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ORIGINAL PAPER

Expression of ER, Ki-67 and CylinD1 in the Pre-cancerous Breast of Chinese Patients

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Abstract To investigate the expression and association of ER, Ki-67 and cyclinD1 in usual ductal hyperplasia(UDH), atypical ductal hyperplasia (ADH) and ductal carcinoma in situ(DCIS) in the breast. The study included 56 cases of pre-cancerous lesions which were surgically excised at Qi Lu Hospital of Shangdong University. Immunohistochemistry was used to determine the expression of ER, Ki-67 and cyclinD1 and double-labelling immunofluorescence technique was used to observe the coexpression of ER and Ki-67. The expression and distribution of ER-positive cells were significantly different in UDH, ADH and DCIS. The ER-positive cells were much more in UDH than in normal TDLUs (terminal duct lobular units). The distribution of ER-positive cells interspersed amid ER-negative cells within UDH. However , the ER positive cells showed marked increases in ADH and low grade nuclear DCIS ($P<0.05$), distributing in almost all constituent cells. The expression of ki-67 and cyclinD1 were significantly different between UDH and DCIS ($P<0.05$) , and a positive correlation was found between expression of Ki-67 and morphological classification of pre-cancerous lesions ($r=0.3522$, $P<0.05$) as well as cyclinD1 ($r=0.3901$, $P<0.05$). Double-labelling immunofluorescence showed that there was no coexpression of ER and Ki-67 in normal breast tissue. The coexpression of the two markers was found in ADH and increased in DCIS. Overexpression of ER, Ki-67 and cyclinD1 significantly accompanies the transition of normal cells and UDH to ADH and DCIS. The coexpression of ER and ki-67 may present the early change in carcinogenesis of breast cancer.

Keywords Pre-cancerous lesions · Breast · ER · Ki-67 · CyclinD1

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Introduction

Oestrogen is thought to be important in the pathogenesis of breast cancer. Oestrogen acts on breast cells via the oestrogen receptor (ER) , which belongs to a large family of nuclear receptors. The ER is a transcription factor which when bound to oestradiol binds DNA and regulates expression of oestrogen-responsive genes. Exposure to estrogen is an important determinant of the risk of breast cancer , the mechanisms of carcinogenesis in the breast caused by oestrogen include the metabolism of estrogen to genotoxic, mutagenic metabolites and the stimulation of tissue growth. Together, these processes cause initiation, promotion, and progression of carcinogenesis [1]. Epidemiological and experimental evidence suggest that breast cancer risk is related to the duration of estrogen exposure during puberty, the early postmenopausal period, and the menopausal period. Estrogen is also associated with epithelial proliferation in noncancerous breasts during the menstrual cycle and in pregnancy. It has been suggested that ER positivity in benign breast epithelium could be a risk factor for breast malignancy because the presence of

ER is thought to render cells susceptible to proliferation stimulus of estrogen [1–3]. Several other studies have shown a very tight association between Ki-67 immunoreactivity and the cell cycle, with expression beginning in the mid to late G1, rising through S phase and G2 to reach maximum in mitosis. CyclinD1 overexpression is commonly seen in breast cancer cell lines and in vitro can cause activation of the estrogen receptor (ER) and gene transcription even in the presence of tamoxifen [2, 3]. The regulation of ER expression in the pre-cancerous lesions of breast is rarely reported, we hereby presented a detailed immunohistochemical and double-labeling immunofluorescence study of ER and Ki-67 as well as cylinD1 in usual ductal hyperplasia (UDH, also referred to as HUT—hyperplasia of usual type), atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS) of breast to investigate the growth regulation of ductal epithelium by ER.

