 on protein expression of cell cycle-related proteins in BT549 and Bcap37 cells by Western Blot. D. The grayscale analysis of overexpression Egr-1 affecting cell cycle-associated protein expression levels in BT549 and Bcap37 cells, ***P*<0.01, ****P*<0.001, n=3.

2B). Furthermore, we sought to explore whether Egr-1 overexpression could affect cell apoptosis in BC cells. Annexin V-FITC binding assay revealed that the apoptotic rate in Egr-1 groups was higher compared with NC groups in BC cells (**Figure 2C**). These data indicate that Egr-1 suppresses cell proliferation and promotes cell apoptosis in BC cells.

Egr-1 arrests cell cycle progression in BC cells

Tumorigenesis is the result of cell cycle deregulation and cell division out of control [11, 12]. The effect of Egr-1 on cell cycle progression was detected by PI staining. After transfected with pcDNA3.1-Egr-1 for 24 h (hours), the cells were harvested and the cell cycles were detected by flow cytometry. The results (**Figure 3A**, **3B**) showed that the percentage of cells in the G0/G1 phase (DNA pre-synthesis) significantly increased in Egr-1 group versus the NC group, whereas the percentage of cells in S phase (DNA synthesis) significantly decreased (*P*< 0.05), with cells in G2/M phase remained insignificant (*P*>0.05). The findings indicate that Egr-1 can affect the cell cycle of BC and arrest the process of G0/G1 phase.

Effects of Egr-1 on cell cycle-related proteins in BC cells

To explore the effect of Egr-1 on cell cycle-related proteins in BC, Real-time Quantitative PCR (qPCR) was applied to detect the mRNA level of CyclinDs (CyclinD1, CyclinD2 and CyclinD3), CyclinA, CyclinBs (CyclinB1, CyclinB2 and CyclinB3) and CyclinE in BT549 and Bcap37 cells. The mRNA levels of CyclinDs were significantly decreased in Egr-1 group compared with the NC group (*P*<0.05), with the mRNA levels of CyclinA, CyclinBs and CyclinE intact (*P*>0.05; **Figure 4A**, **4B**). Consistently, the protein levels of CyclinDs were significantly decreased after the overexpression of Egr-1 (*P*<0.05) and the expression of CyclinA, CyclinBs and CyclinE protein level showed no significant change (*P*>0.05; **Figure 5C**, **5D**). These results indicate that the overexpression of Egr-1 can decrease the expression of CyclinDs in BC cells and the inhibitory effect occurs before transcription.

Egr-1 binds to the promoters of CyclinDs

As depicted afore, Egr-1 can suppress the expression of CyclinDs, we further explored the molecular mechanism of Egr-1 down-regulating the expression of CyclinDs. The plasmids of pcDNA3.1-Egr-1 were co-transfected with the constructed pGL3-CyclinD1, pGL3-CyclinD2 and pGL3-CyclinD3 luciferase reporter plasmids into BT549 and Bcap37 cells and pcDNA3.1 and pGL3 plasmids were transfected as controls. Compared with the control group, the activities of pGL3-CyclinD1, pGL3-CyclinD2 and pGL3-CyclinD3 were significantly up-regulated in Egr-1 overexpressed groups (*P*<0.05; **Figure 5A**, **5B**), suggesting that Egr-1 can bind to the promoters of CyclinDs.

Discussion

BC accounts for the first female carcinoma which is a serious threat to women's health and life. Although in recent years the prevention screening, early detection and combined with surgical resection, radiotherapy and chemotherapy, endocrine therapy and targeted therapy have made a cross-era progress [13-17], effectively improving the 5-year survival rate of BC but the treatment of drug resistance and relapse is still seriously restricting the quality of life of patients with breast carcinoma [18, 19]. Therefore, it is meaningful to find new molecular markers and drug targets to be intervented effectively which can reduce the recurrence rate and mortality rate of BC patients.

