Oestrogen-induced genes in ductal carcinoma *in situ*: their comparison with invasive ductal carcinoma

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Abstract

It is well known that oestrogens play important roles in both the pathogenesis and development of invasive ductal carcinoma (IDC) of human breast. However, molecular features of oestrogen actions have remained largely unclear in pure ductal carcinoma *in situ* (pDCIS), regarded as a precursor lesion of many IDCs. This is partly due to the fact that gene expression profiles of oestrogen-responsive genes have not been examined in pDCIS. Therefore, we first examined the profiles of oestrogen-induced genes in oestrogen receptor (ER)-positive pDCIS and DCIS (DCIS component (DCIS-c)) and IDC (IDC component (IDC-c)) components of IDC cases ($n=4$ respectively) by microarray analysis. Oestrogen-induced genes identified in this study were tentatively classified into three different groups in the hierarchical clustering analysis, and 33% of the genes were predominantly expressed in pDCIS rather than DCIS-c or IDC-c cases. Among these genes, the status of *MYB* (C-MYB), *RBBP7* (RBAP46) and *BIRC5* (survivin) expressions in carcinoma cells was significantly higher in ER-positive pDCIS ($n=53$) than that in ER-positive DCIS-c ($n=27$) or IDC-c ($n=27$) by subsequent immunohistochemical analysis of the corresponding genes ($P<0.0001$, $P=0.03$ and $P=0.0003$ respectively). In particular, the status of C-MYB immunoreactivity was inversely ($P=0.006$) correlated with Ki67 in the pDCIS cases. These results suggest that expression profiles of oestrogen-induced genes in pDCIS may be different from those in IDC; and C-MYB, RBAP46 and survivin may play important roles particularly among oestrogen-induced genes in ER-positive pDCIS.

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Introduction

Breast cancer is the most common malignant neoplasm in women worldwide. In particular, the incidence of ductal carcinoma *in situ* (DCIS) has been markedly increasing possibly due to advancements in population-based mammographic screening for detection (Li *et al.* 2005), and ~20% of breast carcinoma cases actually present as pure DCIS (pDCIS) without invasive components at the time of diagnosis in many countries (Kepple *et al.* 2006, Tsikitis & Chung 2006). This pDCIS is in general considered as

a precursor lesion of invasive ductal carcinoma (IDC). It has been demonstrated that approximately half of untreated pDCIS progresses to IDC with marked variability in the latency of the progression (Cuzick 2003) and up to 80% of IDC were also reported to contain at least small foci of DCIS component (DCIS-c) distinct from the IDC component (IDC-c) if carefully evaluated (Ellis *et al.* 2003). Therefore, it has become very important to examine the biological features of pDCIS to identify the possible molecular mechanisms related to the acquisition of invasive

properties and subsequently to improve clinical outcome of early breast cancer patients.

It is well known that oestrogens play important roles in the progression of breast carcinoma through an interaction with oestrogen receptor (ER). ER is expressed in approximately two-thirds of IDC, and endocrine therapy has been administered in these patients in order to suppress the intratumoural oestrogen actions. A great majority of pDCIS was also reported to express ER in their parenchymal cells (Wiechmann & Kuerer 2008), and the results of National Surgical Adjuvant Breast Project (NSABP) B-24 trial did demonstrate that adjuvant tamoxifen therapy was clinically effective in ER-positive pDCIS and reduced the recurrence of noninvasive breast carcinomas by 27% (Cuzick 2003). Pathological and biological responses to preoperative tamoxifen therapy in ER-positive pDCIS patients has been also reported (Chen et al. 2009).

ER is well known to activate the transcription of various target genes in a ligand-dependent manner, and various oestrogenic functions are also characterised by expression profiles of these genes in oestrogen target cells. Various oestrogen-responsive genes have been also identified in IDC (Frasor et al. 2003), and an analysis of these genes can greatly contribute to the understanding of molecular functions of oestrogen actions, such as cell proliferation, anti-apoptosis, invasion, metastasis, recurrence and resistance to endocrine therapy, in IDC (Suzuki et al. 2012). However, expression profiles of oestrogen-responsive genes have not necessarily been examined in pDCIS to the best of our knowledge. Therefore, it has still remained unclear whether oestrogen actions and/or effectiveness of endocrine therapy in pDCIS could be the same as that in IDC.

Therefore, in this study, we first examined expression profiles of oestrogen-induced genes in carcinoma tissues of breast cancer patients and demonstrated different expression profiles of oestrogen-induced genes in ER-positive pDCIS from ER-positive DCIS-c or IDC-c following an isolation of the corresponding cells under light microscopy using laser-capture dissection. Subsequent microarray analysis indicated that *MYB* (C-MYB), *RBBP7* (retinoblastoma suppressor (Rb)-associated protein 46 (RBAP46)) and *BIRC5* (survivin) were predominantly expressed in pDCIS compared with DCIS-c and IDC-c among these oestrogen-induced genes. Therefore, we subsequently immunolocalised these gene products in ER-positive pDCIS tissues in order to further characterise their oestrogenic actions.

