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Intratatumoral concentration of estrogens and clinicopathological changes in ductal carcinoma *in situ* following aromatase inhibitor letrozole treatment

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Background: Estrogens have important roles in ductal carcinoma *in situ* (DCIS) of the breast. However, the significance of presurgical aromatase inhibitor treatment remains unclear. Therefore, we examined intratumoral concentration of estrogens and changes of clinicopathological factors in DCIS after letrozole treatment.

Methods: Ten cases of postmenopausal oestrogen receptor (ER)-positive DCIS were examined. They received oral letrozole before the surgery, and the tumour size was evaluated by ultrasonography. Surgical specimens and corresponding biopsy samples were used for immunohistochemistry. Snap-frozen specimens were also available in a subset of cases, and used for hormone assays and microarray analysis.

Results: Intratumoral oestrogen levels were significantly lower in DCIS treated with letrozole compared with that in those without the therapy. A great majority of oestrogen-induced genes showed low expression levels in DCIS treated with letrozole by microarray analysis. Moreover, letrozole treatment reduced the greatest dimension of DCIS, and significantly decreased Ki-67 and progesterone receptor immunoreactivity in DCIS tissues.

Conclusion: These results suggest that estrogens are mainly produced by aromatase in DCIS tissues, and aromatase inhibitors potently inhibit oestrogen actions in postmenopausal ER-positive DCIS through rapid deprivation of intratumoral estrogens.

The incidence of ductal carcinoma *in situ* (DCIS) has been markedly increasing during the past two decades, and now represents 25–30% of all mammographically detected breast cancers (Chen *et al*, 2009). Ductal carcinoma *in situ* has a 14–53% risk of invasive carcinoma over a period of ≥ 10 years after

biopsy (Erbas *et al*, 2006), and it is currently treated with simple mastectomy or breast-conserving surgery, with or without radiation therapy, to prevent local recurrence (Burstein *et al*, 2004; Dixon *et al*, 2007). On the other hand, survival rate of DCIS is ~ 98 –99% (Burstein *et al*, 2004), and it is also important to

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consider minimising of aggressive treatments in DCIS patients (Chen *et al*, 2009).

Estrogens have an important role in the development and progression of invasive breast carcinoma. Estrogens are locally synthesised in the invasive breast carcinoma tissues from circulating inactive steroids by oestrogen-producing enzymes, such as aromatase (conversion from circulating androstenedione to estrone or testosterone to estradiol), steroid sulphatase (hydrolysis of circulating estrone sulphate to estrone) and 17 β -hydroxysteroid dehydrogenase type 1 (17 β HSD1; conversion of estrone to estradiol) (Suzuki *et al*, 2005). Oestrogen actions are mediated through oestrogen receptor (ER), and endocrine therapies, such as aromatase inhibitors (i.e., letrozole, anastrozole and exemestane) and tamoxifen, are used in patients with ER-positive invasive carcinoma to block the oestrogen actions. Results of recent clinical trials demonstrated that presurgical aromatase inhibitor treatment for postmenopausal women with ER-positive invasive breast cancer reduced the tumour volume, and the conversion rate from mastectomy to breast-conserving surgery was 48–88% and was superior to tamoxifen (Takei *et al*, 2011).

Intratumoral production of estrogens has also been demonstrated in DCIS tissues (Shibuya *et al*, 2008). Also, a great majority of DCIS expressed ER (Wiechmann and Kuerer, 2008). These findings indicate the importance of oestrogen actions in DCIS, and aromatase inhibitors might be effective for postmenopausal women with ER-positive DCIS as well as invasive carcinoma. Neoadjuvant aromatase inhibitor therapy in DCIS patients has been reported only by two groups (Chen *et al*, 2009; Bundred *et al*, 2010), to the best of our knowledge. These were immunohistochemical studies, and they showed significant decrease in Ki-67 and progesterone receptor (PR) immunoreactivity in ER-positive DCIS tissues after the treatment. On the other hand, aromatase expression in DCIS has been reported to be significantly lower than in invasive carcinoma (Shibuya *et al*, 2008), and importance of aromatase has not yet been fully clarified in DCIS. No information is available regarding effects of aromatase inhibitor on intratumoral oestrogen levels, molecular oestrogen actions or tumour size in DCIS, and biological and/or clinical significance of aromatase inhibitor thus remains largely unclear in DCIS. Therefore, in this study, we first examined intratumoral concentration of estrogens in DCIS with letrozole treatment. Moreover, we examined expression profiles of oestrogen-induced genes and several clinicopathological factors in DCIS tissues after letrozole treatment, and compared these findings with those at baseline.

MATERIALS AND METHODS

Patients and tissues. This study was a non-randomized uncontrolled trial, and registered at University hospital Medical Information Network Clinical Trial Registry (UMIN-CTR) (<http://www.umin.ac.jp/ctr/index/htm>) (ID: UMIN000003101). The main inclusion criteria was the diagnosis of ER-positive DCIS in postmenopausal patients by biopsy, and letrozole treatment was performed before the surgery in these patients.

