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Original Article

Up-regulation of cyclin E in breast cancer via estrogen receptor pathway

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Abstract: It is well known that cell cycle dysregulation plays an important role in breast cancer. The mechanism, however, is not fully understood. In this study, we aimed to explore whether estrogen and estrogen receptor pathway play a role in the regulation of cell cycle protein cyclin E expression, and whether the expression of cyclin E is associated with breast cancer prognosis. We first examined the level of cyclin E expression in breast cancer by immunohistochemistry. Benign fibroadenoma was used as controls. Next we cultured MCF-7 cells with different concentration of 17β-estradiol or tamoxifen for 48 hours. Then we employ qRT-PCR to determine changes of cyclin E in MCF-7 cells. Cyclin E is overexpressed in breast cancer and its expression is associated with the status of estrogen receptor and lymph node metastasis. After treatment with 17β-estradiol, the gene expression of cyclin E was enhanced, and as the concentration increased, the enhancement increased. After treatment with tamoxifen, the gene expression of cyclin E was inhibited, and as the concentration decreased, the inhibition increased. We demonstrated that estrogen induces, while tamoxifen inhibits cyclin E expression. This indicate that estrogen receptor pathway play a critical role in cell cycle dysregulation in breast cancer.

Keywords: Breast cancer, cyclin E, 17β-estradiol, tamoxifen

Introduction

Breast cancer is the most common cancer among women and remains the 2nd leading cause of cancer death in females. Studies have shown that dysregulation of cell cycles plays a critical role in breast cancer development and metastasis, but the mechanisms of cell cycle dysregulation in breast cancer are not completely understood.

Cell cycle is composed of G1, S, G2 and M phases. Normal cells progress to each phase under the tight control of a series of regulatory factors, including cyclin family. Previous studies have demonstrated that cyclin E, as the main promoting factor in G1/S phase transition, is associated with the occurrence, development and prognosis of various types of tumors, including breast cancer [1-3]. Cyclin E is a type of nucleoprotein, the gene of which is located at 19q12-13 and encodes 395 amino acids. It binds to cyclin-dependent kinase 2 (CDK2) to form cyclin E/CDK2 complex, which participates in the phosphorylation of retinoblastoma protein in G1 and S phases, accelerates S phase and affects the initiation of DNA replication. Hillon NK et al. analyzed 114 cases of breast cancer, and found that the mortality rate of patients with cyclin E overexpression was significantly higher than patients with low expression of cyclin E [4], although another study by Barkhem et al. failed to demonstrate the connections between cyclin E and prognosis using multivariate analysis [5, 6]. One aim of our current study is to explore whether cyclin E is overexpressed in breast cancer and whether its expression is associated with nodal metastasis, pathological stage, and the status of estrogen receptor.

Both endogenous and extrinsic estrogens play important roles in the occurrence and development of breast cancer. Estrogen promotes breast cancer via two ways, its toxic metabolite and estrogen receptor signaling pathway. Tamoxifen is a type of selective estrogen receptor modulator that inhibits the classical estro-