Materials and methods

Patients and tissues

Two sets of tissue specimens were used in this study. The first set is composed of eight specimens of ER-positive breast carcinoma (four pDCIS and four IDC cases) obtained from Japanese women (age: 51–77 years in pDCIS, and 49–75 years in IDC) who underwent surgical treatment from 2003 to 2008 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. One IDC patient was premenopausal, and the others were postmenopausal. All the IDC specimens used in this study contained both DCIS-c and IDC-c, and the patients did not receive chemotherapy, irradiation or hormonal therapy before the surgery. All the cases examined in this study were associated with nuclear grade 1 or 2, and their ER labelling index (LI) was ranged from 40 to 96% in pDCIS, 35 to 100% in DCIS-c and 42 to 100% in IDC-c respectively. These specimens were stored at -80°C for subsequent microarray analysis. The second set is composed of 80 specimens of ER-positive ductal carcinoma of human breast (53 pDCIS and 27 IDC cases) obtained from Japanese female patients who underwent surgical treatment from 1995 to 2008 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. These patients also did not receive chemotherapy, irradiation or hormonal therapy before the surgery. The median age of these patients was 61 years (range 39–80 years) for pDCIS and 55 (range 32–84 years) for IDC, and all the cases of IDC contained both DCIS-c and IDC-c in this study. All the specimens were fixed in 10% formalin and embedded in paraffin wax.

The entire resected surgical specimen was sectioned into slices with 3–5 mm thickness, and all the slices were histologically evaluated by surgical pathologists. In this study, pDCIS was defined when DCIS-c was detected but no foci of stromal invasion in carcinoma were detected in all the slides of the cases evaluated. In the first set, thinner section stained with haematoxylin and eosin was prepared from the frozen specimen, and histological features of these lesions were confirmed.

Research protocols for this study were approved by the Ethics Committee at Tohoku University Graduate School of Medicine (accession no. 2009-107).

Laser-capture microdissection/microarray analysis

Gene expression profiles of breast carcinoma cells in the first set of the specimens (four pDCIS, four DCIS-c and four IDC-c samples) were examined using microarray analysis. Laser-capture microdissection

(LCM) was conducted using the MMI Cellcut (Molecular Machines and Industries, Fluhhofstrasse, Glattbrugg, Switzerland). Briefly, breast carcinomas were embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetechnical Co., Tokyo, Japan) and sectioned at a thickness of 10 μ m. Breast carcinoma cells were dissected under the light microscopy and laser transferred from these frozen sections. The total RNA ($\sim$ 200 ng) was subsequently extracted from these cell fractions isolated by LCM using the RNeasy Micro Kit (Qiagen). In IDC cases, carcinoma cells were separately collected in DCIS-c and IDC-c. Whole Human Genome Oligo Microarray (G4112F (ID: 012391)), Agilent Technologies (Waldbronn, Germany), containing 41 000 unique probes, was used in this study, and sample preparation and processing were performed according to the manufacturer's protocol. In this study, we focused on the expression of 51 genes identified to be oestrogen-induced ones in MCF7 breast carcinoma cells by Frasar *et al.* (2003) (two genes corresponding *PPP2R1B* were included in this analysis). Hierarchical clustering analysis was performed using the Cluster and TreeView programs (the software copyright Stanford University 1998–1999, <http://rana.stanford.edu>) to generate tree structures based on the degree of similarity, as well as matrices comparing the levels of expression of individual genes in each specimens.

Immunohistochemistry

Immunohistochemical analysis was performed in the second set (53 pDCIS and 27 IDC cases) described above. Monoclonal antibodies for ER (6F11), progesterone receptor (PR; 1A6) and Ki67 (MIB1) were purchased from NovoCastra (Newcastle upon Tyne, UK), Chemicon (Temecula, CA, USA) and DAKO (Carpinteria, CA, USA) respectively. Rabbit polyclonal antibodies for human epidermal growth factor receptor-2 (HER2; A0485) were obtained from DAKO. In addition, rabbit polyclonal antibodies for C-MYB (EPR718(2)), RBAP46 (EPR5082) and survivin (NB500-201) were purchased from Epitomics (Burlingame, CA, USA) and Novus Biologicals (Littleton, CO, USA) respectively.