Ten postmenopausal women with ER-positive DCIS were enrolled in this study; they underwent breast-conserving surgery from 2010 to 2011 at Tohoku University Hospital (Sendai, Japan), Tohoku Kosai Hospital (Sendai, Japan), Miyagi Cancer Center Hospital, (Natori, Japan), and Nihonkai General Hospital (Sakata, Japan) (range of age; 52–74). All patients gave written informed consent. The patients' characteristics are summarised in Table 1. They received oral letrozole (Femara; Novartis Pharma (Tokyo, Japan)), 2.5 mg daily for 3–27 weeks before breast-conserving surgery. Information about the tumour size evaluated by ultrasonography (US) was available in nine cases both before

Table 1. Characteristics of 10 patients examined in this study

Case	Age	Menopausal status	Diagnosis at baseline	Diagnosis after the letrozole treatment	Period of the letrozole treatment (weeks)
1	69	Post	DCIS (ER +)	DCIS	15
2	57	Post	DCIS (ER +)	DCIS	14
3	74	Post	DCIS (ER +)	IDC (pT1mic (<1mm))	14
4	52	Post	DCIS (ER +)	DCIS	4
5	57	Post	DCIS (ER +)	DCIS	3
6	62	Post	DCIS (ER +)	DCIS	7
7	68	Post	DCIS (ER +)	DCIS	10
8	58	Post	DCIS (ER +)	IDC (pT1a (2mm))	27
9	67	Post	DCIS (ER +)	DCIS	14
10	72	Post	DCIS (ER +)	IDC (pT1mic (<1mm))	14

Abbreviation: IDC = invasive ductal carcinoma. ER + : case showed >10% of ER-positive cells.

and after letrozole treatment in their charts. The entire resected surgical specimen was sectioned into slices with 3–5 mm thickness, and all the slices were histologically evaluated. At post-treatment diagnosis, seven cases were diagnosed as pure DCIS, whereas small invasive foci were detected in three cases. In all the cases, both biopsy at baseline and surgical specimens were available in the formalin-fixed and paraffin-embedded tissues. Snap-frozen specimens were also available in a subset of cases, and these specimens were used for hormone assays (baseline samples: $n = 2$, post-treatment samples: $n = 8$) and/or microarray analysis (baseline samples: $n = 2$, post-treatment samples: $n = 5$).

As untreated controls for hormone assay, six snap-frozen specimens of ER-positive DCIS were also used in this study. These were obtained from postmenopausal women who underwent surgical treatment from 2001 to 2004 at Tohoku University Hospital and Tohoku Kosai Hospital, Sendai, Japan. These patients did not receive any neoadjuvant therapy.

The research protocol was approved by the Ethics Committee at Tohoku University School of Medicine and other institutional review boards.

Liquid chromatography/electrospray tandem mass spectrometry (LC-MS/MS). Concentrations of estrone, estradiol and androstenedione were measured by LC-MS/MS analysis in ASKA Pharma Medical Co., Ltd (Kawasaki, Japan), as described previously (Takagi *et al*, 2013a). Briefly, tissue specimens were homogenised in 1 ml of distilled water, and steroid fraction was extracted with diethyl ether. In this study, we used an LC (Agilent 1100, Agilent Technologies, Waldbronn, Germany) coupled with an API 4000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Ontario, Canada) operated with electron spray ionisation in the positive-ion mode, and the chromatographic separation was performed on Cadenza CD-C18 column (3 \times 150 mm, 3.5 mm, Imtakt, Kyoto, Japan). In our present study, the lower limits of quantification (LLOQ) of estrone, estradiol and androstenedione were 0.05, 0.03 and 2.0 pg per assay, respectively.

LASER capture microdissection (LCM)/microarray analysis.

Gene expression profiles of breast carcinoma cells were examined using microarray analysis. LCM was conducted using the MMI Cellcut (Molecular Machines and Industries, Glattbrugg, Switzerland). Briefly, breast carcinomas were embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetech Co., Tokyo, Japan) and sectioned at a thickness of 10 μm . Breast carcinoma cells were dissected under the light microscopy and laser transferred from these frozen sections. The total RNA (~ 200 ng) was subsequently extracted from these cell fractions isolated by LCM using the RNeasy Micro Kit (QIAGEN, Mannheim, Germany). SurePrint G3 Hm GE 8×60 K Microarray Kit (G4851A, ID 028004; Agilent Technologies) 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 profiles of two gene lists that were previously reported as oestrogen-induced genes in invasive breast carcinoma cell line MCF-7 (i.e., Frasor's (Frasor *et al*, 2003) and Creighton's (Creighton *et al*, 2006) lists that consisted of 50 and 63 genes, respectively). Among these, two genes in Frasor's list and three genes in Creighton's list were not available in our present study. If a gene was represented multiple times on the platform, the probe with strongest positive correlation with ESR1 (ER) was selected (Takagi *et al*, 2013a).

Immunohistochemistry. Monoclonal antibodies for ER (6F11), progesterone receptor (PR; 1A6), and Ki-67 (MIB-1) were purchased from Novocastra (Newcastle upon Tyne, UK), Chemicon (Temecula, CA, USA), and DAKO (Carpinteria, CA, USA) respectively. Monoclonal antibody for aromatase (clone #677/H7) was kindly provided by Dr Evans DB, Novartis, Basel, Switzerland. Rabbit polyclonal antibodies for HER2 (A0485) were purchased from DAKO, C-MYB (EPR718(2)) and RBAP46 (EPR5082) from Epitomics (Burlingame, CA, USA), and survivin (NB500-201) from Novus Biologicals (Littleton, CO, USA).

A Histofine Kit (Nichirei Biosciences, Tokyo, Japan), which employs the streptavidin–biotin amplification method, was used in this study. Antigen retrieval was performed by heating the slides in an autoclave at 120 $^{\circ}\text{C}$ 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 ER, PR, HER2, Ki-67, RBAP46 and survivin. Dilutions of primary antibodies used in this study were as follows: ER, 1/50; PR, 1/50; HER2, 1/100; Ki-67, 1/100; aromatase, 1/3000; 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% H_2O_2) and counterstained with hematoxylin.