Materials and Methods

Patients

The study included 56 cases of pre-cancerous lesions which were surgically excised at Qilu Hospital of Shangdong University. The introductal proliferative lesions we obtained from department of pathology of Qilu Hospital following changes: UDH (without atypia, 26), ADH (four), DCIS (26) of low nuclear grade (LNG, eight) and intermediate nuclear grade (ING, nine) and high nuclear grade (HNG, nine). All the diagnoses were made following the Pathology and Genetics of Tumors of Breast of World Health Organization Classification of Tumours [4] and were made by three pathologists.

Immunostaining Procedure

Immunohistochemistry was performed on 4-μm-thick routinely processed paraffin sections. The primary monoclonal antibodies used were directed against ER, Ki-67 and cyclinD1. ER was detected with a rabbit monoclonal anti-ER antibody (clone SP1;Lab Vision, dilution 1:200) and Ki-67 with a mouse monoclonal anti-Ki-67 antibody (clone MIB-1; DAKO, dilution 1:200) and cyclinD1 with a mouse monoclonal anti-cylinD1 antibody (clone DSC-6; DAKO, dilution 1:200). Sections were de-waxed, and endogenous peroxidase was blocked by immersing the slides in a 3% solution of hydrogen peroxide in methanol for 10 min. This was followed by a step of antigen retrieval, for all three antibodies , in which the slides were immersed in 0.01 M/L citrate buffer solution (pH 6.0) and placed in a microwave oven for 15 min. After a tap water bath, the sections were covered with non-immune horse serum for 30 min. After draining serum, the sections were covered with the primary antibodies overnight at 4°C in a humidity chamber. After that sections were rinsed with TBS (Tris-buffered saline) before incubated with the biotinylated second antibody for 40 min at 37°C in a humidity chamber. After rinsing with TBS, the streptavidin–peroxidase complex reagent (StrepABComplex/HRP Duet, DAKO) was applied for 30 min at 37°C. After washing, DAB was applied for 5 min under microscope. Sections were then immersed in running tap water, counterstained with haematoxylin for 1 min, followed by tap water bath, immersion in a series of alcohol baths of increasing concentrations and then xylene, then applied the coverslips. Negative controls in which the primary antibody was omitted and positive controls of ER, Ki-67 and cyclinD1, positive breast carcinoma of varying staining intensities, were included in each batch of immunohistochemistry.

Dual Immunofluorescence Staining

Double-lablling immunofluorescence technique was performed by the application of 100 μl of a mixture of both primary antibodies including the monoclonal rabbit anti-human ER antibody (clone SP1; Lab Vision, dilution 1:200) and mouse anti-human Ki-67 antibody (clone MIB-1; DAKO, dilution 1:200) for 90 min at 37°C in a humidity chamber. Thereafter this was followed by the application of 100 μl of a mixture of both secondary antibodies for 45 min. The secondary antibodies used were Cy3-conjugated sheep anti-rabbit antibody (SIGMA C2306) and biotinylated goat anti-mouse antibody (DAKO E0433). The slides were then incubated with FITC–streptavidin (fluorescein isothiocyanate–streptavdin, DAKO F0422) for 45 min to visualize the anti-mouse antibody. The slides were assessed by Nikon eclipse E600. All incubations were at room temperature and rinse in PBS (phosphate-buffered saline) were performed in between.

Assessment of Immunostaining

Ductal proliferations were assessed for the numbers and percentage of ER-positive , Ki-67-positive and cyclinD1-positive cells within lesions counted through microscopy under high power, at random every 1,000 epithelial cells. Before counting each field was masked to remove from the analysis all elements. Assessment of benign components (UDH) of mastectomy specimens was restricted to ostensibly normal tissue, if any of the pre-cancerous lesions including ADH and DCIS were present, they were not included. In order to maximize consistency of scoring, only nuclei showing moderate or strong staining were regarded as positive. The results are expressed as mean±standard deviation (mean±SD). The data were analysed by Pearson

correlation coefficient and one-way analysis of variance (OANOVA) for significance ($P<0.05$; Tables 1 and 2) using SPSS 10.0 software for Windows.