A Histofine Kit (Nichirei Biosciences, Tokyo, Japan) that employs the streptavidin–biotin amplification method was used in this study. Antigen retrieval was performed by heating the slides in an autoclave at 120 °C for 5 min in antigen retrieval solution (pH 9.0; Nichirei Biosciences) for C-MYB immunostaining or citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6.0)) for immunostaining of other

antibodies. Dilutions of primary antibodies used in this study were as follows: ER, 1/50; PR, 1/50; HER2, 1/100; Ki67, 1/100; C-MYB, 1/50; RBAP46, 1/1000 and survivin, 1/1000. The antigen–antibody complex was subsequently visualised with 3,3'-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6) and 0.006% H₂O₂) and counterstained with haematoxylin. As a positive control, human IDC tissue was used for C-MYB (McHale *et al.* 2008) and survivin (Barnes *et al.* 2006) immunostaining, and a cellblock of MCF7 breast carcinoma cells was used for RBAP46 (Creekmore *et al.* 2008). Normal rabbit IgG was used instead of the primary antibody, as a negative control in this study.

Immunohistochemical evaluation

Immunoreactivity of ER, PR and Ki67 was detected in the nucleus, and their immunoreactivity was evaluated in counting more than 1000 carcinoma cells for each case. The percentage of immunoreactivity, i.e. LI, was subsequently determined. Cases with ER LI of more than 1% were considered ER-positive breast carcinoma in this study (Hammond *et al.* 2010). HER2 immunoreactivity was evaluated according to the grading system proposed in HercepTest (DAKO), and strongly circumscribed membrane-immunoreactivity of HER2 present in more than 30% carcinoma cells were considered positive (Wolff *et al.* 2007). Both C-MYB and RBAP46 immunoreactivities were detected in the nuclei of carcinoma cells and were evaluated by employing the H-scoring system (McCarty *et al.* 1985). Briefly, C-MYB- and RBAP46-positive carcinoma cells were classified into three groups according to immunointensity (i.e. strongly, moderately or weakly positive cells), and H scores were subsequently generated by adding together 3 $\times$ % of strongly positive cells, 2 $\times$ % of moderately positive cells, 1 $\times$ % weakly positive cells, and 0 $\times$ % of negative cells (range 0–300). Survivin immunoreactivity was detected in the cytoplasm of carcinoma cells and was semi-quantitatively evaluated by modified H-scoring system (Mehta *et al.* 2012), in which the percentage of cytoplasmic immunoreactivity was categorised as 0 (no expression), 10 (up to 10%), 20 (10–20%) until 100 (90–100%), and giving a possible range of 0–300.

Statistical analysis

An association of various clinicopathological factors among three carcinoma components (pDCIS, DCIS-c and IDC-c) was evaluated using a Kruskal–Wallis test or a cross-table with the χ^2 test. An association between C-MYB, RBAP46 and survivin immunoreactivity and

clinicopathological factors was evaluated by a cross-table using the χ^2 test. An association of clinicopathological factors between two components of IDC cases was evaluated using a Wilcoxon signed-ranks test. The statistical analyses were performed using the JMP Pro version 9.02 (SAS Institute, Inc., Cary, NC, USA), and *P* values of <0.05 were considered significant in this study.

Results

Expression profiles of oestrogen-induced genes in pDCIS compared with those of DCIS-c and IDC-c

We first surveyed expression profiles of oestrogen-induced genes in isolated carcinoma cells of pDCIS using microarray analysis which was focused on oestrogen-induced genes reported by Frasor *et al.* (2003), in order to examine the characteristics of oestrogenic actions in pDCIS. Fifty-one oestrogen-induced genes examined were tentatively classified into three groups (i.e. Groups A, B and C) depending on the hierarchical clustering analysis (Fig. 1). In addition, isolated and examined pDCIS carcinoma cells were clustered among the cases examined. Results demonstrated that the genes in Group C were predominantly expressed in pDCIS rather than in DCIS-c or IDC-c, and the genes in Group A were predominantly expressed in DCIS-c and/or IDC-c. Genes classified into Group B were expressed regardless of the carcinoma types. No significant clustering of samples was detected in association with nuclear grade, menopausal status and ER LI of the cases examined in this study.

As shown in Table 1, no significant differences of characteristics were detected between Groups A and C in this study.

Clinicopathological features of pDCIS, DCIS-c and IDC-c

We then evaluated an association of various clinicopathological parameters among pDCIS (*n*=53), DCIS-c (*n*=27) and IDC-c (*n*=27), which were examined in this study. Nuclear grade (*P*=0.68), ER LI (*P*=0.94), PR LI (*P*=0.87) and HER2 status (*P*=0.33) were not significantly different among these three groups, but Ki67 LI was significantly (*P*<0.0001) lower in pDCIS than that in DCIS-c and IDC-c (Table 2). No significant differences of patients' age (*P*=0.43) and menopausal status (*P*=0.34) were detected between pDCIS and IDC patients in this study. HER2 positive status in our study (45% in pDCIS, 33% in DCIS-c and 30% in IDC-c) was consistent with that of a previous report (Park *et al.* 2006).