Immunoreactivity of ER, PR, Ki-67, C-MYB and RBAP46 was detected in the nucleus, and their immunoreactivity was evaluated in counting > 1000 carcinoma cells for each case. The percentage of immunoreactivity, that is, labelling index (LI), was subsequently determined. Cases with ER LI of $> 10\%$ were considered ER-positive breast carcinoma in this study. Aromatase immunoreactivity was detected in the cytoplasm, and the cases that had $> 10\%$ of positive carcinoma cells were considered positive (Takagi *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 $> 30\%$ carcinoma cells were considered positive (Wolff *et al*, 2007). Survivin immunoreactivity was detected in the cytoplasm of carcinoma cells, and was semi-quantitatively evaluated by modified LI system (Mehta *et al*, 2011), in which the percentage of cytoplasmic immunoreactivity was scored as 0 (no expression), 10 (up to 10%), 20 (10–20%) until 100 (90–100%).

RESULTS**Intratumoral concentration of estrogens in DCIS tissues treated with letrozole.**

We first examined tissue concentration of sex steroids in DCIS treated with letrozole by LC-MS/MS. The median with min–max value of intratumoral estrone concentration was 0.23 (0–1.1) pg g^{-1} in DCIS tissues treated with letrozole, which was significantly lower (0.0003-fold and $P = 0.0008$) than that without the therapy (76.5 (11.9–159.3) pg g^{-1}) (Figure 1A).

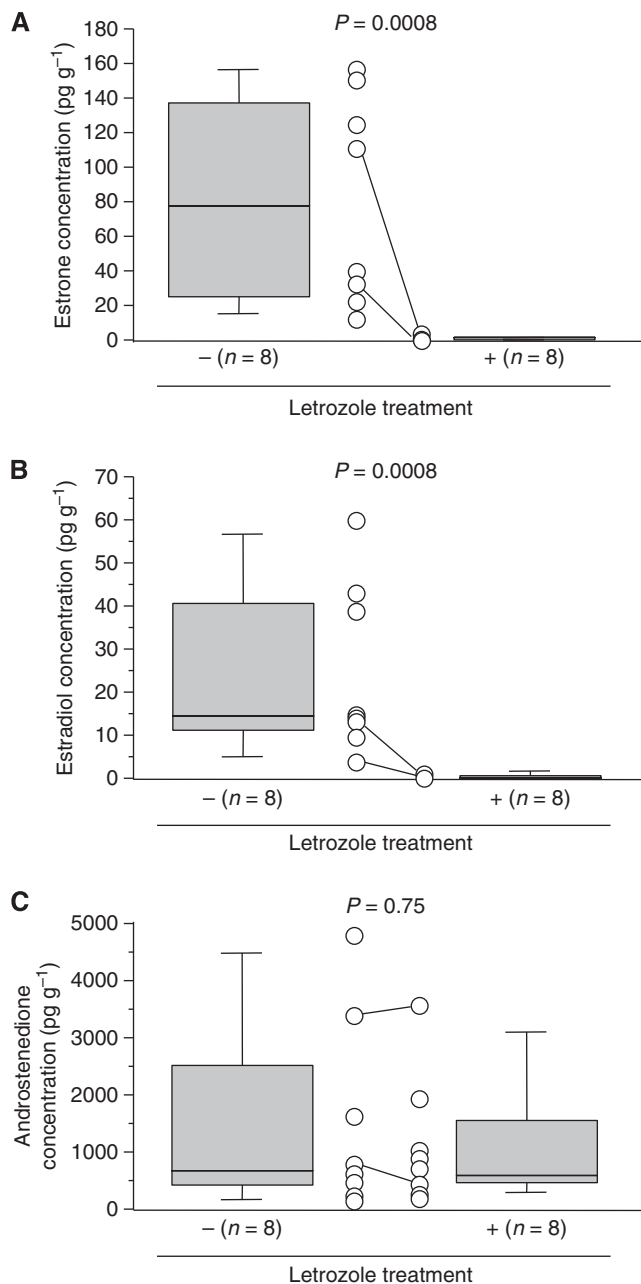


Figure 1. Tissue concentration of estrone (A), estradiol (B) and androstenedione (C) in DCIS with or without letrozole treatment using LC-MS/MS analysis. A horizontal line in the box plot illustrated the median value, and grey box denotes the 75th (upper margin) and 25th percentiles of the values (lower margin), respectively. The upper and lower bars indicate the 90th and 10th percentiles, respectively. Each value was shown in an open circle, and the paired values from the same patient were connected in a line ($n = 2$). The statistical analyses were performed using a Mann–Whitney *U*-test. *P*-values < 0.05 were considered significant, and indicated in bold.

Tissue concentration of estradiol was also significantly lower (0.015-fold and $P=0.0008$) in DCIS treated with letrozole (0.23 ($0-2.0$) pg g^{-1}) than the untreated group (14.3 ($3.1-62.7$) pg g^{-1}) (Figure 1B). On the other hand, androstenedione level of DCIS was not significantly different ($P=0.75$) between letrozole-treated (586 ($228-3563$) pg g^{-1}) and untreated (687 ($128-4864$) pg g^{-1}) groups in this study (Figure 1C).

