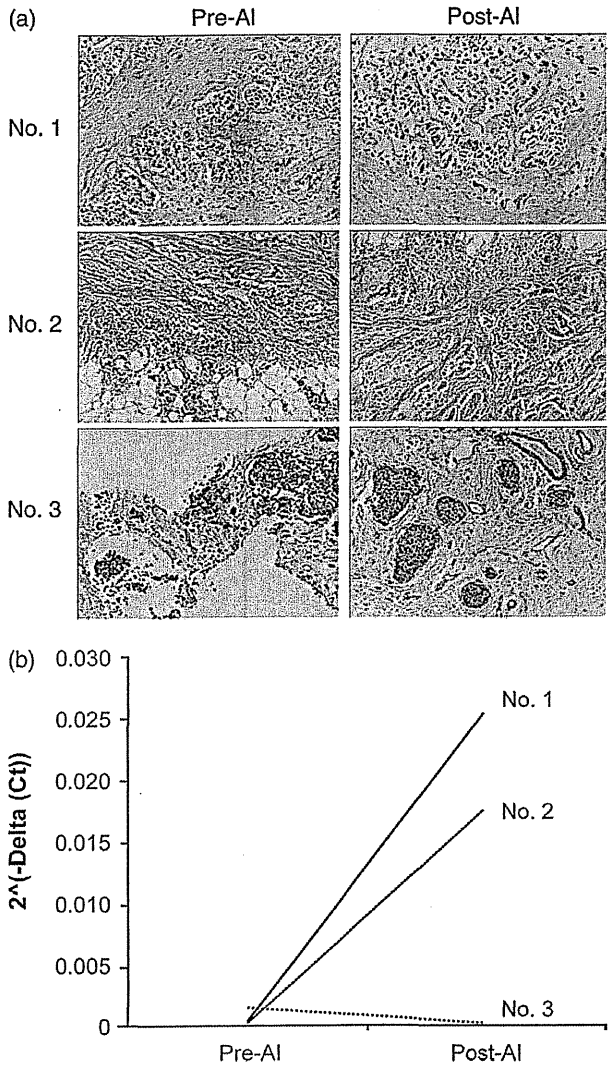

Figure 2. RT-PCR analysis of *let-7f* expression alterations in paired breast cancer specimens receiving 3 months of neoadjuvant AI treatment. (a) Representative presentation of haematoxylin and eosin staining of specimens 1–3; pre-AI (left) and post-AI (right). (b) Individual changes of *let-7f* expression by letrozole treatment were summarized. Restoration of *let-7f* was detected in two out of three cases receiving letrozole treatment. *U6* probe was used for normalization of expression levels.

and tumour suppressors [8,16,17]. Therefore, an analysis of miRNA profile alterations following AI treatment will contribute to the elucidation of its molecular mechanisms. However, few studies have reported the potential involvement of miRNA in the treatment of AIs [18,19]. Masri *et al* reported that *miR-128a* was up-regulated in letrozole-resistant breast cancer cell lines [18,19] but they studied only breast cancer cells resistant to AI. Maillot *et al* [10] reported miRNA profiling in breast cancer specimens treated by tamoxifen combined with exemestane. Therefore, our present study is the first one to demonstrate alterations of miRNA expression as a result of only AI treatment in breast cancer cells.

Genetic changes occur at the mRNA level with AI treatment as Mackay *et al* recently reported that the *aromatase* mRNA itself was down-regulated by AI treatment in breast cancer cells [6]; however, the mechanism for this phenomenon is unknown. We hypothesized that the reason for this finding may be due to the alterations of the miRNA profile caused by AI treatment. To verify this hypothesis, we performed a miRNA PCR array on a letrozole-treated breast cancer cell line. Several miRNAs were altered by letrozole treatment and among these miRNAs, we specifically detected the restoration of *let-7f*, a tumour-suppressor miRNA. The *let-7* family, to which *let-7f* belongs, is suppressed in various human malignancies [20], and its restoration to normal levels has been reported to suppress cancer growth [21–23]. In our present study, *let-7f* was also demonstrated to suppress cell proliferation and migration *in vitro*. These findings are supported by the recent results by Yu *et al*, who showed that *let-7* overexpression resulted in decreased breast tumour-initiating cells (BT-ICs) [24], and Zhao *et al*, who recently demonstrated decreased cell proliferation by *let-7b* and *let-7i* in breast cancer cells [25]. It then became important to evaluate whether these alterations occur *in vivo*. We therefore evaluated the changes of *let-7f* in patients who received neoadjuvant letrozole therapy and correlated the changes of *let-7f* with therapeutic response; Ki-67 (MIB-1 labelling index) was used as a marker of therapeutic efficacy [26]. Two out of three cases which showed increased levels of *let-7f* expression resulted in a marked decrease of Ki-67 (%) following letrozole treatment. The remaining case did not show any change in Ki-67 (%) as this case showed a very low Ki-67 index initially (Supporting information, Supplementary Figure 1). Although the number of clinical cases studied in our study was too small to draw any conclusions, these results suggest that the actions of letrozole on the *aromatase* gene via *let-7f* restoration may be contributing to oestrogen depletion and decreased tumour cell proliferation effects of AIs. Moreover, this may explain the improved relapse and recurrence rates with AI treatment in ER+ breast cancer patients. Further investigations including the study of more patients undergoing neoadjuvant therapy are required for clarification of this interesting hypothesis.

We hypothesized that the *aromatase* gene would be directly targeted by *let-7f*. This was subsequently verified by our experiments. First, an increased level of *let-7f* was detected as a result of letrozole treatment, while the levels of *aromatase* mRNA were decreased in these breast cancer cells. Second, *let-7f* expression was negatively correlated with the level of *aromatase* mRNA expression in both MCF-7 cells and breast cancer tissues. Third, a significant inverse correlation was detected between *let-7f* and aromatase protein expression in FFPE and frozen breast cancer tissues by immunohistochemistry and western blotting. miRNA is preserved excellently in FFPE tissues; therefore they were used in this study [27]. Fourth, transfection of *let-7f* decreased the activities of the luciferase reporter containing the 3'UTR sequence of the *CYP19A1* gene. These results all indicated that

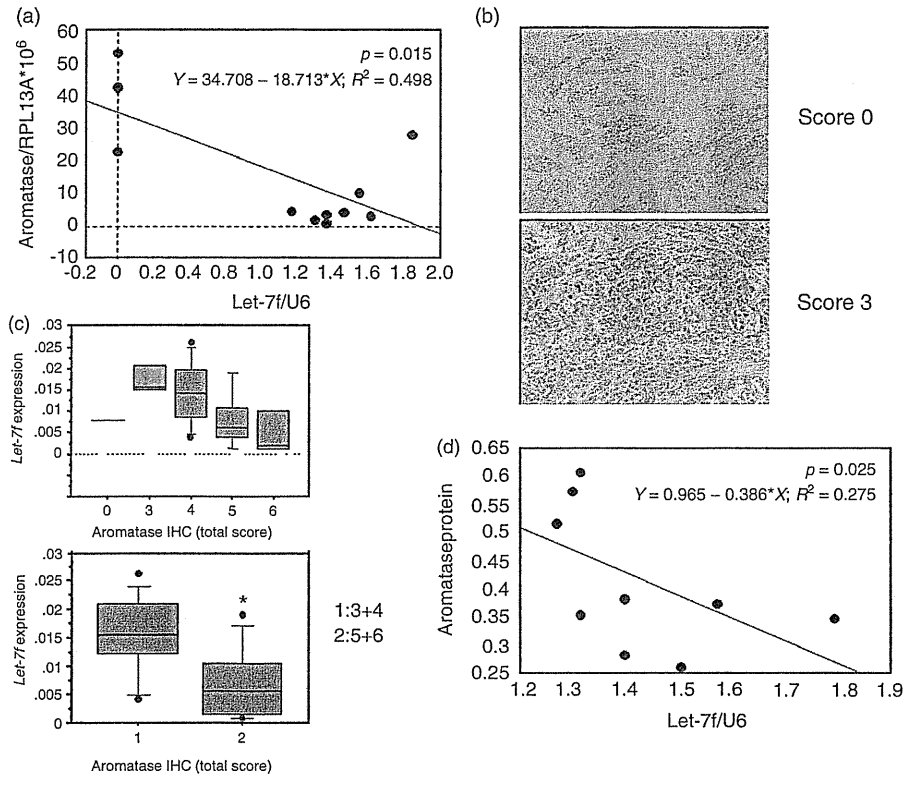

Figure 3. (a) Inverse correlation between *aromatase* mRNA expression and *let-7f* in clinical cases. *Aromatase* mRNA expression was demonstrated as a fraction of *RPL13A* by quantitative RT-PCR. *Let-7f* expression was examined using qRT-PCR and normalized to *U6* probe. (b) Relative immuno-intensity of aromatase positive cells was demonstrated: 0, no immunoreactivity, to 3, intense immunoreactivity. (c) Aromatase immunoreactivity was evaluated as a total score = proportion score + relative immuno-intensity score [13]. Expression of *let-7f* in breast cancer cells was evaluated using LCM and qRT-PCR and subsequently compared with aromatase immunoreactivity. *Let-7f* expression was negatively correlated with aromatase immunoreactivity. $*p < 0.05$. See the Materials and methods section for details. (d) Expression of *let-7f* in frozen breast cancer specimens was evaluated by qRT-PCR and compared with the aromatase protein level using western blotting. Immuno-intensity of aromatase was quantified using an LAS-1000 image analyser and evaluated as a ratio of b-actin in each sample.