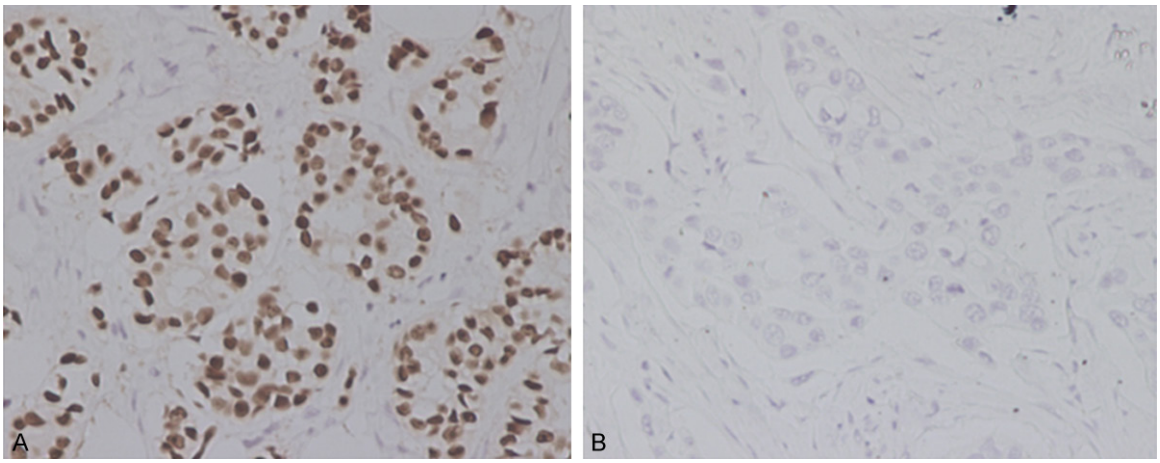

Figure 1. Staining of cyclin E of breast cancer. A. Positive staining. B. Negative staining. Samples were treated with DAB staining kit (Wuhan Boshi Biotech., China) before being washed with tap water for 15 min, followed by counter-staining with hematoxylin for 1 min. The samples were then washed with tap water for 15 min until returning to blue. Finally, the samples were dehydrated, dried, and cover-slipped for study under the microscope.

Table 1. Positive rate of cyclin E in breast cancer and benign breast fibroadenoma

Types of cancers	Total No.	No. of positive patients	Positive rate (%)
Breast cancer	40	17	42.5
Breast fibroadenoma	40	5	12.5

Table 2. Relationship between pathological factors and the expression of cyclin E in breast cancer and benign breast fibroadenoma

Pathological factors	Cyclin E positive	Cyclin E negative
Pathological types		
Infiltrating ductal carcinoma	10	13
Other types	7	10
Age (years)		
≤ 50	5	6
> 50	12	17
Lesion locations		
Left side	8	10
Right side	9	13
Lymph node metastasis		
Yes	12	4
No	9	15
Estrogen receptor		
Negative	8	5
Positive	2	25

gen receptor signaling pathway. It binds to estrogen receptor and alters its conformation, thus suppresses the formation of estrogen receptor dimer and nuclear translocation. Tamoxifen has been widely used since 1870s as an endocrine therapeutic drug for breast cancer. In this study, we also aimed to study the role of estrogen and estrogen receptor pathway in the regulation of cyclin E. We hypothesize that increased cyclin E expression in breast cancer is induced by estrogen pathway.

Materials and methods

Samples

All cases were obtained from patients who underwent mastectomy at the Fifth People's Hospital of Jinan between January 2008 and December 2012, including 40 cases of invasive breast cancer and 40 cases of fibroadenoma. The diagnosis was confirmed using Hematoxylin and Eosin (HE) staining by two pathologists. Tissue samples were immediately frozen in liquid nitrogen after resection and stored at -80°C until use. None of the patients had previous neoadjuvant therapy. Written informed consent was obtained from all patients. Collections and using of tissue samples were approved by the Ethics Committee of Shandong University.

Immunohistochemistry

Cyclin E monoclonal antibody and S-P kits were purchased from Sunbio Biotechnology (Shang-

Table 3. Quantitative data of Cyclin E induction by 17β-estradiol

17β-estradiol concentration (mol/l)	Blank	× 10^{-7}	× 10^{-8}	× 10^{-9}
β-actin	20.4121	20.5745	20.9289	20.8464
	20.2459	20.7249	20.9314	20.6687
	20.2514	20.7816	20.9573	20.6775
Cyclin E	27.3281	26.644	27.1512	27.0055
	27.1214	26.5899	26.918	26.9808
	27.1356	26.4488	26.9582	26.8615
Δt	6.916	6.069	6.222	6.159
	6.875	5.865	5.987	6.312
	6.884	5.667	6.001	6.184
ΔΔt	0.000	-0.846	-0.694	-0.757
	0.000	-1.011	-0.889	-0.563
	0.000	-1.217	-0.883	-0.700
2-ΔΔt	1.000	1.798	1.617	1.690
	1.000	2.015	1.852	1.478
	1.000	2.325	1.845	1.625
Means	1.000	2.046	1.771	1.597

Note: MCF-7 cells were treated with 17β-estradiol (10^{-7} mol/l, 10^{-8} mol/l and 10^{-9} mol/l) for 48 h. Cyclin E mRNA was measured using Fermentas qRT-PCR system (Thermo Scientific, USA). Human β-actin was used as internal reference.