Expression profiles of oestrogen-induced genes in DCIS treated with letrozole. In order to examine the effect of letrozole treatment on molecular functions of estrogens in DCIS, we then performed microarray analysis in DCIS tissues, and compared the expression profiles of oestrogen-induced gene between baseline ($n=2$) and post-treated ($n=5$) DCIS tissues by scatter plot analysis. In this analysis, when the expression ratio of a gene in the letrozole-treated samples when compared with that in the baseline samples was >2.0 or <0.5 , we determined higher or lower expression in the letrozole treatment, respectively. In the Frasor's list, 44 out of 48 (92%) genes examined showed lower expression in the letrozole treatment group, and no gene showed higher expression compared with the baseline (Figure 2A). Paired samples at baseline and post-treatment were available in two cases (Case 4 and Case 8), and similar tendency was detected in each case (Figure 2B and C). When we used Creighton's list to confirm these findings, 58 out of 60 (97%) genes showed lower expression in the post-treatment group (Figure 2D). The microarray data of Figure 2A and D are provided in Supplementary Table S1.

Change of tumour size in DCIS patients after letrozole treatment. We next evaluated an association of various clinico-pathological parameters in DCIS treated with letrozole, and compared these findings to their baseline. The greatest dimension of DCIS evaluated by US was decreased by 18% after the treatment (baseline: 11 ($6-22$) mm and post-treatment: 9 ($3-28$) mm), although P -value did not reach a significant level ($P=0.17$)

(Figure 3A). When we classified the patients into two groups according the median value of the treatment duration (94 days), the tumour size was significantly (0.81-fold and $P=0.043$) decreased after treatment (9 ($3-16$) mm) compared with baseline (11 ($6-22$) mm) in the longer treatment group (Figure 3B), whereas these were similar (0.95-fold and $P=0.85$) in the shorter duration group. Relative tumour size after letrozole treatment compared with baseline was 27% smaller in the longer treatment group (73 ($50-90$) %) when compared with the shorter group (100 ($50-255$) %), although it was not significant ($P=0.18$) (Figure 3C).

DCIS lesions were also evaluated by magnetic resonance (MR) imaging after letrozole treatment in the seven patients. Among these, three cases showed higher signal intensity within the lesions compared with surrounding normal breast tissue on fat-suppressed T2-weighted MR imaging (Figure 3D).

Morphological and immunohistochemical features of DCIS after letrozole treatment. Morphologically, DCIS tissues after letrozole treatment frequently showed increased periductal fibrosis or infiltration of foam cells compared with their baseline, as report by Chen *et al* (2009) (Figure 4). Necrosis was not prevalent in DCIS tissues following letrozole treatment.

Associations of various pathological parameters in 10 paired DCIS tissues before and after letrozole treatment are summarised in Table 2. PR LI (Figure 5B) and Ki-67 LI (Figure 5C) were significantly decreased after letrozole treatment compared with the baseline ($P=0.018$ and $P=0.0050$, respectively). On the other hand, nuclear grade, comedo necrosis, ER LI (Figure 5A), HER2 and aromatase immunoreactivity (Figure 5D) were not changed after the letrozole treatment in this study.

Results of our microarray analysis demonstrated that a great majority of oestrogen-induced genes showed lower expression level in DCIS tissues after letrozole treatment. Among these genes examined, RBAP46, C-MYB and survivin were recently reported to be highly expressed in DCIS (Ebata *et al*, 2012). Therefore, we next

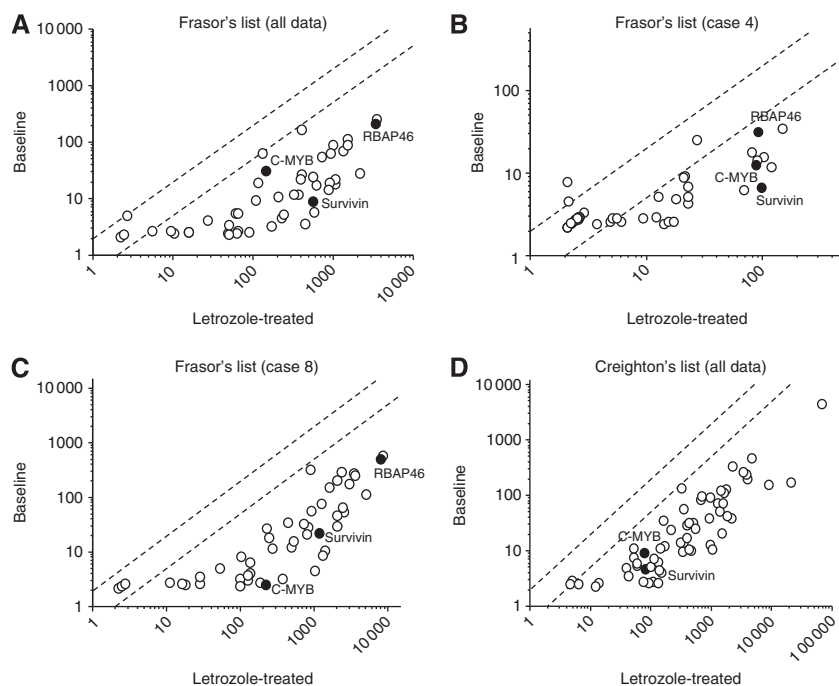


Figure 2. Scatter plot analysis of microarray data of estrogen-induced genes listed by Frasor *et al* (2003) (A–C) and Creighton *et al* (2006) (D). Genes with an expression ratio, post-treatment to baseline, of >2.0 or <0.5 are located outside the diagonal dotted line. A and D summarise all the values (baseline group; $n=2$, and post-treatment group; $n=5$), while (B) and (C) showed the paired data obtained from Case 4 and Case 8, respectively. Locations of estrogen-induced genes, for which immunohistochemistry was performed (i.e., RBAP46, C-MYB and survivin), were noted as closed circles.

