Early growth responsive gene 3 in human breast carcinoma: a regulator of estrogen-mediated invasion and a potent prognostic factor

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Abstract

Early growth responsive gene 3 (EGR3) is a zinc-finger transcription factor and plays important roles in cellular growth and differentiation. We recently demonstrated estrogen-mediated induction of EGR3 in breast carcinoma cells. However, EGR3 has not yet been examined in breast carcinoma tissues and its significance remains unknown. Therefore, in this study, we examined biological functions of EGR3 in the breast carcinoma by immunohistochemistry, *in vitro* study, and nude mouse xenograft model. EGR3 immunoreactivity was detected in carcinoma cells in 99 (52%) out of 190 breast carcinoma tissues and was associated with the mRNA level. EGR3 immunoreactivity was positively associated with lymph node status, distant metastasis into other organs, estrogen receptor α , or EGR3 immunoreactivity in asynchronous recurrent lesions in the same patients, and was negatively correlated with tubule formation. EGR3 immunoreactivity was significantly associated with an increased risk of recurrence and adverse clinical outcome by both uni- and multivariate analyses. *Egr3*-expressing transformant cell lines derived from MCF-7 Tet-Off cells (Eg-10 and Eg-11) significantly enhanced the migration and invasion properties according to the treatment of doxycyclin, but did not significantly change the cell proliferation. Moreover, Eg-11 cells injected into athymic mice irregularly invaded into the adjacent peritumoral tissues, although Clt-7, which was stably transfected with empty vector as a control, demonstrated a well-circumscribed tumor. Eg-11 cells were significantly associated with invasive components and less tubule formation in the xenograft model. These results suggest that EGR3 plays an important role in estrogen-mediated invasion and is an independent prognostic factor in breast carcinoma.

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Introduction

Breast carcinoma is one of the most common malignancies in women worldwide. Human breast tissue is a target for estrogens, and these sex steroids play an important role in development of hormone-dependent breast carcinomas (Thomas 1984, Vihko & Apter 1989). The biological effects of estrogens are

mediated through an initial interaction with estrogen receptor (ER) α and/or β , members of a nuclear receptor superfamily (designated NR3A1 and NR3A2 respectively). ERs function as dimers, and activate transcription in a ligand-dependent manner by binding to estrogen responsive elements (EREs) located in the promoter region of various target genes (Tsai & O'Malley 1994). A variety of estrogenic functions

are characterized by the expression of these genes (Hayashi *et al.* 2003), and therefore, it is very important to examine the expression and roles of estrogen responsive genes to obtain a better understanding of estrogenic actions in human breast cancer.

Early growth responsive gene 3 (EGR3) belongs to the EGR family of zinc-finger transcription factors, and shares a common sequence termed the EGR responsive element with other members involved in DNA binding and transactivation (Patwardhan *et al.* 1991, O'Donovan *et al.* 1999). Previous studies revealed that EGR3 was involved in the development of muscle spindle (Tourtellotte & Milbrandt 1998, Tourtellotte *et al.* 2001) and thymocyte proliferation (Xi & Kersh 2004), indicating that EGR3 plays important roles in cellular growth and differentiation. We have recently demonstrated that EGR3 was induced by estradiol in MCF-7 breast carcinoma cells from the cDNA microarray analysis (Inoue *et al.* 2004). These findings suggest a possible role for EGR3 in estrogen-dependent human breast carcinomas. However, EGR3 has not been examined in human breast carcinoma tissues, and its biological and clinical significance remains unknown. Therefore, in this study, we examined biological functions of EGR3 in the breast carcinoma using immunohistochemistry, *in vitro* study, and nude mouse xenograft model. From these results, here, we first report that EGR3 is a regulator of estrogen-mediated invasion, and is a potent prognostic factor in human breast carcinomas.