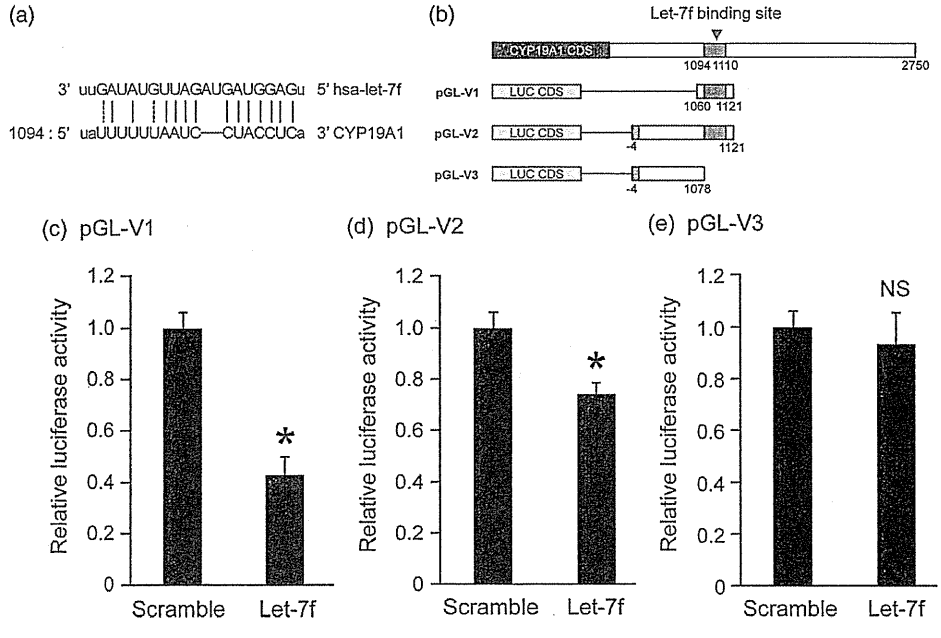

Figure 4. *Let-7f* directly targeted the *aromatase* gene (*CYP19A1*). (a) *Let-7f* target prediction: 3'UTR base pairing between *let-7f* and *CYP19A1*. *Hsa-let-7f/CYP19A1* alignment provided by http://www.microrna.org/. (b) Constructs of the vector, pGL-V1, -V2, and -V3, are demonstrated. *Let-7f* significantly inhibited luciferase activity with (c) pGL-V1 and (d) pGL-V2, which included the *CYP19A1* binding site within 3'UTR. (e) Luciferase activity was not inhibited when pGL-V3, which did not include the binding site, was inserted. $*p < 0.05$ versus scramble siRNA. NS = not significant. All experiments were performed in triplicate independently.

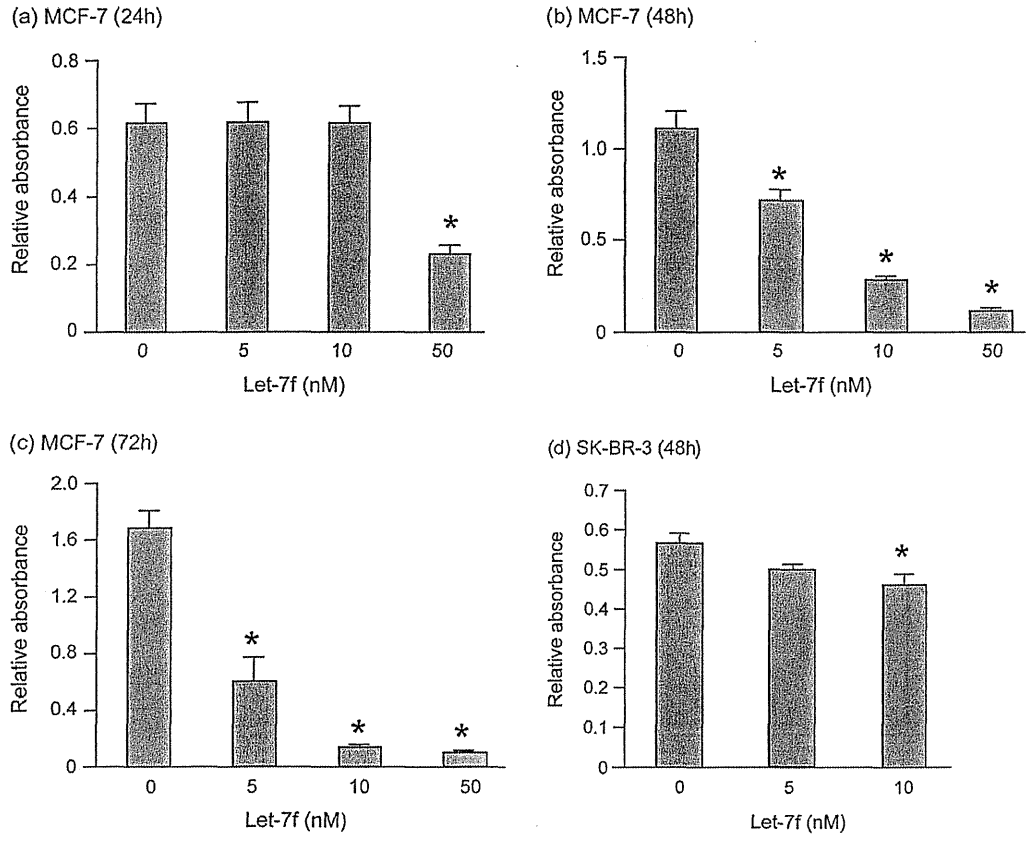

Figure 5. Effects of *let-7f* on cell proliferation in MCF-7 (a–c) and SK-BR-3 (d) cell lines using the WST-8 assay. $*p < 0.05$ compared with scramble siRNA. MCF-7: (a) 24 h, (b) 48 h, and (c) 72 h; and SK-BR-3: (d) 48 h after transfection. Cell proliferation was strongly inhibited by *let-7f*.

CYP19A1 was negatively regulated by *let-7f* in its expression. This may also explain why the *aromatase* gene itself is suppressed by AI treatment via translational suppression by *let-7f*.

We present here two theories which may explain the mechanism of *let-7f* up-regulation by letrozole. The first theory is that *let-7f* restoration by letrozole may be a secondary phenomenon following oestrogen withdrawal and tumour inhibition by AI. Genetic changes by AI were reported earlier at the mRNA level by Mackay *et al*, who showed that many oestrogen-regulated genes were secondarily altered by AI treatment [6]. At the miRNA level, our results as well as previous reports demonstrate that *let-7* is a multifunctional miRNA possessing tumour-suppressing effects as well as oestrogen-regulating features [10]. Therefore, *let-7* up-regulation may simply be a manifestation of the secondary genetic changes that occurred in the tumour environment by AI. The second theory is that letrozole may primarily and directly influence the miRNA expression profiles of cancer cells; therefore, the tumour-inhibiting properties of letrozole are due to *let-7* restoration by letrozole. The extent of our study is too limited to explain whether this possible direct interaction of cancer cells and letrozole is due to the unique molecular structure of letrozole or other mechanisms. Studies of letrozole-treated cases linked with clinical outcome should clarify this.

There is increasing evidence suggesting the importance of miRNAs in modulating sensitivity and resistance to therapy in various human malignancies [7]. For instance, the suppression of *miR-21* was reported to sensitize MCF-7 cells to the anti-cancer agent topotecan [28]. Similar studies have been reported in the case of tamoxifen [29] and have highlighted the importance of miRNAs in endocrine therapy as well as conventional chemotherapy. Resistance to AIs is currently a major clinical problem in AI therapy and many approaches to address this have been reported in the literature. The results of our present study and a previous report by Garcia-Casado *et al* imply the importance of the 3'UTR region of *CYP19A1* in relation to AI resistance [30]. Garcia-Casado *et al* reported that cases showing polymorphisms of rs4646 in the 3'UTR of the *CYP19A1* gene presented with a poor clinical response to letrozole and short progression-free survival [30]. Data from our study confirmed the actual *let-7f* binding region among the 3'UTR of *CYP19A1*. Taken together, these data point to the possibility that genetic alteration in the 3'UTR region leads to either the inability of *let-7f* to bind to *CYP19A1* or a less efficient rate of *CYP19A1* translation inhibition. This would result in weaker tumour-suppressing properties for letrozole. Our study is of clinical significance in this way as genetic alterations in the 3'UTR region of *CYP19A1* may be the key to explaining AI resistance.