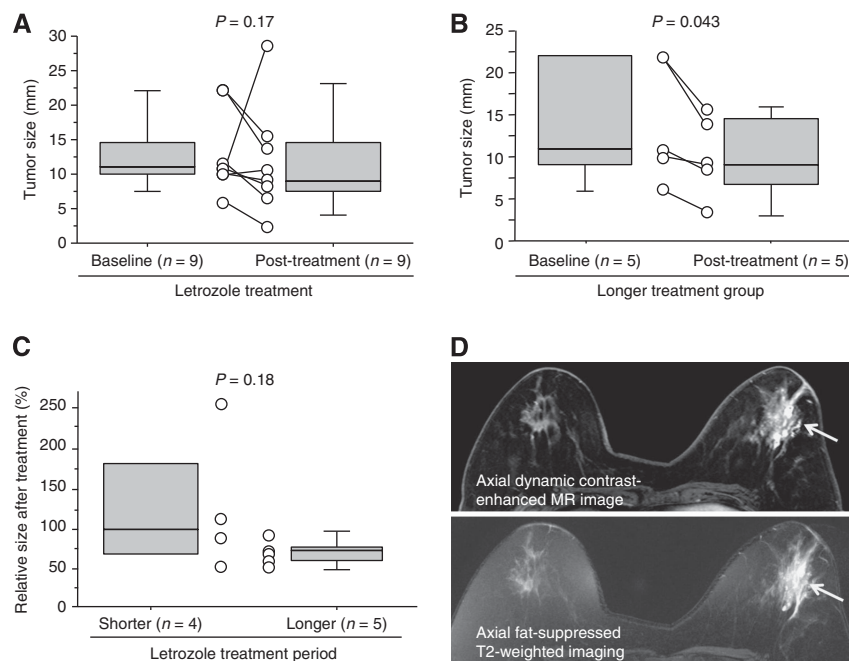


Figure 3. Clinical response of DCIS after letrozole treatment. (A–C) Tumor size of DCIS patients treated with letrozole evaluated by ultrasonography (US). The median value of the greatest dimension was illustrated by a horizontal line in the box plot, and grey box denotes the 75th (upper margin) and 25th percentiles of the values (lower margin), respectively. The upper and lower bars indicate the 90th and 10th percentiles, respectively. Each value was shown in an open circle, and the paired values from the same patient were connected in a line. The statistical analyses were performed using a Wilcoxon signed rank test (A, B), and a Mann–Whitney *U*-test (C). *P*-value <0.05 was considered significant, and indicated in bold. (D) Representative MR images after letrozole treatment (Case 5). Axial dynamic contrast-enhanced MR images obtained in early phase represented a segmental non-mass-like lesion in the left breast (arrow) (upper panel), and corresponding axial fat-suppressed T2-weighted imaging showed strong high signal intensity within the lesion (arrow), compared with that in the contralateral right breast (lower panel).

performed immunohistochemistry for these proteins to confirm the results of microarray analyses. As shown in Table 2, RBAP46 (Figure 5E), C-MYB (Figure 5F) and survivin (Figure 5G) LIs were significantly lower in DCIS tissues after the letrozole treatment ($P = 0.017$, $P = 0.013$ and $P = 0.017$, respectively).

Changes of PR and Ki-67 LIs between baseline and post-treatment samples were further examined according to several clinicopathological factors. As shown in Table 3, Ki-67 LI in the post-treatment group was significantly lower in a group positive for aromatase immunoreactivity at baseline ($P = 0.035$), but not in the aromatase-negative group ($P = 0.46$). Post-treatment diagnosis and period of letrozole treatment did not significantly change the PR or Ki-67 LI both at baseline and post-treatment in this study.

DISCUSSION

This is the first report to evaluate intratumoral oestrogen concentrations in DCIS tissues treated with aromatase inhibitor. In this study, median values of estrone and estradiol in DCIS treated with letrozole were 0.6 and 1.5% of those not treated with letrozole ($P = 0.0008$), respectively. Aromatase immunoreactivity in DCIS tissue was not changed after letrozole treatment in this study. Previous studies demonstrated that intratumoral oestrogen concentrations were markedly suppressed in invasive breast carcinoma treated with aromatase inhibitors. For instance, treatment with anastrozole for 15 weeks suppressed tissue estrone and estradiol levels by 83 and 89%, respectively, compared with baseline levels (Geisler *et al*, 2001), and letrozole treatment for 16 weeks suppressed pretreatment tumour levels of estrone and

estradiol by 91 and 98%, respectively (Geisler *et al*, 2008). The results of our present study were in good agreement with these previous results, and suggest that intratumoral oestrogen concentrations of DCIS were remarkably deprived by aromatase inhibitor to a similar extent as in invasive carcinoma, nevertheless aromatase protein remained in the tissues.

Duration of letrozole treatment was variable from 3–27 weeks in our present study, but both estrone and estradiol concentrations in DCIS tissues were almost negligible regardless of the treatment period. Recently, we reported that invasive breast carcinoma tissues of patients who received exemestane treatment for 2 weeks maintained relatively high concentration of estradiol (35% of that without this mode of therapy, and $P = 0.56$ between these two groups), and speculated that other oestrogen-producing enzymes, such as steroid sulphatase and 17β HSD1, may interrupt the rapid decrease in the intratumoral estradiol level (Takagi *et al*, 2010). Although no data are currently available on the influence of short-term letrozole therapy on estradiol concentration in the invasive carcinoma patients to the best of our knowledge, our present results in DCIS seem superior to those in the invasive carcinoma, and these may suggest that estrogens are mainly synthesised by aromatase in DCIS rather than other enzymes.