Materials and methods

Patients and tissues

About 190 specimens of invasive ductal carcinoma of the breast were obtained from female patients who underwent mastectomy from 1984 to 1992 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. Breast tissue specimens were obtained from patients with a mean age of 53.5 years (range 22–82). The patients did not receive chemotherapy or irradiation prior to surgery. About 62 patients received tamoxifen therapy after the surgery. The mean follow-up time was 102 months (range 3–157 months). The histological grade and tubule formation of each specimen was evaluated based on the method of Elston & Ellis (1991). Asynchronous recurrent lesions of the breast carcinoma were also available for examination in 13 cases (breast, 3 cases; lymph node, 3 cases; skin, 2 cases; liver, 2 case; lung, 1 case; bone, 1 case; and chest wall, 1 case). All specimens were fixed in 10% formalin and embedded in paraffin wax.

Thirty-one specimens of invasive ductal carcinoma were obtained from patients who underwent mastectomy in 2000 in the Departments of Surgery at Tohoku University Hospital and Tohoku Kosai Hospital, Sendai, Japan. Specimens for RNA isolation were snap-frozen and stored at -80°C , and those for immunohistochemistry were fixed in 10% formalin and embedded in paraffin wax. Informed consent was obtained from all patients prior to their surgery and examination of specimens used in this study.

Research protocols for this study were approved by the Ethics Committee at both Tohoku University School of Medicine and Tohoku Kosai Hospital.

Antibodies

A rabbit polyclonal antibody for EGR3 (C-24 (sc-191)) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). This antibody was raised against a peptide mapping at the carboxy terminus of human *EGR3*. The EGR3 antibody specially recognized human EGR3 by immunoblotting and immunohistochemistry, and was non-cross-reactive with EGR1, EGR2, or Wilms' tumor proteins (data from Santa Cruz Biotechnology). Monoclonal antibodies for ER α (ER1D5), progesterone receptor (PR; MAB429), and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), and DAKO (Carpinteria, CA, USA) respectively. Rabbit polyclonal antibodies for ER β (06-629) and HER2 (A0485) were obtained from Upstate Biotechnology (Lake Placid, NY, USA) and DAKO respectively.

Immunohistochemistry

A Histofine Kit (Nichirei, 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°C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6.0)). Dilutions of primary antibodies used in this study were as follows: EGR3, 1/500; ER α , 1/50; ER β , 1/50; PR, 1/30; HER2, 1/200; and Ki-67, 1/50. The antigen–antibody complex was visualized 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. As a negative control, normal mouse or rabbit IgG was used instead of the primary antibodies. Immunohistochemical preabsorption test was also performed for EGR3 immunohistochemistry using the blocking peptide (sc-191 P; Santa Cruz Biotechnology).

Scoring of immunoreactivity and statistical analysis

EGR3, ER α , ER β , PR, and Ki-67 immunoreactivity was detected in the nucleus, and the immunoreactivity was evaluated in more than 1000 carcinoma cells for each case, and subsequently the percentage of immunoreactivity, i.e. labeling index (LI), was determined. Cases with EGR3 or ER α LI of more than 10% were considered EGR3- or ER α -positive breast carcinomas in this study, according to a report on ER α (Goldhirsch *et al.* 2005).

An association between EGR3 immunoreactivity and clinicopathological factors was evaluated using a Student's *t*-test, cross-table using the χ^2 -test, or correlation coefficient (*r*) and regression equation. Overall and disease-free survival curves were generated according to the Kaplan–Meier method and the statistical significance was calculated using the log-rank test. Uni- and multivariate analyses were evaluated by a Cox's proportional hazard model using PROC PHREG in our SAS software.