Figure 1 Hierarchical clustering analysis of mRNA expression levels focused on oestrogen-induced genes identified by Frasor *et al.* (2003). Colour of blocks represents relative mRNA expression level of each gene compared with the average in 12 breast carcinoma samples (four pDCIS, four DCIS-c and four IDC-c). Gene symbols in each gene were listed. Gene-performed immunohistochemistry was noted in red. Two genes corresponding *PPP2R1B* were coloured green.

Immunolocalisation of C-MYB, RBAP46 and survivin in pDCIS

Results of the microarray analysis demonstrate different expression profiles of oestrogen-induced genes in pDCIS compared with those in DCIS-c and IDC-c. We then performed immunohistochemistry for three representative oestrogen-induced genes (C-MYB (*MYB*), RBAP46 (*RBBP7*) and survivin (*BIRC5*)) in the breast carcinoma tissues in Group C towards further confirmation of the findings.

As demonstrated in Fig. 2A, C-MYB was immunolocalised in the nuclei of carcinoma cells, and its H-score was significantly (*P*<0.0001) higher in pDCIS than that in DCIS-c or IDC-c (Fig. 2B). RBAP46 immunoreactivity was also detected in the nuclei of carcinoma cells (Fig. 2C), and its immunoreactivity was significantly (*P*=0.03) higher in pDCIS (Fig. 2D).

Table 1 Comparison of characteristics of genes between Groups A and C

Characteristic of genes	Number of genes		P value
	Group A (n=15)	Group C (n=16)	
First time of significant upregulation by oestrogen			
4 h	7 (47%)	11 (69%)	0.51
8 h	1 (7%)	0 (0%)	
24 h	5 (33%)	4 (25%)	
48 h	2 (13%)	1 (6%)	
Major biological function			
Cell cycle and apoptosis	6 (40%)	5 (31%)	0.34
Growth factors, cytokines and hormones	1 (7%)	3 (19%)	
Receptors and signal transduction proteins	2 (13%)	5 (31%)	
Transcription factors and transcriptional coregulators	6 (40%)	3 (19%)	

Data of characteristics of genes were taken from a report by Frasor *et al.* (2003). Data are presented as the number of cases and percentage. Two genes corresponding *PPP2R1B* were excluded in this table, because these were classified into both Groups A and C.

Survivin was immunolocalised in the cytoplasm of carcinoma cells, and some nuclei of the carcinoma cells were also immunohistochemically positive for survivin (Fig. 2E). Relative survivin immunoreactivity was significantly ($P=0.0003$) higher in pDCIS than that in DCIS-c or IDC-c (Fig. 2F).

As shown in Table 3, when we divided the cases into two groups according to several important pathological factors, such as nuclear grade, HER2 status and ER LI, C-MYB immunoreactivity was significantly higher in pDCIS than that in DCIS-c or IDC-c regardless of the status. Similar tendency was also detected in RBAP46 and survivin immunoreactivities; but P values did not reach significant levels in some groups.

As two genes corresponding *PPP2R1B* were classified into different groups (i.e. Groups A and C) in the microarray analysis (Fig. 1), we performed immunohistochemistry of PPP2R1B (also known as a protein phosphatase 2, regulatory subunit A, β (PP2A-A β)) in these cases. PPP2R1B immunoreactivity was detected in the breast carcinoma cells (Supplementary Figure S1A, see section on supplementary data given at the end of this article), but its immunointensity was generally weak and was not significantly different among the pDCIS, DCIS-c and IDC-c groups examined in this study (Supplementary Figure S1B, see section on supplementary data given at the end of this article).

Association between C-MYB, RBAP46 and survivin immunoreactivity and various clinicopathological parameters in pDCIS

Results of both microarray and immunohistochemical analyses described earlier indicated that C-MYB, RBAP46 and survivin were abundantly expressed in pDCIS. As demonstrated in Table 4, when 53 pDCIS cases examined were tentatively classified into two different groups according to the median value of C-MYB H-score, the status of C-MYB immunoreactivity was inversely ($P=0.006$) associated with Ki67 LI in pDCIS cases. No other significant association was detected between C-MYB immunoreactivity and other clinicopathological parameters of the patients examined, such as patients' age, menopausal status, nuclear grade, comedo necrosis, ER LI, PR LI and HER2 status. The status of RBAP46 immunoreactivity was not significantly associated with any clinicopathological parameters examined (Table 5), while the status of survivin immunoreactivity was positively associated with patients' age ($P=0.002$; Table 6). Association between PPP2R1B immunoreactivity and clinicopathological parameters in pDCIS cases is summarised