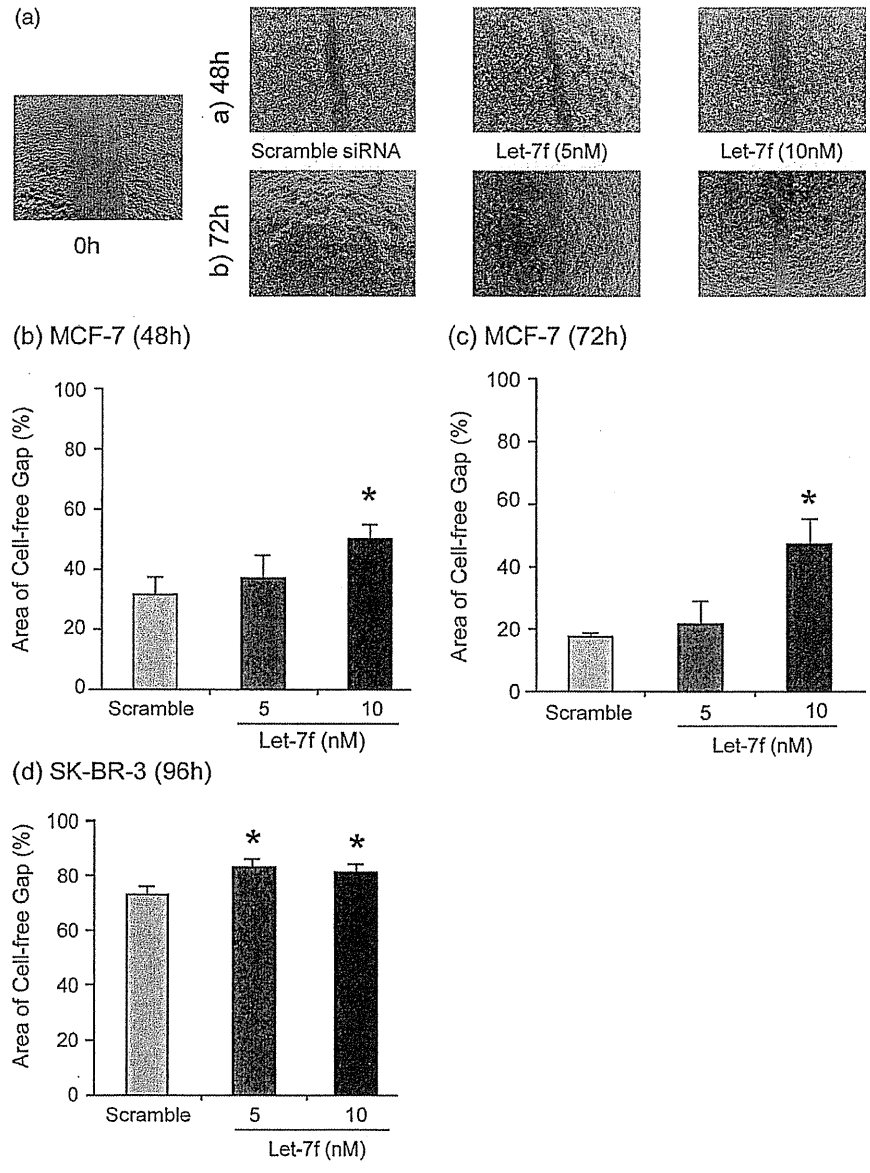

Figure 6. Effects of *let-7f* on cell migration in MCF-7 (a–c) and SK-BR-3 (d) cell lines using a modified wound healing assay. (a) Representative illustration of MCF-7. (b, c) *Let-7f* transfection reduced the area of the cell-free gap after 48 and 72 h in MCF-7. (d) *Let-7f* transfection reduced the area of the cell-free gap after 96 h in SK-BR-3 compared with scramble siRNA transfection. $*p < 0.05$ versus scramble siRNA.

In conclusion, we firstly demonstrated that *let-7f* expression was up-regulated by AI treatment in both breast cancer cells and clinical specimens, which resulted in decreased cell proliferation of breast cancer cells. The results of our study also revealed that *let-7f* directly targeted *aromatase* gene expression. These results raise the possibility that *let-7* restoration accounts for the tumour-suppressing effects of AI through its actions on *aromatase* mRNA expression.

Acknowledgment

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Author contribution statement

YS and YM conceived and designed the study. YS performed most of the experiments and drafted the manuscript. MSMC, CCPY, TYL, LWCC, TI, HH, and NO collected and stored all the samples. YO, SH, and YM contributed in immunohistochemistry and *in vitro* analyses. TS, KA, JA, and YN performed data analysis and histopathological correlations. HS supervised all experiments. YM and HS edited the manuscript. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Figure S1. Ki-67 proliferation index (%) of pre-AI and post-AI treatment in the patients.

Estrogen receptor α and β in esophageal squamous cell carcinoma

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A gender difference has been reported in the morbidity of esophageal squamous cell carcinoma (ESCC). Estrogens have been proposed to play a role in this difference but the details have not yet been clarified. Therefore, in the present study, we examined the status of estrogen receptor (ER)α and ERβ in 90 Japanese ESCC patients. ERα and ERβ immunoreactivity was detected in the nuclei of ESCC cells (41.1 and 97.8%, respectively). There was a significant positive association between the ERβ H score and histological differentiation ($P = 0.0403$), TNM-pM (LYM) ($P = 0.00164$) and Ki67/MIB1 LI of carcinoma cells ($P = 0.0497$, $r = 0.207$). In addition, the ERβ status of carcinoma cells was significantly correlated with unfavorable clinical outcome of the patients. Multivariate analysis further revealed the ERβ status in carcinoma cells as an independent unfavorable prognostic factor of these patients. We further examined the effects of estrogen treatment on ESCC cell line (ECGI-10) transfected with ERα or ERβ *in vitro*. The number of ECGI-10 transfected with ERβ was increased by estradiol or ERβ specific agonist but estradiol did not exert any effect upon the cell number of ECGI-10 transfected with ERα. In summary, the results of the present study clearly demonstrate that the status of ERβ in ESCC was closely associated with the unfavorable prognosis, possibly through altering cell proliferation of carcinoma cells. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2012.02288.x, 2012)

Human esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignancies, despite recent marked improvement of therapeutic techniques and perioperative management. Results of most epidemiological studies demonstrate prominent gender differences in the prevalence of ESCC. ESCC is generally more common in men than women, with a male to female ratio of approximately 6:1 in Japan.[1] In addition, the prognosis of ESCC patients is reported to be significantly better in women than in men following an adjustment of various clinicopathological factors.[2] Several reported studies also suggest the possible roles of estrogens in development of ESCC.[3,4]

Sex steroids, such as estrogen, are well-known to contribute to physiological maturation and cell proliferation of estrogen dependent tissues, such as breast and endometrial tissues. Estrogens also play important roles in many non-gonadal or non-classical estrogen' receptors' dependent tissues in both men and women through estrogen receptors (ER). Therefore, it is important to examine the status of ER in these tissues. Two different isomers of ER, ERα and β, were first identified by Enmark *et al.*[5] Both ERα and ERβ are encoded by ESR1 located on chromosome 6q25[6] and ESR2 located on chromosome 14q22-24,[7] respectively. Estrogens have also been reported to play pivotal roles in several types of human malignancies, which had not been necessarily considered as classical estrogen-dependent neoplasms, such as those arising in lung,[8,9] urinary bladder[10] and gastrointestinal tract.[11,12] ERwere also reported to be detected in laryngeal and ESCC.[13–17] Nozoe *et al.* report that the group of the patients associated with cytoplasmic ERα-positive/nuclear ERβ-negative status was associated with a significant adverse clinical outcome in 73 cases of ESCC.[13] Kalayarasan *et al.* also report that the status of ERβ in carcinoma cells was correlated with poor histological differentiation and advanced clinical stages in ESCC patients.[15] These reported findings all suggest that estrogens are considered to be at least involved in biological behavior of ESCC through ER present in carcinoma cells. However, it is also true that controversy exists regarding the biological and clinical significance of estrogens in ESCC.[17]

Therefore, in the present study, we attempted to evaluate estrogen actions through ERα and ERβ in ESCC as follows. We first examined the status of ERα and ERβ using immunohistochemistry in 90 Japanese ESCC cases and evaluated their correlation with clinicopathological findings of individual patients, including overall survival or disease-free survival. We further characterized the potential biological functions of ERα and ERβ in ESCC cell lines stably transfected with ERα or ERβ.