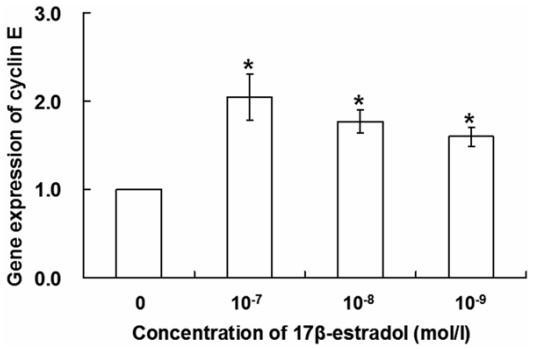

Figure 2. Cyclin E induction by 17β-estradiol. MCF-7 cells were treated with 17β-estradiol (10^{-7} mol/l, 10^{-8} mol/l and 10^{-9} mol/l) for 48 h. Cyclin E mRNA was measured using Fermentas qRT-PCR system (Thermo Scientific, USA). Human β-actin was used as internal reference. Values were normalized to that of the control. Data are expressed as means ± SEM. *, The data were subjected to correlation nanlysis with SPSS and r was 0.551.

hai, China). The test was performed according to the manufacturer's manual. Phosphate-buffered saline was used as negative controls, and positive cancer tissues were used as positive controls.

3, 3'-diaminobenzidine (DAB) staining

Samples were treated with DAB staining kit (Wuhan Boshi Biotech., China) before being washed with tap water for 15 min, followed by counterstaining with hematoxylin for 1 min. The samples were then washed with tap water for 15 min until returning to blue. Finally, the samples were dehydrated, dried, and coverslipped for study under the microscope. Positive staining of cyclin E is confined to the nucleus. The number of positive cells was counted from 5 different areas. From each area, 200 tumor cells were counted, with a total of 1,000 cells in each case. The percentage of positively stained cells was counted. Less than 10% positivity was considered negative, while > 10% positivity was considered positive.

Cell culture

MCF-7 cells were purchased from Biofavor Biotechnology (Wuhan, China) and cultured in phenol red-free a-Minimum Essential Medium (MEM) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 10 μg insulin, under 37°C and 5% CO_2. Log-phase cells were trypsinized and seeded into 6-well plates at a density of 5 × 10^5 per well. Twenty four hours later, cells were washed with phosphate-buffered saline and switched to serum-free, phenol red-free a-MEM medium. Then, 17β-estradiol (10^{-7} mol/l, 10^{-8} mol/l and 10^{-9} mol/l) or tamoxifen (10^{-5} mol/l, 10^{-6} mol/l and 10^{-7} mol/l) were added into the wells and incubated for 48 hours.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using Trizol reagent following manufacturer's manual. Concentration of RNA was measured using a spectrophotometer (Model 752, Shanghai Shunyu Hengping Scientific Instrument Co., Ltd., China). RNA template (4.285 μg) from each sample was mixed with 2 μl Oligo (dT) and 2 μl dNTP, and then diluted to 14.5 μl using RNase-free H_2O. The mixture was incubated at 70°C for 5 min, centrifuged for a short period of time and then