Assessment of Dual Immunofluorescence Staining

Each field was examined under high power for the red (Cy3), green (FITC), using appropriate filters (Nikon filters G-1B, B-1A respectively) according their corresponding absorbance Max to assess the presence and absence of dual-labeled cells. The percentage of coexpression cells (yellow) was calculated in relation to total cell number within pre-cancerous hyperplastic foci and adjacent normal lobules separately.

Results

Distribution of ER+ Cells in HUT, ADH and DCIS

Nuclear staining for ER was restricted to the epithelial cells of ducts and lobules both in the adjacent normal breast tissue and the lesions examined. In normal TDLUs there were single scattered ER+ cells surrounded by ER-cells. In UDH the staining patterns varied. In many areas, ER+ cells were intimately mixed with and separated by ER− cells. But in some zones immunostaining was contiguous. Sometimes there were more ER+ cells at the periphery of a proliferation than the center (Fig. 1a).

In contrast, virtually all cases of ADH were positive and so contiguous expression was observed in all cases (Fig. 1b), the percentages of ER+ cells of all cases were over 80%. Comparing with UDH, there was a statistically difference ($P<0.05$). A similar staining pattern was observed in low nuclear grade DCIS (Fig. 1c). The percentages of ER+ cells in low nuclear grade DCIS were over 80% in seven out of the eight cases , and the distribution pattern was contiguous. The expression of ER was significant difference between low nuclear grade DCIS and UDH ($P<0.05$). The intermediate nuclear grade DCIS expressed weaker ER than UDH, ADH and low nuclear grade DCIS. As to high nuclear grade DCIS, there were even less or no ER positive cells in the lesions (Fig. 1d), only two out of the nine cases with ER+ cells in 40–50%, the others were all below 5% or completely negative, and there was a significant difference in the percentage of ER+ cells compared to the low nuclear grade DCIS and ADH ($P<0.05$). The mean percentage of positive cells (expressed as mean ER+%) and the average number of positive cells (shown as mean±SD) examined in each patient was listed (Table 1).

Expression of Ki-67in UDH, ADH and DCIS

The percentage of Ki-67+ cells were under 2% in 18 out of the 26 UDH cases, the mean percentage of all UDH cases were 3.2%. Of the four ADH cases, one were 16%, and the mean percentage of Ki-67+ cells was 7.2%. The mean percentage of all 26 DCIS cases was 11.3%, five of all were over 10%. There was a significant difference in multiple comparison ($P<0.05$), and showed a significant difference between histological grade of introductal proliferative lesions of breast. In dual comparison, there was a significant difference between UDH and DCIS ($P<0.05$). The mean percentage of positive cells and the average number of positive cells examined in each patient were also listed (Table 2).

Expression of CyclinD1 in UDH,ADH and DCIS

Of the 26 UDH cases, 16 were founded scattered cyclinD1-positive cells in the lesions, and the mean percentage was 2.0%. The mean percentage of ADH was 6.0%. The mean percentage of 26 DCIS cases was 9.9%, and ten cases were over 10%. It was similar to Ki-67 that the expression of cyclinD1 showed a significant difference ($P<0.05$) between histological grade of pre-cancerous lesions of breast, and there was a enhancing trend from UDH to ADH and DCIS. In multiple comparison, there was a significant difference between UDH and DCIS ($P<0.05$; Table 2).