Materials and Methods

Patients and tissue preparation. A total of 90 specimens of thoracic ESCC were obtained from Japanese patients who underwent potentially curative esophagectomy with lymph node dissection from 2000 to 2005 at the Second Department of Surgery at Tohoku University Hospital (Sendai, Japan). These patients had received neither chemotherapy nor irradiation therapy prior to surgery. These 77 men and 13 women had a median age of 63.9 years (range, 37–81 years). A total of 18 specimens of non-neoplastic epithelium were also obtained from these 90 cases. These specimens had been fixed with 10% formalin for 36–48 h at room temperature and embedded in paraffin wax. Relevant clinical data were retrieved from careful review of the patients' charts. Histopathological features of all the tumors were independently reviewed by four of the authors (M. Z., D. T., F. F. and H. S.). The pathological stage of each cancer was defined according to the TNM system and each lesion was graded histologically according to the World Health Organization classification. The median follow-up time was 65.5 months (range, 1–119 months). The research protocol in this study was approved by the Ethics Committee at the Tohoku University School of Medicine (Accesssion No. 2009-453) and informed consent was obtained from each patient before surgery.

Immunohistochemistry. Mouse monoclonal antibodies for ERα(6F11), ERβ(14C8) and Ki-67 (MIB1) were purchased

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from Novocastra (Newcastle, UK), Gene Tex (San Antonio, TX, USA) and DAKO (Carpinteria,CA, USA), respectively.

Serial 4-μm thick tissue sections were deparaffinized with xylene and ethanol. Antigen retrieval was performed by heating the slides in an autoclave at 121°C for 5 min in citric acid buffer (2 mmol/L citric acid and 9 mmol/L trisodium citrate dehydrate, pH 6.0) or instant antigen retrieval H buffer (Mitsubishi Kagaku Iatron, Tokyo, Japan) for ER. Sections were then incubated with 10% normal rabbit serum for the monoclonal antibodies to reduce nonspecific background immunostaining. All incubations were performed for 18 h at 4°C with primary antibodies. The dilutions of primary antibodies were summarized as follows: ERα, 1:50; ERβ, 1:1000; and Ki-67, 1:100. Endogenous peroxidase activity was blocked by immersing the slides in 0.3% hydrogenperoxidase for 30 min at room temperature. The sections were subsequently incubated with biotinylated rabbit antimouse IgG (Histofine Kit; Nichirei, Tokyo, Japan). The localization of ER was then visualized with 3-Amino-9-ethylcarbazole and counterstained with hematoxylin. The other antigen-antibody complex was then visualized with 3.3-diaminobenzidine (1 mmol/L, in 50 mol/L Tris–HCl buffer, pH 7.6 and 0.006% H_2O_2) and counterstained with hematoxylin. ERα positive breast cancer tissue was used as a positive control for ERα. Non-pathologic prostate tissue was used as a positive control for ERβ. As a negative control, normal mouse or rabbit IgG was used instead of the primary antibodies and no specific immunoreactivity was detected in these preparations.

Evaluation of immunoreactivity. We evaluated the nuclear immureactivity of ERα, ERβ and Ki67. The immunoreactivities for ERα and Ki67 were evaluated in approximately 1000 carcinoma cells in each case and the percentage of immunoreactivity (i.e. labeling index [LI]) was determined after counting. In the present study, we used the H score for semi-quantitative analysis of nuclear ERβ immunoreactivity.[18] Approximately 1000 tumor cells were counted in three different representative areas of carcinoma infiltration in each case and the H score was derived from the formula: H score = (percentage of strongly stained nuclei × 3) + (percentage of moderately stained nuclei × 2) + (percentage of weakly stained nuclei × 1). Scoring was performed independently by three of the authors (M. Z., D. T. and F. F.). When the inter-observer differences were <5%, the mean value was obtained as a final value of LI and H score. When the differences were more than 5%, all of the examiners evaluated the immunohistochemical findings simultaneously through multi-headed light microscopy and reached a consensus. When evaluating the possible correlation between ERβ status and clinical outcome of individual patients, the cases were tentatively classified into two groups according to their ERβ H scores (high ERβ, ≥250 H score; low ERβ, 0–249 H score, respectively), because ERβ H scores were found to be distributed in a peak on the boundary of approximately 250 (data not shown). In addition, the median value of the ERβ H score was 231.2, near the value of 250. The selection of the median value as the cut-off point was reported to secure objectivity because it is not determined as an "optimal" cut-off point.[19,20]

Cell culture and chemicals. Human ESCC cell line EC-GI-10 was provided by RIKEN Bioresource Center (Tsukuba, Japan). TE-1, TE-4 and TE-8 were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). All ESCC cell lines were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) with 10% FBS (Nichirei, Tokyo, Japan). In this study, cells were cultured with phenol red-free RPMI 1640 containing 10% dextran coated charcoal treated FBS for 3 days before the experiment. 17β-estradiol was purchased from Sigma-Aldrich. An ERα selective agonist (propyl-pyrazole-triol; PPT),[21] an ERβ selective agonist (diarylpropionitrile [DPN])[21] and a pure ER antagonist, ICI 182780,[22] were purchased from Tocris Bioscience (Minneapolis, MN, USA).

Stable transfection: establishment of EC-GI-10 cells expressing ERα or ERβ. The transformed ECGI-10 cells expressing ERα (EC-GI-10 + ERα) or ERβ (EC-GI-10 + ERβ) were established to further characterize the biological functions of ER isoforms in ESCC.

Stable transfection was performed according to the methods reported in previous studies with some modifications.[8,23,24] ERα and ERβ expression vectors for ERα (pRc/CMV-ERα) and ERβ (pRc/CMV-ERβ) used in this study were described previously.[8,23,24] Briefly, EC-GI-10 cells were transfected with ERα or ERβ expression vector with Lipofectamine LTX (Invitrogen Life Technologies, Gaithersburg, MD, USA). After 24 h in culture, the cells were subsequently grown in fresh RPMI 1640 supplemented with 10% FBS containing 1 mg/mL geneticin (G418; Sigma-Aldrich) for 2 weeks. Isolated colonies were trypsinized in metal ring cups and the cells were further cultured in the presence of 200 μg/mL G418. As a negative control, empty vector was also transfected in the EC-GI-10 cells. Expression of ERα and ERβ at both mRNA and protein levels in EC-GI-10 cells was examined by quantitative RT-PCR and immunohistochemistry.

RT-PCR. Total RNA was carefully extracted from human ESCC cell line (EC-GI-10, TE-1, TE-4 and TE-8) using the TRIzol (Invitrogen Life Technologies) method. The QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany) was used in the synthesis of cDNA. RT-PCR was carried using the Light-Cycler System (Roche Diagnostics, Manheim, Germany), and ribosomal protein L 13a (RPL13A) was also used as an internal standard. The primer sequences used in this study are as follows: ERα(NM_000125); forward: 5′-AGACACTTTGATCCACCTGA-3′ (cDNA position 1811–1831) and reverse: 5′-CAAGGAATGCGATGAAGTAG-3′ (cDNA position 2080–2100), ERβ (AB006590); forward: 5′-CCTGGCTAACCTCCTGATGC-3′ (cDNA position 1460–1480) and reverse: 5′-ACCCCGTGATGGAGGACTT-3′ (cDNA position 1608–1627) and RPL13A (NM_012423); forward: 5′-CCTGGAGAGAAG AGGAAAGAGA-3′ (cDNA position 487–509) and reverse: 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′ (cDNA position 588–612). The PCR products were purified and subjected to direct sequencing to verify amplification of the correct sequences. Negative controls, in which the reaction mixture lacked cDNA template, were included to exclude the possibility of exogenous contaminant DNA.

cDNA of known concentrations for target genes and the housekeeping gene RPL13A were used to generate standard curves for real-time quantitative PCR to determine the quantity of target cDNA transcript. The Ct (cycle threshold) values were used to calculate the gene-specific input mRNA amounts according to the calibration curve method. The mRNA level in each case was represented as a ratio of RLP13A and was evaluated as a ratio (%) compared with that of each control.[8,20,25]

Cell proliferation assays. EC-GI-10 including its transformants and TE-1, TE-4 and TE-8 were treated with the indicated compounds for 3 days and the cell proliferation was measured by a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] method using Cell Counting Kit-8 (Dojin Kagaku, Kumamoto, Japan).[8,26]

Statistical analysis. The values for ERβ H score, patient age, tumor size, Ki67/MIB1 LI and ERα LI were demonstrated as mean ± SEM. The statistical analyses between ERβ H score and clinicopathological parameters of individual patients were evaluated using the Mann–Whitney *U*-test, the Kruskal–Wallis test, a correlation coefficient (*r*) and regression equations when appropriate.