Table 4. Quantitative data of Cyclin E inhibition by tamoxifen

Tamoxifen concentration (mol/l)	Blank	× 10^{-5}	× 10^{-6}	× 10^{-7}
β-actin	20.4348	20.7533	20.8954	21.1701
	20.448	20.6341	20.9097	20.9317
	20.5483	20.6949	21.1219	20.9829
Cyclin E	26.5699	27.6508	27.5654	27.2633
	26.5998	27.6617	27.8049	27.4156
	26.6192	27.8493	27.703	27.5932
Δt	6.135	6.898	6.670	6.093
	6.152	7.028	6.895	6.484
	6.071	7.154	6.581	6.610
ΔΔt	0.000	0.762	0.535	-0.042
	0.000	0.876	0.743	0.332
	0.000	1.084	0.510	0.539
2-ΔΔt	1.000	0.590	0.690	1.030
	1.000	0.545	0.597	0.794
	1.000	0.472	0.702	0.688
Means	1.000	0.535	0.663	0.837

Note: MCF-7 cells were treated with tamoxifen (10^{-5} mol/l, 10^{-6} mol/l and 10^{-7} mol/l) for 48 h. Cyclin E mRNA was measured using Fermentas qRT-PCR system (Thermo Scientific, USA). Human β-actin was used as internal reference.

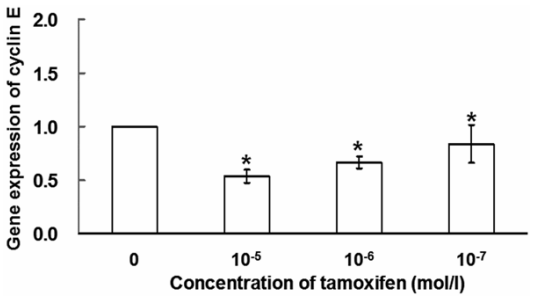

Figure 3. Cyclin E inhibition by tamoxifen. MCF-7 cells were treated with tamoxifen (10^{-5} mol/l, 10^{-6} mol/l and 10^{-7} mol/l) for 48 h. Cyclin E mRNA was measured using Fermentas qRT-PCR system (Thermo Scientific, USA). Human β-actin was used as internal reference. Values were normalized to that of the control. Data are expressed as means ± SEM. *, The data were subjected to correlation nanlysis with SPSS and r was 0.718.

placed on ice. Next, the mixture was mixed with 5 × RT buffer (4 μl), HRP (RRI)/RNase inhibitor (0.5 μl) and M-MLV (1 μl), and diluted to 20 μl using RNase-free H_2O. For cDNA synthesis, the mixture was incubated at 42°C for 60 min and 95°C for 5 min.

Cyclin E gene was amplified using Fermentas qRT-PCR system (Thermo Scientific, USA). Human β-actin was used as internal reference. All primers were synthesized by GenScript Company (Nanjing, China). Cyclin E forward primer was AGTGGCGTTTAAGTCCCCTG, and reverse primer was ATACAAGGCCGAAGCAGCAA. Human β-actin forward primer was AGCGAGCATCCCCCAAAGTT, and reverse primer was GGGCACGAAGGCTCATCATT. PCR reaction system contained 1 μL cDNA, 2 μL dNTP (2.5 mM), 0.25 μL Ex Taq, 2.5 μL Ex Taq E buffer (10 ×), forward primer (0.5 μL) and reverse primer (0.5 μL), and was brought to a final volume of 25 μL by adding RNase free H_2O. PCR procedures are as follows: initial denaturation at 94°C for 4 min, denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 25 s, 30 cycles. After amplification, melting curve analysis was performed.

Statistical analysis

All statistical analyses were performed with SPSS 17.0 for Windows (StatSoft Inc., USA). All results are expressed as means ± SEM. All data were subjected to chi-square test. $P < 0.05$ was considered statistically significant. The data of the Quantitative real-time polymerase chain reaction were subjected to correlation analysis with SPSS.