Cells and chemicals

MCF-7 human breast cancer cell line and LY-2, which is a tamoxifen-resistant MCF-7 cell variant (Paik *et al.* 1994), were cultured in RPMI-1640 (Sigma–Aldrich) with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA). We also used Eg-10 and Eg-11 cells which are *Egr3*-expressing transformants derived from MCF-7 Tet-Off cells (Inoue *et al.* 2004), and Ctl-7 cells which are MCF-7 Tet-Off cells stably transfected with empty vector (Inoue *et al.* 2004). Overexpression of *Egr3* in Eg-10 and Eg-11 cells was dramatically repressed by the treatment of doxycyclin (50 ng/ml; Inoue *et al.* 2004). These cells were also cultured in RPMI-1640 (Sigma–Aldrich) with 10% FBS. All the cells used in this study were cultured with phenol red-free RPMI-1640 medium containing 10% dextran-coated charcoal–FBS for 3 days before treatment of the experiment. Estradiol and tamoxifen were purchased from Sigma–Aldrich, while ICI 182 780 was obtained from Tocris Cookson Inc. (Ellisville, MO, USA).

Real-time PCR

Total RNA was extracted from breast carcinoma tissues or cultured cells using TRIzol reagent (Invitrogen Life Technologies), and a reverse transcription kit (Superscript II Preamplification system; Gibco-BRL) was used in the synthesis of cDNA.

The LightCycler System (Roche Diagnostics GmbH) was used to semi-quantify the mRNA expression levels by real-time PCR (Dumoulin *et al.* 2000). Settings for the PCR thermal profile were as follows: initial denaturation at 95 °C for 1 min followed by 40 amplification cycles of 95 °C for 1 s, annealing at 68 °C (EGR3 and ribosomal protein L 13a (RPL13A)) for 15 s, and elongation at 72 °C for 15 s. Oligonucleotide primers for EGR3 (NM_004430) were designed in different exons to avoid the amplification of genomic DNA, and the primer sequences were FWD: 5'-CTGCCTGACAATCTGTACCC-3' (cDNA position; 416–435) and REV: 5'-GTAGGT-CACGGTCTTGTTGC-3' (cDNA position; 594–613). The primer sequences for RPL13A (NM_012423) were FWD: 5'-CCTGGAGGAGAAGAGGAAAGAGA-3' (cDNA position; 487–509) and REV: 5'-TTGAG-GACCTCTGTGTATTTGTCAA-3' (cDNA position; 588–612; Vandesompele *et al.* 2002). To verify amplification of the correct sequences, PCR products were purified and subjected to direct sequencing. Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA. EGR3 mRNA level was summarized as the ratio of RPL13A mRNA level (%).

Immunoblotting

The cell protein was extracted in triple detergent lysis buffer (LK-18) at 4 °C. About 25 μ g (immunoblotting for EGR3) or 5 μ g (immunoblotting for β -actin) of the protein (whole cell extracts) were subjected to SDS–PAGE (10% acrylamide gel). Following SDS–PAGE, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (Amersham Biosciences). The blots were blocked in 5% nonfat dry skim milk for 1 h at room temperature, and were then incubated with a primary antibody for EGR3 (C-24 (sc-191), Santa Cruz Biotechnology) or β -actin (AC-15 (Sigma #A-5411), Sigma–Aldrich) for 18 h at 4 °C. After incubation with anti-rabbit or anti-mouse IgG horseradish peroxidase (Amersham Biosciences) for 1 h at room temperature, antibody/protein complexes on the blots were detected using ECL plus western blotting detection reagents (Amersham Biosciences). Immunointensity of specific bands was measured by LAS-1000 imaging system (Fuji Photo Film, Tokyo, Japan). Immunointensity of EGR3 in each sample was normalized to that of β -actin, and subsequently, relative immunointensity ratio of EGR3 was summarized as a ratio compared with that of MCF-7 cells in the absence of estradiol or tamoxifen.

Migration assay and invasion assay

Cell migration assay was performed using a 24-well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ, USA) and Chemotaxicell (8 µm pore size; Kurabo, Osaka, Japan). The membrane of Chemotaxicell was coated with 0.3 mg/ml of collagen I (CELLGEN, Tokyo, Japan). After 3 days of the treatment with or without doxycyclin (50 ng/ml) in serum-free RPMI-1640 medium, 5×10^5 cells were plated at the upper chamber, while NIH/3T3 conditioned medium was in the lower chamber. After incubation for 6 h at 37 °C, cells on the upper surface of membrane were removed by wiping with a cotton swab, and those on the lower surface were subsequently fixed with 70% ethanol and stained with hematoxylin and eosin. The migration ability was evaluated as a total number of cells on the lower surface of membrane, which was counted under microscopy.