Relationship Between the Expression of ER,Ki-67 and CyclinD1 and Histological Grade

The mean percentage of Ki-67 positive cells increased and the staining correlated significantly with the morphological classification of intraductal proliferative lesions, from UDH

Table 1 Relationship between ER and morphological classification

	Number of patients	Mean ER+%	Mean±SD
UDH	26	38	425.50±129.64
ADH	4	86	817.25±78.36*,**
DCIS (LNG)	8	72	699.88±181.21*,**
DCIS (ING)	9	51	470.75±378.45
DCIS (HNG)	9	24	237.67±191.32

*$P<0.05$, compared with UDH
**$P<0.01$, compared with high nuclear grade DCIS

Table 2 Relationship between Ki-67, cyclinD1 and morphological classification

	Number of patients	Ki-67		CyclinD1	
		Mean Ki-67+%	Mean±SD	Mean cyclinD1+%	Mean±SD
UDH	26	1.7	31.87±28.56	2.0	24.51±21.23
ADH	4	7.2	70.35±65.47	6.0	58.55±41.97
DCIS	26	11.3	105.06±101.39*	9.9	89.76±83.06*

*$P<0.05$, compared with UDH

to ADH and DCIS, ($r=0.352$, $P<0.05$), as well as cyclinD1+ cells ($r=0.390$, $P<0.05$). In ADH and low nuclear grade DCIS, the expression of ER reached the top comparing with UDH and high nuclear grade DCIS, meanwhile the proliferation of epithelia more active with increasing expression of Ki-67 and cyclinD1.

Co-expression of ER and Ki-67 in Introductal Proliferative Lesions of Breast

In normal TDLUs adjacent to UDH there were scattered ER-positive cells (red) and Ki-67-positive cells (green), no dual-labeled cells (yellow) were observed (Fig. 2a). In UDH a few dual-labeled cells were found (Fig. 2b), but in ADH (Fig. 2c) and DCIS (Fig. 2d) the number of dual-labeled cells were significantly increased. It was obvious that the mutual excluding of ER and Ki-67 began to disappear.

Discussion

Oestrogen is a potent mitogen for normal breast epithelial cells and is believed to be a major contributor to the pathogenesis of breast cancer. Oestrogen regulates normal growth and differentiation of breast epithelium by interacting with ER. In turn, ER influence the expression of oestrogen-responsive genes by acting as a nuclear transcription factor. Investigators have used mainly immunohistochemistry to explore the alteration of ER expression in the progression of introductal proliferative lesions of breast. In healthy premenopausal women, ER is expressed in the majority of TDLUs, but only a minority of the comprising cells (10%) are positive for ER, and they are distributed singly amid ER-negative cells. The expression varies with the phase of the menstrual cycle and is highest in the follicular phase [5]. Shoker et al. [6] have demonstrated a clear correlation between age and the occurrence of a