Table 2 Association of various clinicopathological parameters among pDCIS, DCIS-c and IDC-c

Parameter	pDCIS (n=53)	DCIS-c (n=27)	IDC-c (n=27)	P value
Nuclear grade ^a				
Grades 1 + 2	44 (83%)	24 (89%)	24 (89%)	0.68
Grade 3	9 (17%)	3 (11%)	3 (11%)	
ER LI (%)	81 (12–100)	80 (15–100)	80 (8–100)	0.94
PR LI (%)	40 (0–100)	40 (0–100)	40 (0–100)	0.87
HER2 status ^a				
Negative	29 (55%)	18 (67%)	19 (70%)	0.33
Positive	24 (45%)	9 (33%)	8 (30%)	<0.0001
Ki67 LI (%)	4 (1–12)	8 (1–23)	12 (1–32)	

P value <0.05 was considered significant and is in boldface.

^aData are presented as the number of cases and percentage. All other values represent the median (min–max).

Figure 2 Immunohistochemistry for C-MYB (A and B), RBAP46 (C and D) and survivin (E and F) in the breast cancer cases. Immunoreactivity of C-MYB (A) and RBAP46 (C) was detected in nuclei of carcinoma cells in pDCIS. Survivin was immunolocalised in the cytoplasm of carcinoma cells in pDCIS and was also positive in some nuclei of the carcinoma cells (an arrow; E). Bar = 50 μ m respectively. Relative immunoreactivity of C-MYB, RBAP46 and survivin in pDCIS, DCIS-c and IDC-c was summarised in B, D and F respectively. Data are represented as box and whisker plots. Briefly, the median value is represented by a horizontal line in each box, and the 75th (upper margin) and 25th (lower margin) percentiles of the values are demonstrated. The upper and lower bars indicate the maximum and minimum values respectively. In F, the median value of relative survivin immunoreactivity in DCIS-c was 100. Statistical analysis was carried out using the Kruskal–Wallis test. *P* values < 0.05 were considered significant and were indicated in bold letter.

in [Supplementary Table S1](#), see section on [supplementary data](#) given at the end of this article.

Association between clinicopathological parameters and three oestrogen-induced proteins in DCIS-c and IDC-c

As summarised in [Table 7](#), Ki67 LI was significantly lower ($P=0.04$) in DCIS-c than that in IDC-c, but no significant differences between clinicopathological parameters of the patients and the status of immunoreactivity of C-MYB, RBAP46 and survivin were detected between DCIS-c and IDC-c of 27 IDC patients in this study.

Discussion

pDCIS is generally considered as a precursor lesion of IDC. Two different models have been proposed to explain the possible mechanisms of transition from pDCIS to IDC, i.e. theories of linear progression or parallel disease ([Wiechmann & Kuerer 2008](#)). In the former model, low-grade pDCIS lesions are considered to progress to high-grade pDCIS lesions and then to become IDC ([Carter et al. 1988](#), [Bodian et al. 1993](#), [Lakhani et al. 1999](#)). In the latter model of hypothesis, low-grade pDCIS lesions progress to low-grade IDC and high-grade pDCIS lesions to high-grade IDC ([Sontag & Axelrod 2005](#), [Wiechmann & Kuerer 2008](#)). Accumulating data including chromosomal-alteration studies support the parallel disease theory ([Hwang et al. 2004](#), [Irvine & Fentiman 2007](#)), and the great majority of molecular alterations detected in breast carcinoma, including *ESR1* which codes for ER, can be clearly detected already in pDCIS, whether high or low grades ([Nofech-Mozes et al. 2005](#), [Burkhardt et al. 2010](#)). In this study of ER-positive breast carcinoma, both ER and PR LIs in pDCIS were similar to those in IDC-c or DCIS-c, which is considered to be compatible with parallel disease theory of development. [Shibuya et al. \(2008\)](#) also previously demonstrated that various oestrogen-producing enzymes were abundantly expressed in pDCIS, and intratumoural oestrogen concentration was similar between pDCIS and IDC ([Shibuya et al. 2008](#)). Therefore, oestrogens are considered to play pivotal roles in pDCIS as well as in IDC.

Results of our present study also demonstrated that Ki67 LI was significantly lower in ER-positive pDCIS than that in ER-positive IDC. Antibody Ki67 recognises cells located in all the phases of cell cycle except for G_0 (resting) phase ([Gerdes et al. 1983](#)), and Ki67 LI is closely correlated with the cell proliferation activity of the tissues ([van Diest et al. 2004](#)). Ki67 was also reported as a prognostic factor in pDCIS ([van Diest et al. 2004](#)) as well as in IDC ([de Azambuja et al. 2007](#)), and increased Ki67 was associated with negative ER status of breast carcinoma ([Burkhardt et al. 2010](#)). All these findings suggest that oestrogen actions are more associated with cell proliferation of breast carcinoma in IDC than in pDCIS.