Overall survival (OS) and disease-free survival (DFS) curves of the patients examined were generated according to the

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Kaplan–Meier method and statistical significance was calculated using the log-rank test. Cox's proportional hazard model was used for both univariate and multivariate analyses.

The proliferation activity was evaluated as a ratio (%) compared to that of controls (no treatment with either estradiol for 72 h). The statistical analyses between relative luciferfase activity and each agent were evaluated using the Kruskal–Wallis test and the Scheffe test.

Statistical differences were examined using StatView 5.0 J software (SAS Institute, Cary, NC, USA) and values of $P < 0.05$ were considered statistically significant.

Results

Immunohistochemistry: ERα and ERβ status in non-neoplastic esophagus and esophageal squamous cell carcinoma. Both ERα and ERβ immunoreactivity were detected in the nuclei of non-neoplastic basal layer cells of the esophagus (Fig. 1a,b). ERα immunoreactivity was detected in the nuclei of carcinoma cells in 38/90 ESCC (Fig. 1c). The mean value of the ERα labeling index in 90 ESCC was 22.3 ± 3.2 (range, 0–90). ERβ immunoreactivity was detected in the nuclei of carcinoma cells with a variety of immunointensity in 88/90 ESCC (Fig. 1d). The mean value of the ERβ H score in 90 ESCC was 208.9 ± 7.4 (range, 0–295).

Correlation between the status of ERα immunoreactivity and clinicopathological variables in 90 esophageal squamous cell carcinoma patients. Associations between ERα status and clinicopathological variables of the patients examined are summarized in Table 1. No significant association was detected between ERα status and age, gender, tumor size, depth of tumor invasion, presence of lymph node metastasis, TNM stage, lymphatic invasion, venous invasion, infiltrative growth pattern and the Ki67/MIB1 Labeling index status of the patients examined.

Correlation between the status of ERβ immunoreactivity and clinicopathological variables in 90 esophageal squamous cell carcinoma patients. Associations between ERβ status and clinicopathological variables of the individual patients examined are summarized in Table 1. There was a statistically significant positive association between ERβ H score and tumor differentiation ($P = 0.0403$) and TNM-pM (LYM) ($P = 0.0164$). There was also a weak but statistically significant positive correlation between the ERβ H score and Ki67/MIB1 LI ($P = 0.0497$, $r = 0.207$). No significant association was detected between ERβ immunoreactivity and age, gender, tumor size, depth of tumor invasion, presence of lymph node metastasis, TNM stage, lymphatic invasion, venous invasion or infiltrative growth pattern of the patients examined in the present study.

Correlation between ERα status and clinical outcome in 90 esophageal squamous cell carcinoma patients. The OS and DFS curves of the patients examined are summarized in Figure 2(a, b), respectively. The patients with positive nuclear ERα immunoreactivity in carcinoma cells were by no means associated with better survival or favorable clinical outcome (log-rank test: OS, $P = 0.4660$; DFS, $P = 0.3468$).

Correlation between ERβ status and clinical outcome in 90 esophageal squamous cell carcinoma patients. The OS and DFS curves of the patients examined are illustrated in Figure 2(c, d), respectively. In the present study, the patients with high nuclear ERβ immunoreactivity were significantly associated with shorter survival or adverse clinical outcome (log-rank test: OS, $P = 0.0017$; DFS, $P = 0.0005$). Results of univariate analysis (Table 2) demonstrated that pathological stage (OS, $P = 0.0003$; DFS, $P = 0.0006$), ERβ status in the nucleus of carcinoma cells (OS, $P = 0.0025$; DFS, $P = 0.0010$), tumor size (OS, $P = 0.0485$; DFS, $P = 0.0366$) and infiltration type (OS, $P = 0.0200$; DFS, $P = 0.0416$) were all significant prognostic factors for OS and/or DFS in 90 ESCC examined in our study. A subsequent multivariate analysis did reveal that ERβ status (OS, $P = 0.0010$; DFS, $P = 0.0007$) was an independent prognostic factor for OS and DFS of these patients, as well as pathological stage (OS, $P = 0.0019$; DFS, $P = 0.0091$) and infiltration type (OS, $P = 0.0185$; DFS, $P = 0.0328$).

Establishment of EC-GI-10 esophageal squamous cell carcinoma cells expressing ERα or ERβ. The transformed EC-GI-10 ESCC cells expressing ERα (EC-GI-10 + ERα) or ERβ (EC-GI-10 + ERβ) were established because the parental EC-GI-10 cells and TE-1, TE-4 and TE-8 cells examined in this study expressed only miniscule amounts of ERα or ERβ at mRNA levels (data not shown). Estradiol administration (1 pmol/L–100 nmol/L) did not change the number of cells even after 72 h in all the cell lines, TE-1, TE-4, TE-8 and EC-GI-10 (data not shown). As a control, we also isolated a clone

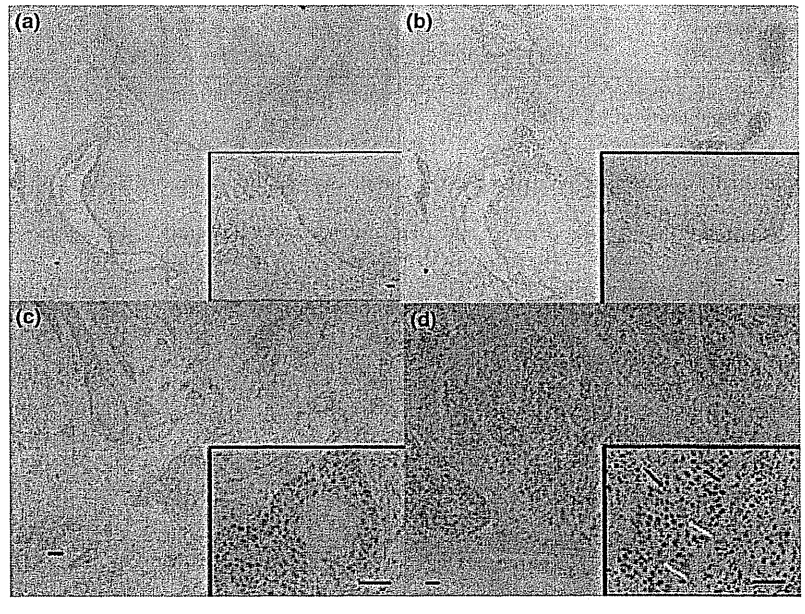

Fig. 1. Representative illustrations of immunohistochemistry in human esophageal squamous cell carcinoma. (a) Estorogen receptor α (ERα) immunoreactivity was detected in the nuclei of non-neoplastic epithelial cells. (b) Estorogen receptor β (ERβ) immunoreactivity in the nuclei of non-neoplastic epithelial cells. (c) ERα immunoreactivity in esophageal squamous cell carcinoma (ESCC) case. (d) ERβ immunoreactivity in ESCC case. Black arrows represent marked immunoreactivity (+++) of ERβ and white arrows represent weak immunoreactivity (+) of ERβ. Bar represents 100 μm.

Table 1. Correlation between ERα and ERβ and clinicopathological variables in 90 esophageal squamous cell carcinoma patients