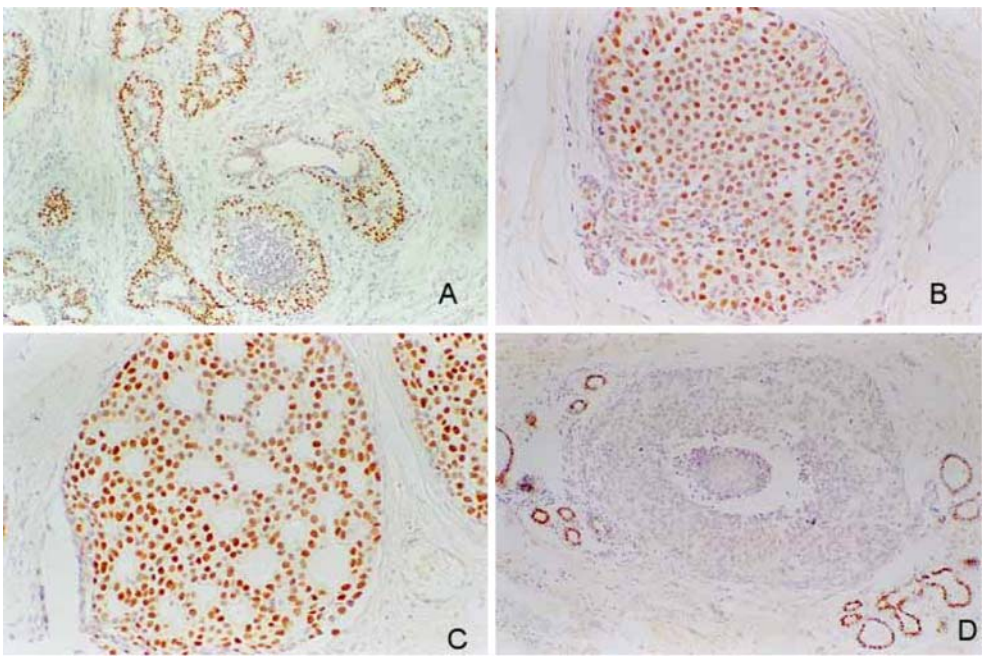

Fig. 1 Expression of ER in precancerous lesions of breast. **a** ER+ cells in a case of UDH. Note that most of the ductal epithelial cells in the periphery are positive, ×4. **b** ER+ cells in a case of ADH. Almost 100% of the constituent cells of the lesion are ER-positive, ×10. **c** A case of low grade nuclear DCIS showing ER positivity similar to ADH, ×10. **d** High grade nuclear DCIS showing estrogen receptor-negative cells, ×4

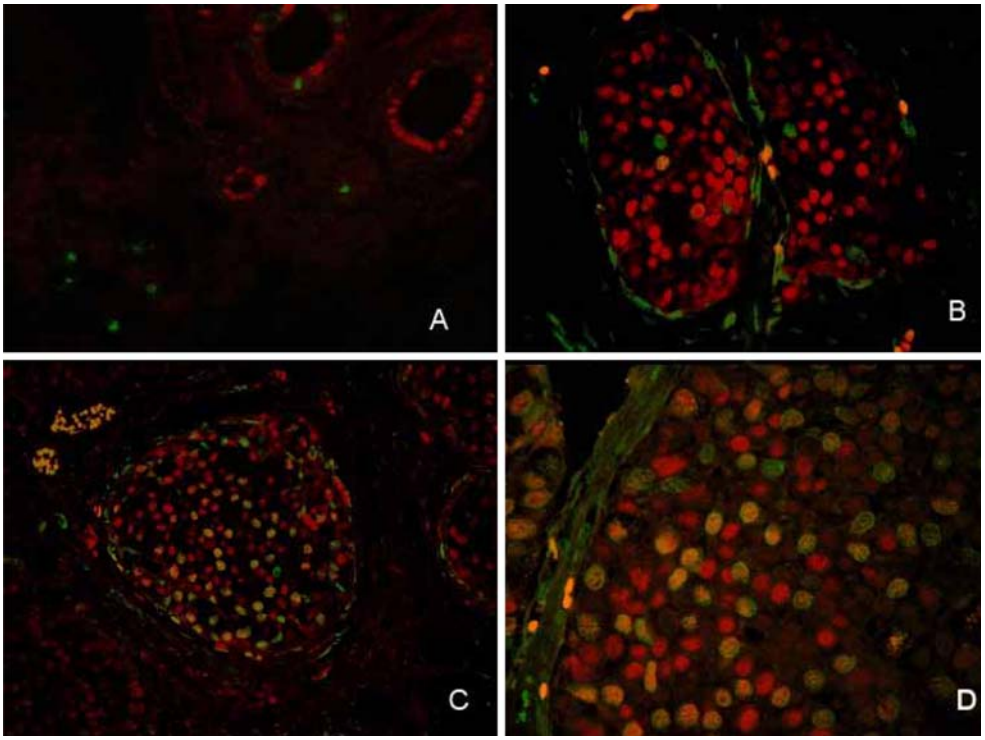

Fig. 2 Coexpression of ER and Ki-67 in precancerous lesions of breast. **a** Indirect immunofluorescence for ER (red), Ki-67 (green) in normal TDLUs, ×20. **b** Indirect immunofluorescence for ER (red), Ki-67 (green) and dual-labeled cells (yellow) in UDH, ×20. **c** Indirect immunofluorescence for ER (red), Ki-67 (green).and dual-labeled cells (yellow) in ADH, ×10. **d** Indirect immunofluorescence for ER (red), Ki-67 (green) and dual-labeled cells (yellow) in DCIS, ×40