This is the first study to demonstrate expression profiles of oestrogen-induced genes in pDCIS compared with IDC. Results of our present microarray analysis did reveal that one-third of oestrogen-induced genes were predominantly expressed in pDCIS, while the other one-third of the genes mainly in IDC and the rest in both categories with equivalent frequency.

Table 3 Statistical associations of C-MYB, RBAP46 and survivin immunoreactivity among pDCIS, DCIS-c and IDC-c cases according to several pathological parameters

Parameter	C-MYB immunoreactivity	RBAP46 immunoreactivity	Survivin immunoreactivity
Nuclear grade			
Grades 1 + 2	<0.0001	0.04	0.001
Grade 3	0.008	0.5	0.3
HER2 status			
Negative	<0.0001	0.02	0.01
Positive	0.01	0.73	0.02
ER LI (%)			
8–79	0.0003	0.06	0.01
80–100	0.0002	0.20	0.008

Data are presented as *P* values. *P* values <0.05 were considered significant and are in boldface.

These findings suggest that oestrogenic actions in pDCIS were different from those in IDC, even if the carcinoma cells expressed ER and intratumoural oestrogen was present at a significant level in both of these lesions. Among the genes predominantly expressed in IDC (Group A in Fig. 1), *EGR3* (early growth-responsive gene 3) was reported to play a pivotal role in the process of oestrogen-mediated invasion in breast cancer, and its expression was associated with adverse clinical outcome of the patients with ER-positive IDC (Suzuki *et al.* 2007). In addition, the kinetochore-bound protein kinase *BUB1* (budding uninhibited by benzimidazoles 1) is also considered to play possible role in the process of breast tumourigenesis (Klebig *et al.* 2009), and its mRNA expression was also reported to be positively associated with clinical recurrence in ER-positive IDC patients (Suzuki *et al.* 2012). *MYC* (C-MYC) was also reported to be associated with poor prognosis or adverse clinical outcome of ER-positive breast cancer patients (Chen & Olopade 2008). Robanus-Maandag *et al.* (2003) reported that *MYC* amplification may drive transition from pDCIS to IDC in human breast (Robanus-Maandag *et al.* 2003), although some conflicting data were reported in the literature (Burkhardt *et al.* 2010). These findings suggest that oestrogen-mediated transactivation is considered to vary among the target genes, and the genes promoting aggressive biological or clinical behaviour of breast carcinoma cells may be more efficiently induced by oestrogen in IDC. However, immunoreactivity of C-MYB, RBAP46 and survivin was not associated with ER LI in pDCIS cases in this study, and previous studies have demonstrated that the expression of these molecules was regulated by several factors (for instances, microRNA-150 downregulated C-MYB in liver cancer stem cells (Zhang *et al.* 2012), RBAP46 functioned as a downstream target gene of WT1 (Guan *et al.* 1998), and genetic variants of the survivin

promotor were associated with survivin expression (Xu *et al.* 2004)). Therefore, factors other than oestrogen may also be involved in the different expression profiles of oestrogen-induced genes in pDCIS from IDC. Our experiments serve as a starting point for clarifying the molecular features of oestrogen actions in pDCIS, and further examination is required.

We first identified C-MYB, RBAP46 and survivin as oestrogen-induced proteins predominantly expressed in pDCIS compared with IDC in this study. Among these three genes identified by gene profilings, a nuclear transcription factor C-MYB regulates differentiation and proliferation in various types of cells (Oh & Reddy 1999), and expression of *C-MYB* mRNA was

Table 4 Association between C-MYB immunoreactivity and clinicopathological parameters in pDCIS

Parameter	C-MYB immunoreactivity		<i>P</i> value
	High (<i>n</i> =26)	Low (<i>n</i> =27)	
Patients' age	61 (48–80)	61 (39–80)	0.91
Menopausal status ^a			
Premenopausal	7 (30%)	3 (56%)	0.14
Postmenopausal	19 (70%)	24 (44%)	
Nuclear grade ^a			
Grades 1 + 2	20 (77%)	24 (89%)	0.25
Grade 3	6 (23%)	3 (11%)	
Comedo necrosis ^a			
Absent	11 (42%)	7 (26%)	0.21
Present	15 (58%)	20 (74%)	
ER LI (%)	84 (13–100)	80 (12–100)	0.77
PR LI (%)	40 (6–93)	46 (0–100)	0.72
HER2 status ^a			
Negative	14 (54%)	15 (56%)	0.90
Positive	12 (46%)	12 (44%)	
Ki67 LI (%)	3 (1–10)	6 (2–12)	0.006

Fifty-three pDCIS cases were classified into two (i.e. high and low) groups according to the median value of C-MYB immunoreactivity. *P* value <0.05 was considered significant and is in boldface.