The cell invasion assay was performed by a modified migration assay. In this experiment, upper surface of the membrane of Chemotaxicell was coated with 80 µg/cm² of Matrigel basement membrane matrix (BD Biosciences, Two Oak Park, MA, USA; Albini et al. 1987, Taniguchi et al. 1989). About 5×10^5 cells at the upper chamber were incubated with 24 h at 37 °C, and the invasion ability was subsequently evaluated as the total number of cells on the lower surface of membrane.

Cell proliferation assay and apoptosis analysis

The status of cell proliferation of cells was measured using a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) method (Cell Counting Kit-8; Dojindo, Kumamoto, Japan). The apoptotic status of cells was evaluated by an apoptosis screening kit (Wako, Osaka, Japan), which employed a modified TdT-mediated dUTP nick-end labeling (TUNEL) method. Optical densities (OD=450 nm for cell proliferation assay and OD=490 nm for apoptosis analysis) were obtained with a Model 680 microplate reader (Bio-Rad Laboratories). The cell number and apoptosis index were calculated according to the following equation: (cell OD value after test materials treated/vehicle control cell OD value), and subsequently evaluated as a ratio (%) compared with that at 0 day after the treatment.

Athymic mouse xenograft model

Eg-11 and Ctl-7 cells were resuspended in phenol-red free Matrigel (Becton Dickinson; 1×10^7 (0.1 ml)/site)

and placed on superior side of BALB/c-nu/nu athymic female mice (5 weeks of age; Charles River Laboratories, Tokyo, Japan). Tumor tissues were resected after 2 months, and were subsequently fixed in 10% formalin and embedded in paraffin wax.

Results

Immunohistochemistry for EGR3 in breast carcinoma tissues

Immunoreactivity for EGR3 was detected in the nuclei of invasive ductal carcinoma cells (Fig. 1A). A mean value of EGR3 LI in the 190 breast carcinoma tissues examined was 19.1% (range 0–96%), and a number of EGR3-positive breast carcinomas (i.e. EGR3 LI of more than 10%) were 99 out of 190 cases (52%). EGR3 immunoreactivity was weakly and focally detected in epithelial cells of morphologically normal glands (Fig. 1B). Immunohistochemical preabsorption test for EGR3 demonstrated no specific immunoreactivity in a negative control (Fig. 1C). We also examined mRNA expression of EGR3 in 31 cases of invasive ductal carcinoma tissues using real-time PCR. EGR3 mRNA expression level was significantly ($P=0.003$, $r=0.52$) correlated with the EGR3 immunoreactivity in these cases examined (Fig. 1D).

Associations between EGR3 immunoreactivity and clinicopathological parameters in 190 breast carcinomas were summarized in Table 1. EGR3-positive breast carcinoma was significantly associated with synchronous lymph node status ($P=0.002$), distant metastasis into other organs ($P=0.02$), ERα status ($P=0.01$), ERα LI ($P=0.02$), or EGR3 immunoreactivity in asynchronous recurrent lesions in the same patients ($P=0.03$). On the other hand, a negative correlation was detected between EGR3 immunoreactivity and tubule formation ($P=0.01$). In this study, there were no significant correlations between EGR3 immunoreactivity and other clinicopathological parameters, including the patient age, menopausal status, clinical stage, tumor size, histological grade, ERβ LI, PR LI, HER2 status, and Ki-67 LI. Similar tendency was detected when EGR3 immunoreactivity was evaluated as a continuous variable (i.e. LI; Table 1).