contiguous pattern of ER expression in the cells of TDLUs. Studies evaluating ER expression in hyperplastic lesions have unanimously concluded that these lesions significantly overexpress ER and that progressive alteration in ER accompanies the transition of normal cells to hyperplastic lesions and in situ carcinoma. In general, UDH lesions contain more ER-positive cells than normal TDLUs but maintain the usual relation between ER expression and age. In sharp contrast to UDH, however, almost all cases of ADH, low nuclear grade DCIS show marked increases in ER expression in almost 100% of constituent cells and, more importantly, the relation to age is lost [5–8]. ER expression in DCIS generally varies with the differentiation of the constituent cells. Although low nuclear grade DCIS invariably shows ER positivity in the majority of cells, only approximately 30% of high nuclear grade DCIS lesions show ER positivity [5, 9–11].

It is therefore conceivable that an increase in ER-positive cells in the non-neoplastic breast, particularly if in contiguity, could represent a pre-cancerous change. This is an attractive hypothesis because such breasts may be more susceptible to the effects of oestrogen [6].

In this study, we have confirmed the findings of others [5–7] that only a small minority of epithelial cells in the normal TDLUs are ER-positive cells and that they are generally surrounded by ER-negative cells, and we have found that the progressive alteration of ER positivity accompanies the transition from normal TDLUs to UDH, ADH and DCIS. In this process the patterns of the distribution of ER-positive cells are significantly different as well as the numbers and intensity. We think that there is a dynamic change of ER-positive cells in the progress from normal TDLUs to UDH, ADH and DCIS, with the introductal epithelial proliferation of breast, the ER expression increases gradually. Some studies [12] have revealed a mutation of ER gene in ADH and DCIS. ADH and DCIS lose control of the epithelial hyperplasia because of the overexpression of mutational ER, finally, the mutation leads to malignant change accompanied with abnormity of regulation cell cycle and proliferation.

In normal breast and the majority of benign hyperplastic lesions, the expression of ER and Ki-67 mutually excludes evidenced by the lack of dual immunostaining for ER and Ki-67 antigen. But the negative association is lost in UDH associated with an increased risk of carcinoma and all ADH and DCIS lesions that overexpress ER evidenced by the coexpression of ER and Ki-67, which indicates dysregulation of ER-positive proliferative cells [13–19].

Our findings support that UDH, ADH and DCIS are different introductal proliferative and pre-cancerous lesions of breast, they have different relative risk of developing invasive carcinoma because the dysregulation of proliferation and differentiation in different gene level. As pre-cancerous lesion, ADH has exhibited some characters of DCIS in biological phenotypes evidenced by the similar expression of ER, Ki-67 and cyclinD1. The coexpression of ER and Ki-67 farther indicates the dysregulation of proliferation in ADH and DCIS, and this may eventually

lead to the greater loss of control of cell division and function which characterize in situ and invasive breast carcinomas, in support of this contention is the abnormal expression of cylinD1. In our study, there is a similar expression of ER, Ki-67 and cyclinD1 in ADH and low nuclear grade DCIS. In ADH and low nuclear grade DCIS the expression of ER reaches the top comparing with UDH and high nuclear grade DCIS, meanwhile the proliferation of epithelia becomes more active, and the mutual excluding of ER and Ki-67 begins to disappear and the coexpression increases. All these indicate that ADH and low nuclear DCIS are likely to be real neoplastic lesions, and that ADH is early neoplastic lesion or the beginning of neoplastic lesion, we should pay more attention to it. In some UDH and ADH, the ER immunostaining is very helpful when differential diagnosis should be needed.

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