Egr-1 is a nuclear protein belonging to the EGR family and functions as a transcriptional regulator [20]. Egr-1 has a low level in variety of cancers such as colon cancer, ovarian cancer and liver cancer compared with normal tissues, and up-regulating Egr-1 could attenuate the cell migration of cancer cells [21-23]. In breast can-

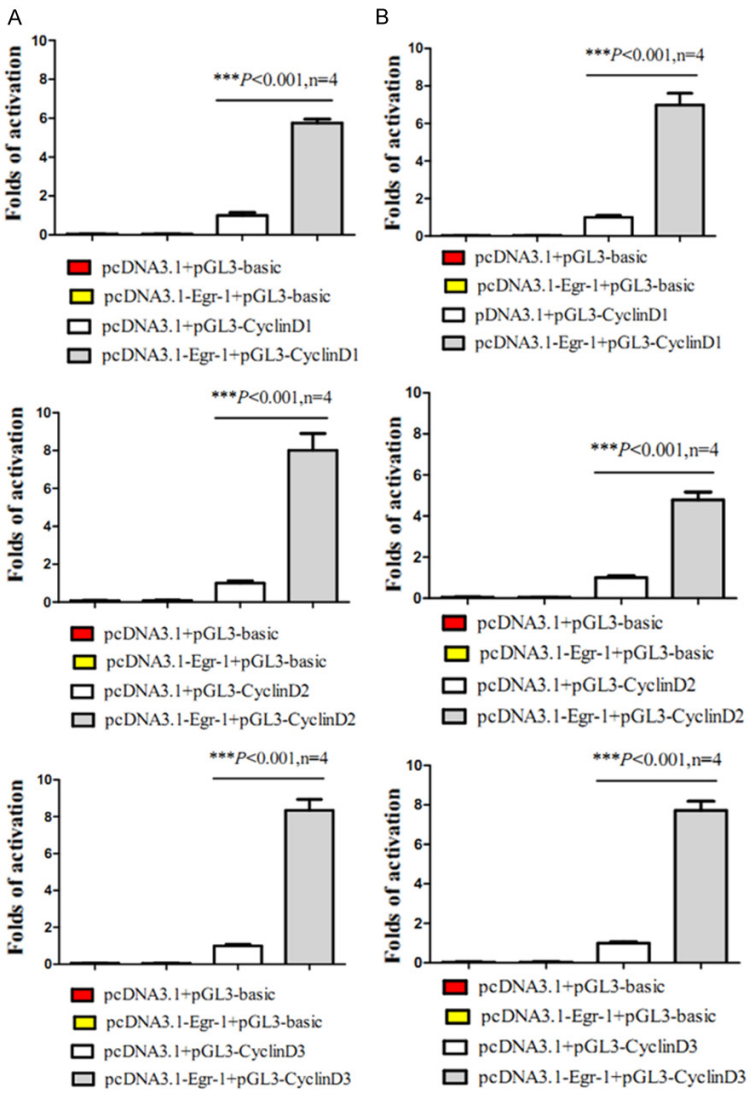

Figure 5. Egr-1 binds to the promoters of CyclinDs. A. The effect of Egr-1 on the activity of CyclinD1, CyclinD2 and CyclinD3 promoters in BT549 cells was significantly higher compared with the NC group, ***P<0.001, n=4. B. The effect of Egr-1 on the activity of CyclinD1, CyclinD2 and CyclinD3 promoters in Bcap37 cells was significantly higher compared with the NC group, ***P<0.001, n=4.

cer, we found that the expression of Egr-1 was down-regulated in BC cells compared with MCF10A. The BT549 and MDA-MB-231 cell lines with lack of expression of ER, PR and Her2 have low Egr-1 expression. The similar results could be found in MCF-7 cell line with ER+, PR+/PR- and Her2-. These results indicate that ER+ insignificantly affects the expression of Egr-1, which is inconsistent with the previous report [24]. The Bcap37 cell line is ER-/ER+ while the status of PR and Her2 are still unclear [25] and the Egr-1 expression in Bcap 37 cell line is significantly reduced than other BC cells. Therefore, the relationship between Egr-1 and the expressions of ER, PR and Her-2 need more exploration.

Recent studies have shown that Egr-1 could bind to the promoters of p21 further inhibited the cell proliferation of human gastric cancer cells [26]. Tao W, Shi JF, Zhang Q, Xue B, Sun YJ and Li CJ [27] reported that Egr-1 knockdown using RNA interference technology significantly promoted BC cells growth, indicating the tumor suppressor nature of Egr-1. Consistently, our results revealed the overexpression of Egr-1 could inhibit the cell proliferation of BC. Despite its known role as a tumor-suppressor in several other types of human cancers, Egr-1 was reportedly overexpressed in the majority of aggressive tumorigenic [28] prostate cancer cells and observations supported the notion that Egr-1 contributed to prostate cancer progression [29-31]. Therefore, more understanding of Egr-1 related signaling networks in cancer required further investigations.