Correlation between EGR3 immunoreactivity and clinical outcome of the breast carcinoma patients

In order to examine an association between EGR3 immunoreactivity and prognosis precisely, we excluded stage IV cases and used stages I to III breast carcinoma patients ($n=169$) in the following analyses. EGR3 immunoreactivity was significantly associated with an

Figure 1 Immunohistochemistry for EGR3 in the invasive ductal carcinoma. (A) EGR3 immunoreactivity was detected in the nuclei of the carcinoma cells. (B) In morphologically normal mammary glands, immunoreactivity for EGR3 was focally and weakly detected in the nuclei of epithelial cells. (C) No significant immunoreactivity of EGR3 was detected in the sections of breast carcinomas in immunohistochemical preabsorption test as a negative control. Bar = 100 μ m. (D) Association between the mRNA level and relative immunoreactivity (LI) of EGR3 in 31 breast carcinoma tissues. Significant positive association was detected ($P=0.0029$, $r=0.517$). EGR3 mRNA level was summarized as the ratio of RPL13A mRNA level (%). Statistical analysis was performed utilizing a correlation coefficient (r) and regression equation.

increased risk of recurrence (Fig. 2A; $P=0.004$ in the log-rank test). Following univariate analysis by COX (Table 2), lymph node status ($P<0.0001$), EGR3 immunoreactivity ($P=0.01$), HER2 status ($P=0.01$), and tumor size ($P=0.04$) were demonstrated significant prognostic parameters for disease-free survival in 169 breast carcinoma patients. A multivariate analysis (Table 2) revealed that only lymph node status ($P=0.0002$) and EGR3 immunoreactivity ($P=0.01$) were independent prognostic factors with relative risks over 1.0.

Similar tendency was detected when EGR3 immunoreactivity was further categorized into three groups (0–9, 10–49, and 50–100% of positive cells; $P=0.01$ in both uni- and multivariate analyses), EGR3 immunoreactivity was evaluated as a continuous variable ($P=0.003$ in both uni- and multivariate analyses), or EGR3 immunoreactivity was evaluated in all the cases from stages I to IV ($n=190$; $P=0.0002$ in univariate analysis and $P=0.002$ in multivariate analysis).

Overall survival curve was demonstrated in Fig. 2B, and a significant correlation was detected between EGR3 immunoreactivity and adverse clinical outcome

of the patients ($P=0.01$ in the log-rank test). Utilizing a univariate analysis (Table 3), lymph node status ($P<0.0001$), histological grade ($P=0.003$), HER2 status ($P=0.004$), EGR3 immunoreactivity ($P=0.01$), and tumor size ($P=0.02$) turned out to be significant prognostic factors for overall survival in this study. Multivariate analysis revealed that lymph node status ($P=0.001$), EGR3 immunoreactivity ($P=0.01$), and histological grade ($P=0.03$) were independent prognostic factors with a relative risk over 1.0, but other factors were not significant in this study (Table 3).

Similar tendency was detected when EGR3 immunoreactivity was categorized into the three groups ($P=0.002$ in both uni- and multivariate analyses), EGR3 immunoreactivity was evaluated as a continuous variable ($P=0.002$ in univariate analysis and $P=0.001$ in multivariate analysis), or EGR3 immunoreactivity was evaluated in stage I to IV cases ($n=190$; $P=0.0003$ in univariate analysis and $P=0.001$ in multivariate analysis).

An association between EGR3 immunoreactivity and clinical outcome of the patients was similar regardless of the ER α status in this study (Fig. 2C and D). About 48 out of 169 patients received tamoxifen therapy after surgery,

Table 1 Association between Early growth responsive gene 3 (*EGR3*) immunoreactivity and clinicopathological parameters in 190 breast carcinomas