Results

Cyclin E is overexpressed in breast cancer and its expression is associated with the status of lymph node metastasis and estrogen receptors

To determine the level of cyclin E expression in breast cancer, immunohistochemistry was performed in breast cancer specimens. Positive staining of cyclin E is confined to the nucleus (**Figure 1A** and **1B**). Benign breast tumor, fibroadenoma, was used as a control. As summarized in **Table 1**, the positive staining rate of cyclin E in breast cancer is 42.5%, compared to only 12.5% in fibroadenoma ($P < 0.05$). Next we studied the relationship between cyclin E expression and various clinical parameters. We found that the expression of cyclin E was not related to pathological types, ages and lesion locations ($P > 0.05$), but was associated with the presence of lymph node metastasis and the expression of estrogen receptors ($P < 0.05$) (**Table 2**).

17β-estradiol increases cyclin E expression in a dose-dependent manner

To study the potential mechanisms that mediate cyclin E overexpression in breast cancer, we treated breast cancer cells (MCF-7) with different doses of 17β-estradiol for 48 hours. Cyclin E mRNA level was measured by qRT-PCR. As shown in **Table 3** and **Figure 2**, cyclin E mRNA level is induced by 17β-estradiol in a dose dependent manner. The data of quantitative real-time polymerase chain reaction were subjected to correlation analysis with SPSS, and r value was 0.551. As the concentration of 17β-estradiol increased, the enhancement increased.

Tamoxifen decreases cyclin E expression in a dose-dependent manner

To test whether estrogen induces cyclin E expression via estrogen receptor pathway, we treated cells with tamoxifen, which blocks the pathway. After 48 hours treatment, cyclin E mRNA level was measured. As shown in **Table 4** and **Figure 3**, tamoxifen inhibits cyclin E expression in a dose dependent manner. The data of quantitative real-time polymerase chain reaction were subjected to correlation analysis with SPSS, and r value was 0.718. As the concentration of tamoxifen decreased, the inhibition increased.

Discussion

Breast cancer, as the 2nd leading cause of death and the most common cancer in women, its mechanisms are not completely understood. Both estrogen receptor pathway and cell signal pathway play important roles in breast cancer development. The relationship between these two pathways, however, is not established. In this paper, we aimed to explore the relationship between these two pathways. We found that cyclin E is overexpressed in breast cancer compared to benign breast tumors (**Table 1**). Its expression is induced by estrogen in vitro, while inhibited by the antagonist of estrogen receptor, tamoxifen (**Figures 1** and **2**). We also found that the expression of cyclin E is not related to pathological types, ages and locations of the lesion, but is strongly associated with the status of lymph node metastasis and the expression of estrogen receptors.

Estrogen stimulates the growth of breast cancer cells by binding estrogen receptor. The main downstream transcriptional signaling of estrogen receptor is c-Myc expression [7]. Myc activates cyclin E/CDK2 in breast cancer cells resulting in cell cycle progression. The role of cyclin E in breast cancer has been previously studied in animal models. Weroha SJ et al. discovered that cyclin E/CDK2 complex participated in all steps of estradiol-induced breat cancerization in rats by stimulating the over-replication of centrosome [8]. Breast cancer cells can also express the homopolymer of cyclin E with low molecular weight [9]. In human, cyclin E has been linked to breast cancer prognosis. There were studies on the relationship between cyclin E and breast cancer prognosis, some of which showed that poor prognosis was related to the high expression of cyclin E [10-13]. Keyomarsi K et al. showed that cyclin E was usually overexpressed in breast cancer, and the activity of cyclin E in breast cancer could be used as predictors for judging tumor aggressiveness and its clinical prognosis [14]. Our results confirmed the overexpression of cyclin E in breast cancer and found that cyclin E is associated with lymph node metastasis and the status of estrogen receptor. The overexpression of cyclin E may be a promising therapeutic target in breast cancer. In clinic, high level of cyclin E was found to be associated with resistance to endocrine therapy and human epidermal growth factor receptor-2 monoclonal antibody therapy [15, 16]. Thus, developing drugs or small molecules that target to lower cyclin E level in breast cancer will be potentially enhance breast cancer therapy.

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Disclosure of conflict of interest

None.

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