Cell cycle regulation is a very complex and fine regulatory process which is close to cell differentiation, growth and death. The molecular characteristics of human malignant tumors are cell cycle regulation out of control while tumor cells affect the expression of cell cycle regulatory proteins through genetic and epigenetic mechanisms, and further cause tumorigenesis through the oncogene activated and tumor suppressor gene silenced [32]. Our results showed that Egr-1 was down-regulated in BC and the results of flow cytometry suggested that Egr-1 arrested the cell cycle progression of BC in the G1 phase.

A number of molecules have been found involved in tumor cell cycle regulation as CyclinA, CyclinB, CyclinD and CyclinE. The most common events are G1 phase cycle protein and CDK inactivation [33]. CyclinD is the starting factor of cell cycle [34]. We found that Egr-1 protein was involved in the regulation of G1 phase to S phase transformation of BC cells. The mechanism may be down-regulating CyclinDs expression, thus decelerating the G1 phase to S phase transition. CyclinD overexpression can activate CDK4 and CDK6 while shortens the G1 phase by CyclinD-CDK4/CDK6 pathway regulating the cell crossing the G1 phase check point. Then we found that Egr-1 could bind to the promoters of CyclinDs further inhibit their transcription activity and decrease the mRNA and protein expression, thereby arrested cell cycle from G1 phase to S phase.

In summary, our study confirmed that Egr-1 was decreased in BC tissues and cells. We also found that Egr-1 could affect the cell cycle of BC and arrest the process of G0/G1 phase. In addition, Egr-1 could bind to the promoters of CyclinDs and further inhibit the transcription activity of CyclinDs. Taken together, Egr-1 could provide a theoretical basis for the development of tumor therapy target drug by regulating the cell cycle in BC.

Materials and methods

Cell culture and reagents

MCF-10A, MCF7, BT549, Bcap37 and MDA-MB-231 cells were obtained from the ATCC. MCF-10A cells were maintained in Dulbecco's modified Eagle's medium/nutrient F12 media supplemented with 10% house serum. BT549 and Bcap37 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. MDA-MB-231 cells were maintained in L15 medium supplemented with 10% fetal bovine serum. Antibodies to the following epitopes and proteins were purchased from the indicated vendors: Egr-1 (Bioworld), CyclinD1 (Santa Cruz Biotechnology) and actin (ZSGB-BIO). CyclinD2, CyclinD3, CyclinA, CyclinE, CyclinB1, CyclinB2 and CyclinB3 antibodies were purchased from Biogot Biotechnology. CCK8 was obtained from Beyotime. Edu Kit was obtained from KeyGEN BioTECH.

Transfection

Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The plasmids (pcDNA-3.1-Egr-1, pcDNA3.1) were constructed by our laboratory. After 24 h of transfection, the efficiency of overexpression was analyzed by Western blot and qPCR.

Immunoblotting

Total cell lysates were solubilized in lysis buffer. Proteins were resolved by SDS-PAGE gels and then proteins were transferred (Bio-Rad) to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% nonfat milk for 2 h at room temperature prior to incubation with indicated primary antibodies. Subsequently membranes were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies (LI-COR Biosciences). Following several washes, chemiluminescent images of immunodetected bands on the membranes were recorded on X-ray films using the enhanced chemiluminescence (ECL) system (Tanon) according to the manufacturer's instructions.

Cell proliferation and apoptosis assay

We detected the cell proliferation through the Cell Counting Kit-8 (CCK-8 kit, Beyotime). 24 h after transfection, cells were plated in a 96-well microplate (Corning Incorporated, New York, USA) in triplicate and incubated at 37°C with 5% CO_2. 10 μl CCK-8 solution with 100 μl serum-free medium (Hyclone) was added to each well at 24 h, 48 h, 72 h and 96 h respectively, followed by incubation for 2 h. The absorbance at 450 nm (OD value) was recorded to gauge the cell viability by a multi-function enzyme-linked analyzer (Biotek Instruments, Winooski, VT, USA). Incorporation of EdU was examined under a fluorescence microscope with fluorescein isothiocyanate-conjugated anti-EdU. The EdU-positive cells were measured. The Annexin V-FITC Kit (FITC Annexin V Apoptosis Detection Kit I, BD, USA) was used to analyze cell apoptosis. Cells were plated in 24 well plates. After 24 h transfection of Egr-1 plasmids, cells were washed twice with ice-cold PBS, then incubated with 200 ul 1×binding buffer containing 5 ul Annexin V-FITC, and then in 300 ul 1×binding buffer containing 5 ul Propidium iodide (PI) for 10 minutes at room temperature in the dark. After incubation, cells were visualized under a florescence microscope. When observed with the microscopy, 5 fields were randomly selected from every sam-

ple and independent observers performed cell counting in a blind fashion. Data shown are from a typical experiment performed in triplicate.