	EGR3 immunoreactivity		<i>P</i> value	EGR3 LI	<i>P</i> value
	+(<i>n</i> =99)	−(<i>n</i> =91)			
Age (years)	52.3±1.2	54.4±1.2	0.23		0.59
Menopausal status					
Premenopausal	46 (24%)	38 (20%)		19.1±2.3	
Postmenopausal	53 (28%)	53 (28%)	0.61	19.2±2.2	0.96
Stage					
I	18 (10%)	26 (14%)		15.8±2.8	
II	56 (30%)	51 (27%)		18.8±2.1	
III	9 (5%)	9 (5%)		16.1±5.2	
IV	16 (8%)	5 (3%)	0.07	30.6±6.4	0.07
Tumor size (mm)	34.6±2.7	30.0±3.4	0.29		0.20
Lymph node status					
Positive	54 (28%)	29 (15%)		24.6±2.8	
Negative	45 (24%)	62 (33%)	0.002	14.9±1.7	0.003
Distant metastasis					
Positive	16 (8%)	5 (3%)		30.6±6.4	
Negative	83 (44%)	86 (45%)	0.02	17.7±1.6	0.01
Histological grade					
1 (well)	26 (14%)	24 (13%)		19.7±3.1	
2 (moderate)	40 (21%)	31 (16%)		21.5±2.7	
3 (poor)	33 (17%)	36 (19%)	0.60	16.3±2.6	0.48
Tubule formation					
1 (>75%)	12 (6%)	22 (12%)		13.2±2.9	
2 (10–75%)	23 (12%)	29 (15%)		14.9±2.5	
3 (<10%)	64 (34%)	40 (21%)	0.01	23.2±2.4	0.20
ERα status					
Positive	78 (41%)	57 (30%)		22.0±2.0	
Negative	21 (11%)	34 (18%)	0.01	12.2±2.3	0.01
ERα LI (%)	47.9±3.4	37.3±3.7	0.02	(<i>r</i> =0.23)	0.001
ERβ LI (%)	16.2±2.3	15.2±2.1	0.82		0.37
PR LI (%)	42.9±3.3	36.9±3.6	0.23		0.33
HER2 status					
Positive	29 (15%)	28 (15%)		15.4±2.5	
Negative	70 (37%)	63 (33%)	0.82	20.8±2.0	0.23
Ki-67 LI (%)	24.8±1.8	24.4±1.9	0.89		0.93
EGR3 immunoreac- tivity in recurrent lesions (<i>n</i> =13)				(<i>r</i> =0.65)	0.02
Positive	9 (69%)	1 (8%)			
Negative	1 (8%)	2 (15%)	0.03		

Data are presented as mean ± 95% confidence interval (95% CI) or the number of cases with percentage. *P* values < 0.05 were considered significant, and described as boldface.

and these cases were ERα-positive breast cancers. The disease-free and overall survival curves in these patients were summarized in Fig. 2E and F respectively. EGR3 immunoreactivity was also associated with an increased risk of recurrence and worse prognosis in the group of breast cancer patients who received tamoxifen therapy (*P*=0.01 and 0.03 in the log-rank test respectively). An association between EGR3 immunoreactivity and clinical outcome of the patients was not significantly changed regardless of the status of adjuvant chemotherapy after surgery in this study (data not shown).

Estrogen-mediated expression of EGR3 in MCF-7 breast carcinoma cells

As shown in Fig. 3A, EGR3 mRNA expression was induced by estradiol in a dose-dependent manner in MCF-7 cells. This induction became significant from 1 nM estradiol (*P*<0.001 versus control (non-treatment with estradiol)), and EGR3 mRNA level of MCF-7 cells treated with 10 nM estradiol (100.0±2.0%) was 14-fold increased when compared with the control level (7.2±2.0%). The estradiol-mediated mRNA expression of EGR3 was suppressed by addition of

Figure 2 Disease-free and overall survival of 169 patients with breast carcinoma according to EGR3 immunoreactivity (Kaplan–Meier method). (A and B) EGR3 immunoreactivity was significantly associated with an increased risk of recurrence ($P=0.0036$, log-rank test) (A) and worse prognosis ($P=0.0090$, log-rank test) (B). (C and D) EGR3 immunoreactivity was significantly correlated with a risk of recurrence regardless of the ER α status ($P=0.0154$ in ER α -positive cases (C), and $P=0.0348$ in ER α -negative cases (D)). (E and F) EGR3 immunoreactivity was significantly associated with an increased risk of recurrence ($P=0.0123$) (E) and worse prognosis ($P=0.0316$) (F) in the 48 patients received tamoxifen therapy.