The results of our microarray analysis demonstrated that a great majority of oestrogen-induced genes (92% in Frasar's list and 97% in Creighton's list) showed <50% expression levels in DCIS treated with letrozole compared with that in a group without this mode of therapy. We confirmed these results by employing immunohistochemistry for three representative genes (i.e., RBAP46, C-MYB and survivin). On the other hand, letrozole treatment did not significantly change ER LI in our study. The lists of oestrogen-induced genes used in this study were identified in

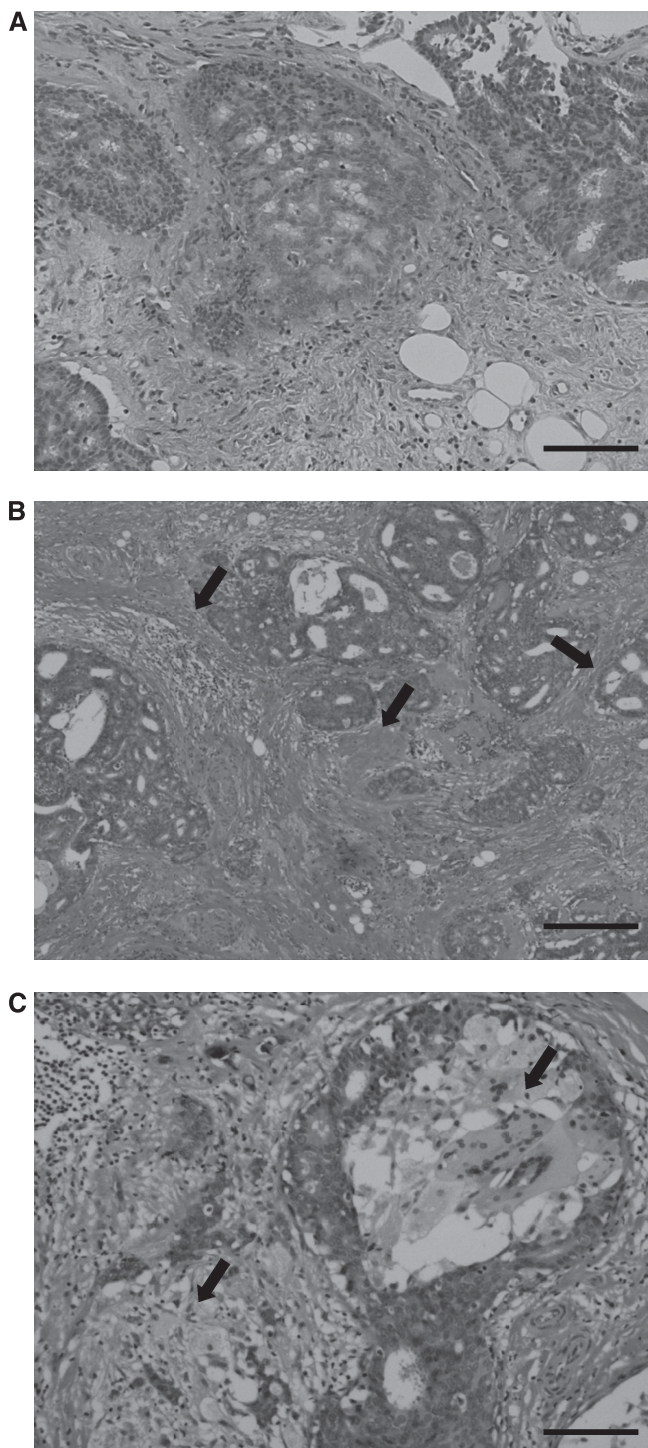


Figure 4. Morphological features of DCIS at baseline (A) and corresponding post-treatment (B, C) (Case 1). Increased periductal fibrosis (B) and infiltration of foam cells (C) were observed after the letrozole treatment as shown by arrows. Hematoxylin and eosin (HE) staining. Bar = 100 µm, respectively. A full colour version of this figure is available at the *British Journal of Cancer* journal online.

MCF-7 invasive breast carcinoma cells (Frasor *et al*, 2003; Creighton *et al*, 2006), as no information is available on oestrogen-induced genes in DCIS cells. The results of our study suggest that a great majority of oestrogen-induced genes identified in the invasive carcinoma cells are also regulated by estrogens in DCIS, and aromatase inhibitor broadly suppresses these expressions consistent with deprivation of intratumoral estrogens even if

Table 2. Change of pathological parameters in 10 paired DCIS tissues obtained before and after the letrozole treatment

Parameter	Baseline	Post- treatment	P-value
Nuclear grade^a			
Grades 1 + 2	8	8	—
Grade 3	2	2	>0.99
Comedo necrosis^a			
Absent	5	5	—
Present	5	5	>0.99
ER LI (%)	97 (15–100)	95 (30–100)	>0.99
PR LI (%)	93 (0–100)	18 (0–83)	0.018
HER2 status^a			
Negative	9	9	—
Positive	1	1	>0.99
Ki-67 LI (%)	10 (4–24)	3 (0–7)	0.005
Aromatase immunoreactivity^a			
Negative	5	5	—
Positive	5	5	>0.99
RBAP46 LI (%)	58 (33–98)	38 (15–62)	0.017
C-MYB LI (%)	35 (7–82)	10 (0–25)	0.013
Survivin LI (%)	30 (0–80)	20 (0–40)	0.017

The statistical analyses were performed using a Wilcoxon signed rank test, and P-values <0.05 were considered significant, and given in bold.
^aData are presented as the number of cases, and all other values represent the median (min–max).