Variable	*n* or mean ± SEM	Nuclear ERα		Nuclear ERβ	
		Labeling		Index H score	
		Mean ± SEM	*P*-values	Mean ± SEM	*P*-values
Age (years)					
<65	48	22.2 ± 4.5	0.5991	201.0 ± 10.3	0.1929
≥65	42	22.4 ± 4.7		218.3 ± 10.5	
Gender					
Male	77	22.6 ± 3.5	0.6298	209.2 ± 7.9	0.7786
Female	13	20.5 ± 9.0		206.8 ± 21.8	
Tumor size (mm)					
<50	54	25.9 ± 4.5	0.2439	208.3 ± 10.2	0.6329
≥50	36	17.0 ± 4.4		209.7 ± 10.6	
TNM-pT					
pT1	29	28.7 ± 6.5	0.4309	202.2 ± 16.5	0.2148
pT2	11	25.8 ± 9.3		206.7 ± 15.4	
pT3	47	19.0 ± 4.1		209.8 ± 9.1	
pT4	3	0.0		266.2 ± 13.3	
TNM-pN					
pN0	36	27.0 ± 5.5	0.3130	200.3 ± 13.1	0.6565
pN1	54	19.2 ± 3.9		214.6 ± 8.7	
TNM-pM					
pM0	81	23.8 ± 3.5	0.3569	203.7 ± 7.9	0.0164**
pM1 (LYM)	9	8.5 ± 6.8		255.4 ± 10.4	
TNM-pStage					
I	19	36.2 ± 8.4	0.2381	198.2 ± 20.8	0.0580
II	31	7.6 ± 5.1		195.5 ± 12.5	
III	31	22.5 ± 5.3		215.3 ± 10.8	
IV	9	8.5 ± 6.8		255.4 ± 10.4	
Differentiation					
Well	17	11.5 ± 4.9	0.0688	188.2 ± 23.4	0.0403**
Mod	59	20.5 ± 4.0		205.2 ± 8.5	
Poorly	14	43.0 ± 9.1		249.4 ± 7.8	
Lymphatic invasion					
Negative	35	24.2 ± 5.8	0.8167	218.3 ± 11.3	0.1288
Positive	55	21.1 ± 3.8		202.9 ± 9.7	
Venous invasion					
Negative	31	26.2 ± 5.5	0.4223	209.7 ± 13.6	0.5810
Positive	59	20.3 ± 4.0		208.4 ± 8.8	
Growth pattern					
Expansive	26	21.3 ± 6.1	0.4961	224.4 ± 12.6	0.1039
Intermediate	52	20.4 ± 4.2		198 ± 10.0	
Infiltrative	12	32.9 ± 8.9		222.4 ± 20.4	
Ki-67 LI (%)	43.7 ± 2.0 (8.1–83.9)	0.8412 (r = 0.021)		0.0497** (r = 0.207)	

Data are presented as mean ± SEM. All other values represent the number of cases. **P-values less than 0.05 were considered significant.
ER, estrogen receptor; LI, labeling index.

tentatively termed EC-GI-10 + CMV, which was stably transfected with empty vector in the EC-GI-10 cells.

Relatively low levels of ERα and ERβ mRNA were detected in EC-GI-10 + CMV cells (Fig. 3A). The levels of ERα and ERβ mRNA were significantly higher in the EC-GI-10 + ERα and + ERβ cells, respectively, than those in EC-GI-10 + CMV cells (Fig. 3A). In immunohistochemistry, ERα and ERβ immunoreactivity was relatively weak or negative in EC-GI-10 + CMV cells (Fig. 3B). Immunoreactivity of ERα but not of ERβ was detected in EC-GI-10 + ERα cells (Fig. 3B) and vice versa in EC-GI-10 + ERβ cells (Fig. 3B).

Effects of ERα or ERβ expression on estradiol, propyl-pyrazole-triol and diarylpropionitrile-mediated cell proliferation in EC-GI-10 cells. The number of EC-GI-10 + ERα cells was significantly decreased following the treatment with PPT (1 μmol/L), but not with estradiol (1 μmol/L) or DPN (1 μmol/L) compared to that administered with vehicle control cells (Fig. 3C, left). Both estradiol (1 μmol/L) and ERβ specific agonist DPN (1 μmol/L) treatments significantly increased the cell proliferation of EC-GI-10 + ERβ cells when compared to that administered with vehicle control cells (Fig. 3C, center). The estradiol-mediated cell proliferation of EC-GI-10 + ERβ cells was significantly suppressed ($P < 0.001$) by the addition of ICI 182780 (100 pmol/L) and reached the control level. There was no significantly difference in the number of EC-GI-10 + CMV cells treated with estradiol, DPN or TTP (Fig. 3C, right). Estradiol-mediated cell proliferation was detected in EC-GI-10 + ERβ cells and was significantly induced following the treatment with 1 nmol/L to 1 μmol/L estradiol (the difference of groups; Kruskal–Wallis, $P = 0.0004$, NC v 1 nM to 1 μM have difference in Scheffe test; Fig. 3D, right). Estradiol decreased the cell proliferation of EC-GI-10 + ERα in the concentration of 1 pmol/L to 1 μmol/L, but this increment did not reach statistical significance (Fig. 3D, left)

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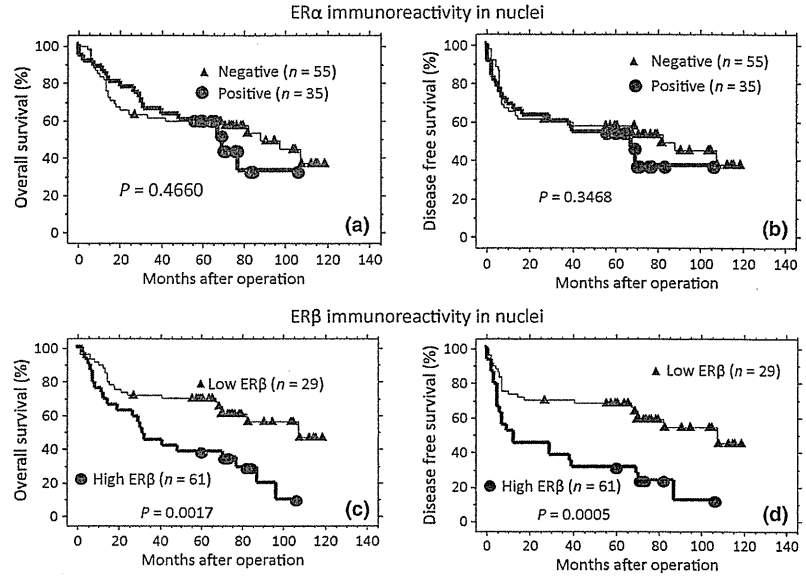

Fig. 2. Overall and disease-free survival (OS and DFS) curves of 90 Japanese patients with esophageal squamous cell carcinoma examined in this study according to the status of estrogen receptor (ER)α and ERβ immunoreactivity (Kaplan–Meier method). Significant difference in survival of the patients was noted between those with high and low ERβ nuclear immunoreactivity (c,d), but significant difference was not detected according to the status of ERα nuclear immunoreactivity (a,b). Cases were tentatively classified into two groups according to results of ERβ H score in the nuclei: high ERβ, >250 H score and low ERβ, <250 H score. High ERβ status in carcinoma cells was significantly associated with unfavorable clinical outcome of the patients examined.

Table 2. Univariate and multivariate analysis of overall survival/disease free survival in 90 esophageal squamous cell carcinoma patients

	Univariate		Multivariate	
	P-value	Relative risk (95% CI)	*P*-value	Relative risk (95% CI)
(A) Overall survival				
Sex (male/female)	0.1337	2.196 (0.785–6.139)		
Age (≥65/<64 years)	0.9419	1.022(0.572–1.824)		
Depth of tumor (pT3,4/pT1,2)	0.0069**	2.418 (1.274–4.589)		
TNM–pN (positive/negative)	0.0105**	2.287 (1.213–4.311)		
TNM–pM (positive/negative)	0.0579	2.191 (0.974–4.927)		
pStage (III,IV/I,II)	0.0003**	3.065 (1.675–5.609)	0.0019**	2.786 (1.459–5.322)
Tumor size (≥50/<50 mm)	0.0485**	1.807 (1.004–3.252)	0.4515	1.279 (0.674–2.427)
Lymphatic invasion (positive/negative)	0.0521	1.866 (0.994–3.503)		
Venous invasion (positive/negative)	0.3368	1.361 (0.726–2.552)		
Growth pattern (infiltrative/expansive + intermediate)	0.0200**	2.428 (1.150–5.126)	0.0185**	2.531 (1.169–5.484)
Histological grade (por/well + mod)	0.3653	1.424 (0.662–3.062)		
Ki67 status†	0.7021	0.997 (0.484–1.540)		
ERα status (positive/negative)	0.4692	1.249 (0.684–2.281)		
ERβ status (high/low)	0.0025**	2.469 (1.374–4.438)	0.0010**	2.754 (1.509–5.027)
Brinkman index (≥400/<400)	0.9389	0.975 (0.504–1.885)		
(B) Disease free survival				
Sex (male/female)	0.1142	2.286 (0.819–6.378)		
Age (≥65/<64 years)	0.9997	1.000 (0.567–1.763)		
Depth of tumor (pT3,4/pT1,2)	0.0124**	2.200 (1.186–4.080)		
TNM–pN (positive/negative)	0.0218**	2.051 (1.110–3.788)		
TNM–pM (positive/negative)	0.1305	1.865 (0.831–4.185)		
pStage (III,IV/I,II)	0.0006**	2.804 (1.560–5.039)	0.0091**	2.304 (1.230–4.314)
Tumor size (≥50/<50 mm)	0.0366**	1.840 (1.038–3.259)	0.3092	1.378 (0.743–2.555)
Lymphatic invasion (positive/negative)	0.1816	1.506 (0.826–2.746)		
Venous invasion (positive/negative)	0.4967	1.236 (0.671–2.276)		
Growth pattern (infiltrative/expansive + intermediate)	0.0416**	2.150 (1.029–4.490)	0.0328**	2.307 (1.071–4.971)
Histological grade (por/well + mod)	0.1013	1.844 (0.887–3.835)		
Ki67LI†	0.6212	0.996 (0.981–1.012)		
ERα status (positive/negative)	0.3563	1.317 (0.734–2.363)		
ERβ status (high/low)	0.0010**	2.627 (0.734–2.363)	0.0007**	2.828 (1.551–5.158)
Brinkman index (≥400/<400)	0.8198	1.079(0.560–2.078)		

**Data considered significant ($P < 0.05$) in the univariate analysis were examined in the multivariate analysis. CI, confidence interval; ER, estrogen receptor; LI, labeling index; mod, moderate; por, poor. ERα status (positive; LI ≥ 10%/negative; LI < 10%). ERβ status (high; H score ≥ 250/low; H score < 250). †Data were evaluated as continuous values and displayed mean ± SEM (range).