^aData are presented as the number of cases and percentage. All other values represent the median (min–max).

Table 5 Association between RBAP46 immunoreactivity and clinicopathological parameters in pDCIS

Parameter	RBAP46 immunoreactivity		P value
	High (n=28)	Low (n=25)	
Patients' age	65 (39–80)	54 (49–77)	0.06
Menopausal status ^a			
Premenopausal	4 (14%)	6 (24%)	0.81
Postmenopausal	24 (86%)	19 (76%)	
Nuclear grade ^a			
Grades 1 + 2	21 (75%)	23 (92%)	0.99
Grade 3	7 (25%)	2 (8%)	
Comedo necrosis ^a			
Absent	9 (32%)	9 (36%)	0.77
Present	19 (68%)	16 (64%)	
ER LI (%)	88 (12–100)	80 (13–100)	0.60
PR LI (%)	44 (6–100)	40 (0–100)	0.19
HER2 status ^a			
Negative	16 (57%)	13 (52%)	0.71
Positive	12 (43%)	12 (48%)	
Ki67 LI (%)	4 (1–12)	4 (2–10)	0.31

Fifty-three pDCIS cases were classified into two (i.e. high and low) groups according to the median value of RBAP46 immunoreactivity.
^aData are presented as the number of cases and percentage. All other values represent the median (min–max).

rapidly stimulated by oestrogen administration in the MCF7 breast carcinoma cells (Frasor *et al.* 2003). C-MYB protein was detected in ER-positive IDC and was associated with a good prognosis in the patients (Guerin *et al.* 1990, Drabsch *et al.* 2007, Deisenroth *et al.* 2010, Thorner *et al.* 2010). Immunohistochemistry for C-MYB in pDCIS has been reported only by McHale *et al.* (2008) to the best of our knowledge, in which C-MYB immunoreactivity in the breast carcinoma containing both pDCIS and IDC was significantly higher than that in normal/hyperplastic epithelium. Results of our present study first demonstrated that C-MYB immunoreactivity was significantly higher in pDCIS than in IDC and was inversely associated with Ki67 LI in pDCIS. Very recently, Thorner *et al.* (2010) reported that stable RNAi knock-down of endogenous *C-MYB* in the MCF7 cells increased tumourigenesis, both *in vitro* and *in vivo*, suggesting a tumour suppressor function in luminal breast cancer subtypes (Thorner *et al.* 2010). Results of our present study are consistent with these previously reported studies, and decreased induction of C-MYB expression by oestrogen may result in the possible acceleration of oestrogen-mediated cell proliferation of breast carcinoma in IDC.

RBAP46, a nuclear protein, was originally identified as histone-binding proteins and its components of protein complexes have been demonstrated to be

involved in the process of histone deacetylation and chromatin remodelling (Zhang *et al.* 1997, Bowen *et al.* 2004). *RBAP46* mRNA expression was reported to be rapidly induced by oestrogens in MCF7 cells (Frasor *et al.* 2003). Results of previous *in vitro* studies demonstrated that RBAP46 modulated oestrogen responsiveness in MCF7 cells in a gene-specific manner through interaction with ER α (Creekmore *et al.* 2008), and RBAP46 was also reported to inhibit an oestrogen-stimulated progression of transformed breast epithelial cells (Zhang *et al.* 2007). However, immunohistochemical evaluation of RBAP46 has not been reported in breast carcinoma to the best of our knowledge. In this study, RBAP46 immunoreactivity was more frequently detected in ER-positive pDCIS than in IDC, which also indicated that RBAP46 may play an important role in the alteration of oestrogen actions in the process of transition from pDCIS to IDC.

Survivin is known as an inhibitor of apoptosis, which prevents cell death by mainly blocking activated caspases (Ryan *et al.* 2006). Survivin mRNA expression was reported to be slowly induced by oestrogen in MCF7 cells (Frasor *et al.* 2003). Immunolocalisation of cytoplasmic survivin has been reported in human breast carcinoma by several groups, with positivity ranging from 56 to 76% of pDCIS cases (Barnes *et al.* 2006, Okumura *et al.* 2008) and 17 to

Table 6 Association between survivin immunoreactivity and clinicopathological parameters in pDCIS