ER expression remains. Among the genes markedly suppressed by letrozole in this study, some were reported to be closely associated with cell proliferation (CCND1 (Stegg and Zhou, 1998) and BUB1 (Takagi *et al*, 2013b)) and invasion (EGR3 (Suzuki *et al*, 2007)) of the breast carcinoma, DCIS recurrence (survivin (Barnes *et al*, 2006)), and transition from DCIS to invasive carcinoma (MYC (Robanus-Maandag *et al*, 2003)), although biological functions remains largely unclear in a majority of oestrogen-induced genes. Various oestrogen functions, including cell proliferation, invasion and metastasis, are mediated by networks and pathways of the oestrogen-regulated genes (Frasor *et al*, 2003, Suzuki *et al*, 2012), and therefore, aromatase inhibitor may inhibit the growth and progression to invasive carcinoma in DCIS.

In our immunohistochemical study, letrozole treatment significantly decreased Ki-67 LI (0.3-fold and $P=0.0050$) and PR LI (0.19-fold and $P=0.018$). Ki-67 LI is well known to reflect the proliferative activity of breast carcinoma (van Diest *et al*, 2004). On the other hand, PR positivity is considered to represent an intact oestrogen-signaling pathway (Dowsett *et al*, 2005) and often used as an indicator of a response to endocrine therapy in the invasive breast carcinoma (Bardou *et al*, 2003), although PR gene was not included in Frasor’s or Creighton’s lists. Previously, Chen *et al* (2009) reported that Ki-67 LI and PR score were significantly lower in DCIS tissues treated with letrozole for 3 months ($n=9$) compared with that in the control group ($P=0.013$ and $P=0.001$, respectively), and Bundred *et al* (2010) also showed that exemestane treatment for 2 weeks significantly reduced Ki-67 and PR compared with placebo group in 22 DCIS patients ($P<0.001$ and $P=0.011$, respectively). Our present results are in good agreement with these previous studies.

Moreover, our results did demonstrate that Ki-67 LI after letrozole treatment was significantly lower (0.25-fold and $P=0.035$) in aromatase-positive cases at baseline than in the negative group. Previous studies reported an association between

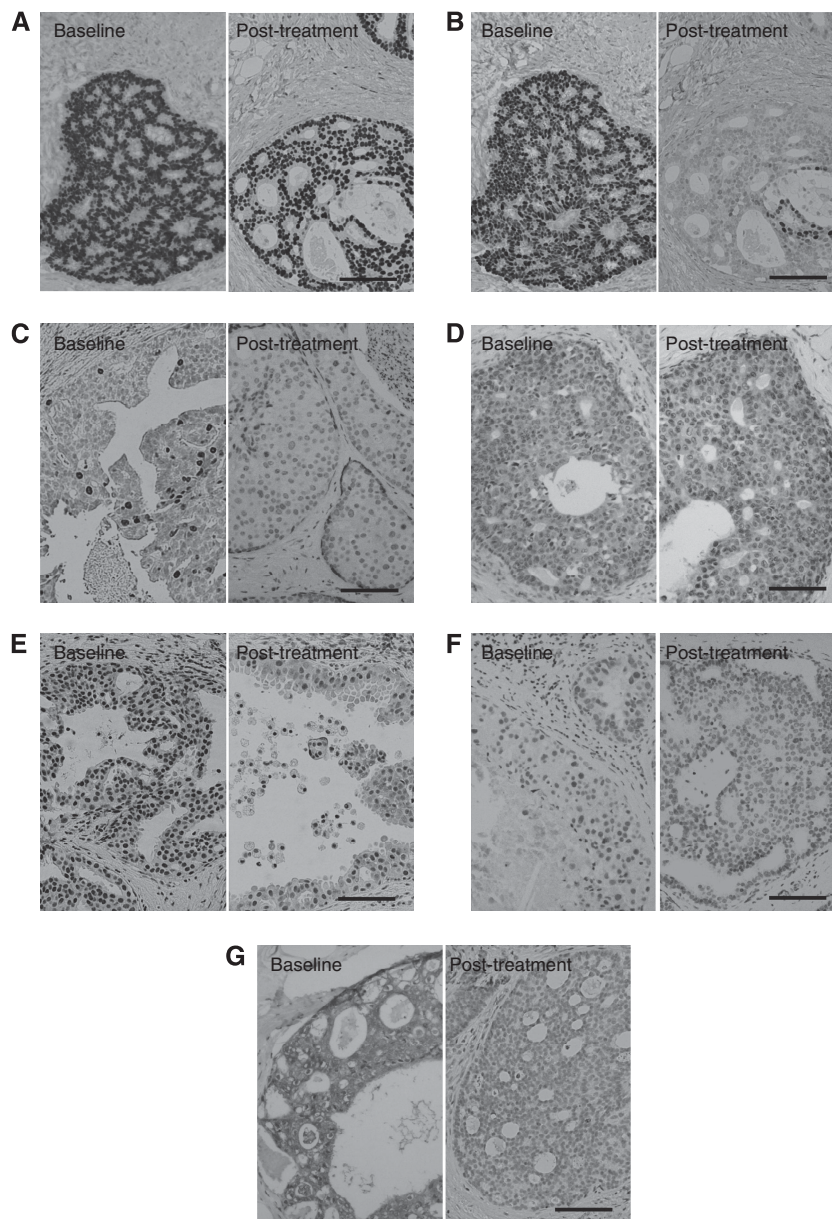


Figure 5. Immunohistochemistry for ER (A), PR (B), Ki-67 (C), aromatase (D), RBAP46 (E), C-MYB (F) and survivin (G) in DCIS at baseline (left panel) and corresponding post-treatment tissues (right panel). Left panels of (A) and (B), and right panels of (A) and (B) were of the same area, respectively. Bar = 100 μm, respectively.