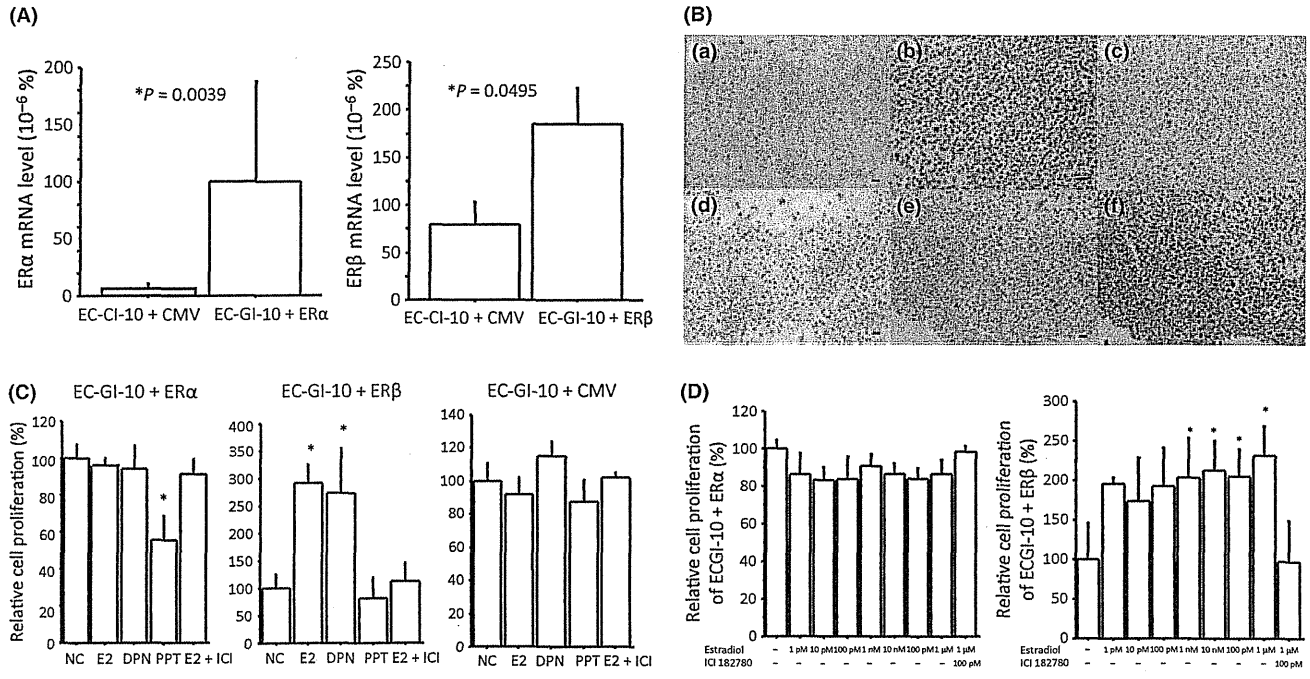

Fig. 3. (A) mRNA level of EC-GI-10 + CMV versus EC-GI-10 + ERα(left), mRNA level of EC-GI-10 + CMV versus EC-GI-10 + ERβ(right), mean ± SD (n = 6). (B) Immunostaining for estrogen receptor (ER) isoforms in the EC-GI-10 transformants. (a,d) EC-GI-10 + CMV; (b,e) EC-GI-10 + ERα; (c,f) EC-GI-10 + ERβ. ERα immunoreactivity was detected in the nuclei of EC-GI-10 + ERα cells (b), ERβ immunoreactivity was detected in the nuclei of EC-GI-10 + ERβ cells (f). No significant immunoreactivity for ER isoforms was detected in EC-GI-10 + CMV cells (a,d). Immunoreactivity was evaluated in the cell blocks specimens. Bar represents 100 μm. (C) Left, EC-GI-10 + ERα cells; center, EC-GI-10 + ERβ cells; right, EC-GI-10 + CMV. Estradiol (1 μmol/L) with or without ER antagonist ICI 182780 (100 pmol/L), ERα agonist PPT (1 μmol/L), ERβ agonist DPN (1 μmol/L) were added to these cells. They were then cultured for 72 h. NC, no changes. The cell proliferation activity was evaluated as a ratio (%) compared with that of controls (no treatment with either estradiol, PPT, DPN or ICI 182780 for 72 h). Mean ± SD (n = 6). *P < 0.05 versus controls (Kruskal–Wallis test and Scheffe test). (D) Effects of the estrogen-mediated proliferation of EC-GI-10 + ERα cells, EC-GI-10 + ERβ cells. The proliferative activity was evaluated as a ratio (%) compared to that of controls (no treatment with either estradiol or ICI 182780 for 72 h). Mean ± SD (n = 6). *P < 0.05 versus controls (Kruskal–Wallis test and Scheffe test).

Discussion

Freedman *et al.* (2010) report that menopausal hormone therapy is significantly associated with lower risk of head, neck and ESCC in the National Institutes of Health-American Association of Retired Persons (NIH-AARP) Diet and Health Study.[27] Bodelon *et al.* also report the significant association between hormone replacement therapy and the risks of developing ESCC in postmenopausal women enrolled in a Women's Health Initiative (WHI) and observational study.[28] They both demonstrate that the women who took both estrogen and progestin had lower risk of developing ESCC than those who took a placebo, but this association was not detected when women took estrogen alone.[28] Further investigations regarding hormone receptor status, including progesterone receptor in ESCC, are certainly required for clarification, but it has become important to examine the details of estrogenic actions, especially the status of ER in development and behavior of ESCC patients.

Enmark *et al.* report the presence of two different isoforms of ER, ERα and β, in many types of human tissues.[5] Subsequent studies confirm that ERα and ERβ are not only expressed in classical estrogen target tissues but are also rather widely distributed in humans. Taylor *et al.* report the presence of ERα and ERβ in normal esophagus squamous epithelium.[29] Nozoe *et al.* report the presence of ERα and ERβ in ESCC patients.[13] Kalayarasan *et al.* report that the status of ERβ is correlated with aggressive behavior in ESCC patients,[15] but different results have been also reported.[17] Therefore, the clinical and biological significance of ERα and ERβ in ESCC has not been clarified.

In the present study, we examined the nuclear immunoreactivity of ERα and ERβ in both squamous cell carcinoma and non-neoplastic squamous cell epithelium of the esophagus. A relatively high level of ERα immunoreactivity was detected in the nuclei of non-neoplastic basal layer cells in normal esophageal mucous. In mammary glands, Khan *et al.* report increased ERα immunoreactivity in normal epithelium obtained from tumor-bearing breasts compared to non-tumor bearing breast.[30] Lawson *et al.* also report that ERα expression is higher in the breast tissue of women from a population at high risk of breast cancer compared with that in the tissue of women associated with a relatively low risk of the disease development.[31] It is also interesting to note that Zhai *et al.* report the loss of ERα expression in the advanced stages of cervical squamous cell carcinoma progression.[32] In the present study, a relatively high level of ERα immunoreactivity was also detected in non-neoplastic esophageal mucosa bearing ESCC compared to that in concomitant or adjacent carcinoma cells. Therefore, in human squamous mucosa, estrogen may help to maintain normal cell cycle or exert a protective effect upon epithelial cells through the ERα. Further investigation is necessary to clarifying this interesting hypothesis, for example through comparison of ERα status of morphologically normal esophageal mucosa in tumor bearing and non-tumor bearing subjects. However, it is also true that the status of ERα immunoreactivity in ESCC was by no means associated with any of the clinicopathological variables of the

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patients examined in this study, including their clinical outcome. In contrast, ERβ status of carcinoma cells was significantly associated with unfavorable clinical outcome of the patients and ERβ status in carcinoma cells also turned out to be an independent unfavorable prognostic factor for the patients (determined using multivariate analysis). Therefore, it has become important to examine the effects of estrogen signals mediated through ERβ in these patients to clarify the possible involvement of estrogens in the biological behavior of ESCC.