Immunohistochemistry

Immunohistochemistry was performed according to the streptavidin-peroxidase (Sp) method using a standard SpKit (Zhongshan biotech, Beijing, China). The tissue slice was incubated with monoclonal mouse anti-Egr-1 antibody (1:100) (Bioworld) overnight at 4°C, and diaminobenzidine (DAB; Zhongshan Biotech, Beijing, China) was used to produce a brown precipitate. The immunoreactivity was assessed blindly by two independent observers using lightmicroscopy (Olympus BX-51) and the image was collected by Camedia Master C-3040 digital camera.

Luciferase reporter assay

The firefly luciferase constructs were generated by insertion of a fragment of human CyclinDs into the pGL3 Luciferase Report vector. BT549 and Bcap37 cells were co-transfected with pcDNA3.1 and pGL3-CyclinD or pcDNA3.1-Egr-1 and pGL3-CyclinD using Lipofectamine 2000 Reagent, respectively. The activities of firefly fluorescence intensity were assessed by Luciferase Reporter Assay System at the end of 48 h transfection (Promega, Beijing, China). Co-transfection with pGL3-basic reporter plasmids was used as negative control.

Quantitative real-time PCR

The PCR primers for cell cycle related proteins were as follows: of CyclinD1, forward 5'-GTCGCTGGAGCCCGTAA-3', reverse 5'-GAGTTGTCGGTGTAG-3', of CyclinD2, forward 5'-TGCAGAAGGACATCC-3', reverse 5'-AGGAACATGCAGACA-3', of CyclinD3, forward 5'-GATGCCAACCTCCTCAACGAC-3', reverse 5'-CTCCTCGCACTTCTGTTCCTC-3', of CyclinA, forward 5'-TTCCAACAACAAACCAAT-3', reverse 5'-GGCAAGGCACAATCTCAT-3', of CyclinB1, forward 5'-AGGCTTTCTCAGATGTAATTC-3', reverse 5'-TCACGACCCATTAGGTATT-3', of CyclinB2, forward 5'-TGCAGCACATGGCCAAGAA-3', reverse 5'-CTTCAGGAGTTTGCTGCTTGCATA-3', of CyclinB3, forward 5'-CATAAAACTCCTTCTGGTGT-3', reverse 5'-TAATCGTCAATAGGGAAAAG-3', of CyclinE, forward 5'-TTTCTTGAGCAACACCCT-3', reverse 5'-GTCACATACGCAAACTGG-3', of GAPDH, forward 5'-CAAAGTTGTCATGGATGACC-3', reverse 5'-CCATGGAGAAGGCTGGGG-3'. Total RNA was extracted from BC tissues and cell lines using TRIzol reagent (ambion; Invitrogen), and reverse transcription was performed using a reverse transcription kit (Cat.no.RR037A; Takara, Tokyo, Japan), following the manufacturers' instructions. qRT-PCR reactions were performed on an ABI 7500 real-time PCR system (Applied Biosystems, Waltham, MA, USA) using 2×SYBR Premix Ex Taq (Cat.No.RR420A, Takara). The levels of expression were normalized by GADPH levels, respectively. Each sample was run in triplicate. The relative expression was calculated using the relative quantification equation (RQ) $=2^{-\Delta\Delta Ct}$.

Human tumor tissue samples

A total of 60 specimens of breast biopsy were obtained from the Affiliated Hospital of Xuzhou Medical University. Seven cases of malignant breast tumor tissues and seven cases of benign breast tumor tissues collected from the Affiliated Hospital of Xuzhou Medical University. Seven cases of BC were confirmed by postoperative pathology and seven cases of breast fibroids. All patients had no anti-cancer treatment, without serious complications of heart and lung. The material was recruited stored at -80°C.

Statistical analysis

Two-tailed Student's t-test was performed to calculate significance in an interval of 95% confidence level. Statistical differences between the means for the different groups were evaluated with Instat 5.0 (GraphPAD software, San Diego, CA) using one-way analysis of variance (ANOVA). And the Pearson Correlation Coefficient was used for correlation analysis. All values are shown as means ± SD and a value of $P<0.05$ was considered statistically significant. For TMA, the association between Egr-1 staining and the clinicopathologic parameters of the BC patients were evaluated by x^2 test.

Acknowledgements

This work was supported by the Science and Technology Department of Jiangsu Province (BK20141149).

Disclosure of conflict of interest

None.

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