aromatase activity in breast carcinoma tissues and the response to treatment with aromatase inhibitors (Bezwoda *et al*, 1987; Miller and O'Neill, 1987), and aromatase immunoreactivity reflected its enzymatic activity in human cancer cell lines (Suzuki *et al*, 1994). Ki-67 was reported as one of the biomarkers associated with subsequent breast carcinoma events after initial DCIS diagnosis (Kerlikowske *et al*, 2010), and Dowsett *et al* (2007) demonstrated that higher Ki-67 expression after endocrine therapy was significantly associated with poor recurrence-free survival in invasive breast carcinoma patients who received presurgical endocrine therapy for 2 weeks, whereas higher Ki-67 expression at baseline was not. Therefore, proliferation of ER-positive/aromatase-positive DCIS may depend more on oestrogen actions compared with that of ER-positive/aromatase-negative cases. Further examinations are required to determine whether aromatase status at baseline may be a useful indicator of a response to aromatase inhibitor treatment or not in postmenopausal ER-positive DCIS patients.

US is known as a useful modality for preoperative evaluation of not only invasive breast carcinoma but also DCIS (Hayashi *et al*, 2012). In our study, the greatest dimension of DCIS evaluated by US was decreased after letrozole treatment, and it was significant (0.81-fold and $P=0.043$) in the longer treatment group (14–27 weeks) but not (0.95-fold and $P=0.85$) in the shorter group (3–14 weeks). Most neoadjuvant studies with aromatase inhibitors in invasive breast carcinoma have treated patients for 3–4 months, and by this time many patients' tumours have responded sufficiently to downstage the surgical procedure required to excise the cancer (Dixon *et al*, 2007). In addition, Krainick-Strobel *et al* (2008) reported that a higher clinical response rate was observed after 8 months of neoadjuvant letrozole therapy compared with treatment for 4 months, and Takei *et al* (2011) suggested that the optimal duration of neoadjuvant aromatase inhibitor therapy was at least 4 months to obtain significant clinical response. Our present results were consistent with these data, and DCIS patients may also need >4 months of

Table 3. PR and Ki-67 LIs before and after the letrozole treatment according to clinicopathological factors in 10 DCIS cases examined

		Aromatase immunoreactivity at baseline		
Parameter	Evaluation point	+	–	P-value
PR LI	Baseline	84 (0–100)	95 (0–100)	0.83
	Post-treatment	36 (0–83)	6 (0–66)	0.68
Ki-67 LI	Baseline	7 (4–24)	10 (8–18)	0.46
	Post-treatment	1 (0–4)	4 (3–7)	0.035
		Post-treatment diagnosis		
Parameter	Evaluation point	DCIS (n = 7)	IDC (n = 3)	P-value
PR LI	Baseline	84 (0–100)	95 (90–100)	0.36
	Post-treatment	6 (0–83)	30 (3–36)	0.91
Ki-67 LI	Baseline	10 (4–24)	12 (7–18)	0.57
	Post-treatment	3 (0–4)	5 (1–7)	0.26
		Period of letrozole treatment		
Parameter	Evaluation point	Shorter (n = 5)	Longer (n = 5)	P-value
PR LI	Baseline	84 (0–100)	95 (0–100)	0.83
	Post-treatment	58 (0–83)	6 (0–36)	0.4
Ki-67 LI	Baseline	10 (4–24)	10 (7–18)	0.83
	Post-treatment	2 (0–4)	4 (1–7)	0.14

Data represent the median (min–max). The statistical analyses were performed using a Mann–Whitney U-test. P-value <0.05 was considered significant, and given in bold.

aromatase inhibitor treatment to obtain its clinical response. Our MR imaging examination frequently revealed high signal intensity within DCIS lesions after letrozole treatment. Strong high signal intensity on fat-suppressed T2-weighted imaging is rare in DCIS (Goto *et al.*, 2012), and it may reflect oedematous stroma (Yuen *et al.*, 2007) and/or accumulation of foam cells in DCIS tissues by letrozole treatment.

In our study, reduction of the tumour size in DCIS patients who received letrozole treatment seems much slower compared with the rapid decrement of intratumoral oestrogen levels, oestrogen-induced gene expressions and Ki-67 LI in the DCIS tissues. Previous studies have demonstrated that apoptotic markers such as cleaved caspase 3 (Chen *et al.*, 2009) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling deoxynucleotide labelling (Bundred *et al.*, 2010), were not significantly changed regardless of aromatase inhibitor treatment in DCIS tissues. Therefore, apoptotic effects of aromatase inhibitors may not be evident in DCIS, which may be partly due to the delayed clinical responses to letrozole in DCIS patients in this study. It awaits further examinations in larger number of patients and longer duration of aromatase inhibitor treatment to clarify clinical significance of aromatase inhibitor in DCIS patients.

In summary, intratumoral concentrations of estrogens were significantly lower in DCIS tissues treated with letrozole compared with those without the therapy, and a great majority of oestrogen-induced genes showed lower expression levels in DCIS tissues after the therapy. Letrozole treatment significantly decreased Ki-67 LI and PR LI in DCIS tissues compared with the baseline, and the tumour size evaluated by US was significantly decreased in the longer duration group in this study (14–27 weeks). These results suggest that estrogens are mainly produced by aromatase in DCIS tissues, and aromatase inhibitors may potentially inhibit the oestrogen-mediated growth and/or progression in postmenopausal ER-positive DCIS cases through rapid deprivation of intratumoral estrogens.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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