Parameter	Survivin immunoreactivity		P value
	High (n=25)	Low (n=28)	
Patients' age	66 (48–80)	54 (39–80)	0.002
Menopausal status ^a			
Premenopausal	4 (16%)	6 (21%)	0.61
Postmenopausal	21 (84%)	22 (79%)	
Nuclear grade ^a			
Grades 1 + 2	19 (76%)	25 (89%)	0.20
Grade 3	6 (24%)	3 (11%)	
Comedo necrosis ^a			
Absent	7 (28%)	11 (39%)	0.39
Present	18 (72%)	17 (61%)	
ER LI (%)	87 (27–100)	80 (12–100)	0.25
PR LI (%)	47 (0–100)	40 (7–100)	0.58
HER2 status ^a			
Negative	12 (48%)	17 (61%)	0.35
Positive	13 (52%)	11 (39%)	
Ki67 LI (%)	4 (1–12)	4 (1–12)	0.80

Fifty-three pDCIS cases were classified into two (i.e. high and low) groups according to the median value of survivin immunoreactivity. *P* value <0.05 was considered significant and is in boldface.
^aData are presented as the number of cases and percentage. All other values represent the median (min–max).

Table 7 Association of clinicopathological parameters and three oestrogen-induced proteins between DCIS-c and IDC-c in 27 IDC patients

Parameter	DCIS-c	IDC-c	P value
Nuclear grade ^a			
Grades 1 + 2	24 (33%)	24 (25%)	0.99
Grade 3	3 (17%)	3 (17%)	
ER LI (%)	80 (15–100)	80 (8–100)	0.97
PR LI (%)	40 (0–100)	40 (0–100)	0.56
HER2 status ^a			
Negative	18 (67%)	19 (70%)	0.77
Positive	9 (33%)	8 (30%)	
Ki67 LI (%)	8 (1–23)	12 (1–32)	0.04
C-MYB immunoreactivity	5 (0–70)	3 (0–70)	0.13
RBAP46 immunoreactivity	69 (0–250)	60 (0–230)	0.80
Survivin immunoreactivity	100 (0–220)	80 (0–150)	0.19

^aData are presented as the number of cases and percentage. All other values represent the median (min–max). P value <0.05 was considered significant and is in boldface.

71% of IDC cases (Tanaka *et al.* 2000, Kennedy *et al.* 2003, Barnes *et al.* 2006, Sohn *et al.* 2006, Al-Joudi *et al.* 2007, Hinnis *et al.* 2007, Kleinberg *et al.* 2007). In particular, Barnes *et al.* (2006) reported that cytoplasmic survivin immunoreactivity was significantly ($P=0.0001$) frequent in pDCIS compared with IDC, which is consistent with results of this study. In addition, Barnes *et al.* also reported that the status of survivin immunoreactivity was significantly correlated with pDCIS recurrence and suggested that survivin was involved particularly in an early event of breast carcinoma development. Therefore, anti-apoptotic effects of oestrogen may play an important role also in pDCIS. Results of our present study also demonstrated a positive association between the status of survivin immunoreactivity and patients' age in pDCIS cases (Table 6). Considering a previous report that polymorphisms in survivin promotor were associated with the age of onset of ovarian cancer (Han *et al.* 2009), some factors other than oestrogen may be involved in the development of pDCIS, but it awaits further investigations for clarification.

Amari *et al.* (1999) examined the loss of heterozygosity in tumours derived from 23 patients, which harboured synchronous lesions of atypical ductal hyperplasia (ADH), DCIS and IDC, and reported that genetic alterations accumulate during cancer progression from ADH to DCIS and finally to IDC (Amari *et al.* 1999). However, several groups reported a close association of molecular features between DCIS-c and IDC-c (Done *et al.* 1998, Half *et al.* 2002, van der Groep *et al.* 2009, Burkhardt *et al.* 2010). In this study, various clinicopathological features and three oestrogen-induced proteins examined were not

significantly different between DCIS-c and IDC-c in ER-positive IDC cases. Therefore, alterations of oestrogenic actions may mainly occur at the possible transition from pDCIS to IDC, rather than the intraductal to invasive growth of cancerous cells. Further examinations are required to clarify molecular features of oestrogen actions in pDCIS, which may also contribute to improved histopathological diagnosis of pDCIS through definitive differentiation from DCIS-c of IDC in the biopsy specimen of human breast.

In summary, we examined the expression profiles of oestrogen-induced genes in pDCIS using microarray analysis to characterise molecular features of oestrogen actions in pDCIS. Results demonstrated that one-third of the genes examined were predominantly expressed in pDCIS rather than DCIS-c or IDC-c of IDC cases. Among these pDCIS-associated genes, *C-MYB*, *RBAP46* and survivin immunoreactivity was significantly higher in pDCIS than that in DCIS-c or IDC-c by subsequent immunohistochemical analysis. In particular, C-MYB immunoreactivity was inversely associated with Ki67 LI in pDCIS cases. These results suggest that expression profiles of oestrogen-induced genes in pDCIS are different from those in IDC, and C-MYB, RBAP46 and survivin may play important roles to characterise the oestrogen actions in pDCIS.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-11-0345>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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