tamoxifen in a dose-dependent manner (Fig. 3B). EGR3 mRNA level in MCF-7 cells treated with 10 nM estradiol and 10 μ M tamoxifen ($21.8 \pm 6.2\%$) was decreased into 22% of that treated with 10 nM estradiol alone ($100.0 \pm 2.0\%$), but its level remained significantly higher ($P<0.001$ and threefold) than the control level (neither estradiol nor tamoxifen; $7.2 \pm 2.0\%$). Tamoxifen (10 μ M) alone did not significantly change the EGR3 mRNA level in MCF-7 cells.

Estradiol also induced EGR3 mRNA expression in LY-2 cells, a tamoxifen-resistant MCF-7 cell variant, in a dose-dependent manner (Fig. 3C), which was

Table 2 Uni- and multivariate analyses of disease-free survival in stages I–III breast cancer patients examined ($n=169$)

Variable	Uni- variate	Multivariate	
	<i>P</i>	<i>P</i>	Relative risk (95% CI)
Lymph node status (positive/negative)	<0.0001	0.0002	3.8 (1.9–7.7)
EGR3 immunoreactivity (positive/negative)	0.01	0.01	2.5 (1.3–4.8)
HER2 status (positive/negative)	0.01	0.08	
Tumor size (≥ 20 mm/ < 20 mm)	0.04	0.48	
Ki-67 LI (≥ 10 / < 10)	0.15		
Adjuvant chemotherapy (no/yes)	0.34		
ER α status (negative/positive)	0.44		
Tamoxifen therapy (no/yes)	0.46		
Histological grade (3/1, 2)	0.78		

Data considered significant ($P<0.05$) in the univariate analyses were described as boldface, and were examined in the multivariate analyses.

significant from 10 pM estradiol ($P<0.05$ versus the control level). The level of EGR3 mRNA in LY-2 cells treated with 10 nM estradiol ($1245 \pm 222\%$) was 31-fold higher than the control level ($39.7 \pm 14.2\%$), and was 12-fold higher than that in MCF-7 cells treated

Table 3 Uni- and multivariate analyses of overall survival in stages I–III breast cancer patients examined ($n=169$)

Variable	Uni- variate	Multivariate	
	<i>P</i>	<i>P</i>	Relative risk (95% CI)
Lymph node status (positive/negative)	<0.0001	0.001	6.0 (2.2–16.5)
Histological grade (3/1, 2)	0.003	0.03	2.5 (1.1–4.7)
HER2 status (positive/ negative)	0.004	0.38	
EGR3 immunoreactivity (positive/negative)	0.01	0.01	3.0 (1.3–7.0)
Tumor size (≥ 20 mm/ < 20 mm)	0.02	0.28	
Ki-67 LI (≥ 10 / < 10)	0.11		
Adjuvant chemotherapy (no/yes)	0.21		
Tamoxifen therapy (no/yes)	0.37		
ER α status (negative/ positive)	0.42		

Data considered significant ($P<0.05$) in the univariate analyses were described as boldface and were examined in the multivariate analyses.