Ivanova *et al.* report on the difference between men and women in the modes of actions of ERβ in lung cancer.[33] Therefore, we initially postulated that variations in expression of ER were due to the differences in the prevalence of gender in ESCC. However, in the present study there were no differences in the status of ER according to the gender of the patients. Therefore, the gender differences in the prevalence of ESCC might be due to differences in the lifestyles of male and female patients (e.g. drinking and smoking), but further investigations are required for clarification.

In the present study, ERβ was detected in the nuclei of ESCC, as in Nozoe *et al.* The differences between the results of the present study and those of Nozoe *et al.* could be due to the ERβ antibodies used and the evaluation method of ERβ immunoreactivity. Nozoe *et al.* determined that ERβ nuclear staining in at least 50% of tumor cells were scored as positive for overexpression, based upon a study of lung cancer by Wu *et al.*[13,34] However, we defined ERβ nuclear positive immunoreactivity or overexpression as the cases with an *H* score of more than 250. The validity of this definition is discussed in the Materials and Methods section. In breast carcinoma, the difference of the cut-off points of the ER status is well-known to be related to the prognosis of individual patients.[35,36] The results of survival analyses of breast carcinoma patients are also known to be different depending on the evaluation method of the ER (e.g. proportion score and intensity score).[35] Further examinations using different ERβ antibodies and evaluation methods are obviously required to clarify the ERβ and prognosis in ESCC.

According to Matsuoka *et al.* and Ueo *et al.*, estrogen prevents cell proliferation of primary ESCC cells, which are reported to be associated with the presence of ER.[3,4] ERβ was not identified in the 1980s and, therefore, results from these previous reports[3,4] could not clarify whether estrogenic signals were mediated in ESCC through ERα, ERβ or both. When both ERα and ERβ are present in the cells and bound to estrogen, estrogen signals through ERβ are generally considered to inhibit ERα-dependent transcription.[37] For instance, estrogen-dependent cell proliferation is reported to be inhibited by transfection of ERβ in breast carcinoma cells.[38] However, several studies state that estrogenic action through ERβ signaling induces rather than suppresses the cell proliferation of carcinoma cells.[8,39,40] For instance, Teng *et al.* demonstrate that the treatment of ERβ specific agonist DPN significantly increased cell proliferation in primary urothelial cells, which predominantly expressed ERβ.[10] They also report that estrogenic signals mediated through ERβ predominantly induce G1/S transition in primary urothelial cells.[41] Hershberger *et al.* report ERβ-dependent cell proliferation through both genomic and non-genomic pathways in lung carcinoma cell line.[42] In the present study, the treatment of PPT, the specific ERα agonist, significantly decreased the number of EC-GI-10 + ERα cells and that of estradiol, and DPN, the specific ERβ agonist, significantly increased the number of EC-GI-10 + ERβ cells. Results of the present study clearly indicate that estrogenic actions through ERβ were predominant in ESCC cells compared to those through the ERα. Therefore, estrogen might also play a pivotal role in the cells in which estrogenic signals are mediated predominantly through ERβ, such as ESCC cells. Interestingly, TGFα, amphiregulin or HB-EGF, all of which are considered EGFR activators, are induced by estradiol treatment in ERα negative/ERβ positive breast carcinoma cell line[43] or ERβ dominantly expressed HNSCC cells.[44] EGFR is also reported to be detected in many ESCC cells,[45,46] and is even considered as a target molecule for a potential target specific therapy in ESCC patients.[47] Therefore, estrogenic effects, including an induction of its target genes, might facilitate the proliferation of ESCC cells through the activation of EGFR signals in ESCC, but further investigations are required for clarification.

In summary, we demonstrated ERα and ERβ expression using immunohistochemistry in human ESCC. The status of nuclear ERβ immunoreactivity in carcinoma cells turned out to be an unfavorable independent prognostic factor in ESCC patients. Results of our immunohistochemical and *in vitro* studies clearly demonstrate that ESCC is an estrogen-dependent human malignancy, as in other human cancers. ERβ might serve as a novel target molecule for ESCC patient therapy.

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Disclosure Statement

The authors have no conflict of interest.

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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

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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, Flughofstrase, 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 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). 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 Frasor *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% H_2O_2) 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×% of strongly positive cells, 2×% of moderately positive cells, 1×% weakly positive cells, and 0×% 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).

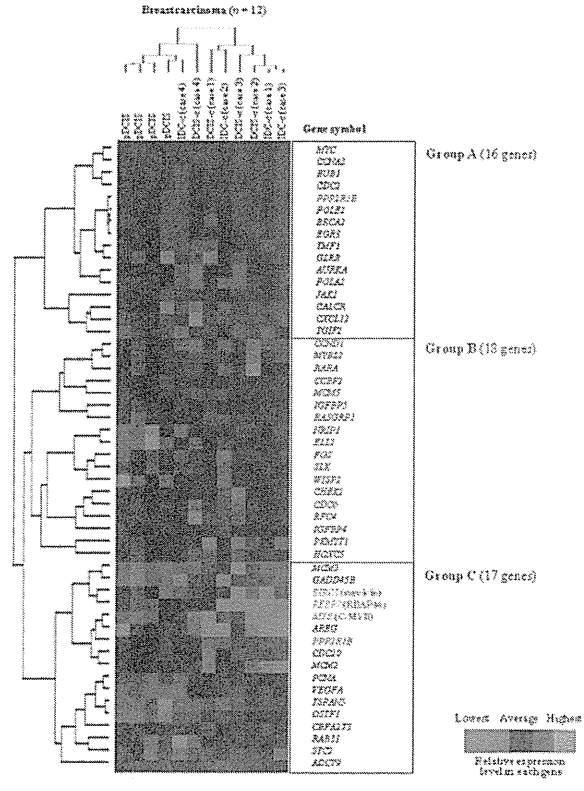

Figure 1 Hierarchical clustering analysis of mRNA expression levels focused on oestrogen-induced genes identified by Frasor *et al.* (2003). Colour of blocks represents relative mRNA expression level of each gene compared with the average in 12 breast carcinoma samples (four pDCIS, four DCIS-c and four 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: Group A ($n=15$)	Number of genes: Group C ($n=16$)	*P* value
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%)	
Growth factors, cytokines and hormones	1 (7%)	3 (19%)	
Receptors and signal transduction proteins	2 (13%)	5 (31%)	0.34
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-Aβ)) 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.
[a]Data are presented as the number of cases and percentage. All other values represent the median (min–max).

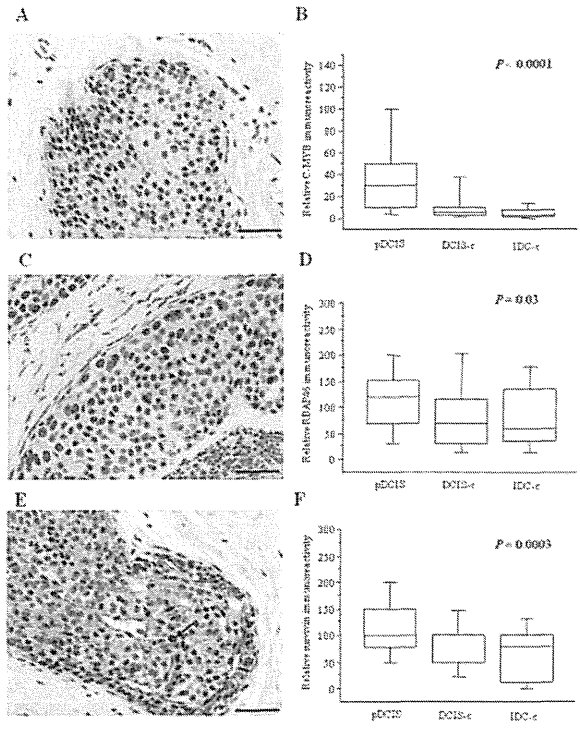

Figure 2 Immunohistochemistry for C-MYB (A and B), RBAP46 (C and D) and survivin (E and F) in the breast cancer cases. Immunoreactivity of C-MYB (A) and RBAP46 (C) was detected in nuclei of carcinoma cells in pDCIS. Survivin was immunolocalised in the cytoplasm of carcinoma cells in pDCIS and was also positive in some nuclei of the carcinoma cells (an arrow; E). Bar=50 μm 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, miroRNA-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

	C-MYB immunoreactivity		
Parameter	High (*n*=26)	Low (*n*=27)	*P* value
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.
[a]Data are presented as the number of cases and percentage. All other values represent the median (min–max).