Figure 3 Induction of EGR3 expression by estradiol in MCF-7 cells. (A and C) MCF-7 (A) or LY-2 (C) cells were treated with indicated concentrations of estradiol for 3 days, and the EGR3 mRNA was evaluated as the ratio of RPL13A mRNA level (%). (B and D) MCF-7 (B) or LY-2 (D) cells were treated with estradiol (10 nM) with indicated concentrations of tamoxifen for 3 days, and the EGR3 mRNA was evaluated as the ratio of RPL13A mRNA level (%). Data are presented as mean $\pm$ s.d. ($n=4$). * $P<0.05$ and *** $P<0.001$ versus control (no treatment with estradiol or tamoxifen for 3 days; left column) respectively. The statistical analyses were performed using a one-way ANOVA and Bonferroni test. (E and F) Immunoblotting for EGR3 in MCF-7 (E) and LY-2 (F) cells. Cells were treated with estradiol (10 nM) and/or tamoxifen (10 μ M) for 3 days. Amount of protein loaded in each lane was 25 μ g (immunoblotting for EGR3) or 5 μ g (immunoblotting for β -actin). Immunointensity of EGR3 in each sample was normalized to that of β -actin, and relative immunointensity ratio of EGR3 was summarized as a ratio compared with that of MCF-7 cells treated without estradiol or tamoxifen (left lane in E).

under the same condition. Tamoxifen dose-dependently suppressed the estradiol-mediated mRNA expression of EGR3 in LY-2 cells (Fig. 3D). EGR3 mRNA level in LY-2 cells treated with 10 nM estradiol and 10 μ M tamoxifen ($278 \pm 35.0\%$) was decreased into 22% of that treated with 10 nM estradiol alone ($1,245 \pm 222\%$), but was still significantly higher ($P<0.001$ and sevenfold) than the control level ($39.7 \pm 14.2\%$). Tamoxifen (10 μ M) did not significantly change the EGR3 mRNA level also in LY-2 cells. Similar tendency was detected at EGR3 protein levels both in MCF-7 and LY-2 cells by immunoblotting analyses (Fig. 3E and F).

Pure anti-estrogen, ICI 182 780, alone did not significantly change EGR3 mRNA level in MCF-7 cells (Fig. 4A). EGR3 mRNA level was slightly increased by addition of estradiol in MCF-7 cells under the treatment with 10 nM ICI 182 780, but no significant association was detected (2.3-fold and $P=0.06$, 10 nM estradiol versus control (Fig. 4B; non-treatment with estradiol).

On the other hand, as shown in Fig. 4C, ICI 182 780 alone significantly inhibited the EGR3 mRNA level of LY-2 cells in a dose-dependent manner, and the EGR3 mRNA level of LY-2 cells treated with 10 μ M ICI 182 780 was decreased into 21% ($7.8 \pm 3.6\%$, $P<0.001$) of the basal level (non-treatment with ICI 182 780; $37.8 \pm 11.8\%$). When LY-2 cells were treated with 10 nM ICI 182 780, the EGR3 mRNA level was significantly induced by estradiol in a dose-dependent manner (43-fold and $P<0.001$, 10 nM estradiol versus control (Fig. 4D; non-treatment with estradiol).

Estradiol (10 nM) did not significantly induce the EGR3 mRNA expression when MCF-7 or LY-2 cells were treated with 10 μ M ICI 182 780 (0.9-fold and $P=0.83$ in MCF-7, and 1.2-fold and $P=0.77$ in LY-2).

Figure 4 Effects of ICI 182 780 on EGR3 mRNA expression in MCF-7 cells. (A and C) MCF-7 (A) or LY-2 (C) cells were treated with indicated concentrations of ICI 182 780 for 3 days in the absence of exogenous estradiol. (B and D) MCF-7 (B) or LY-2 (D) cells were treated with indicated concentrations of estradiol and ICI 182 780 (10 nM) for 3 days. Data are presented as mean $\pm$ s.d. ($n=4$). * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ versus control (non-treatment with ICI 182 780 (A and C) or 10 nM ICI 182 780 in the absence of estradiol (B and D) for 3 days (left column in each figure)). The statistical analyses were performed using a one-way ANOVA and Bonferroni test.

Increased invasion properties in *Egr3*-expressing MCF-7 Tet-Off cells

In order to further characterize the biological functions of EGR3 in breast carcinoma cells, we then employed Eg-10 and Eg-11 *Egr3*-expressing transformants derived from MCF-7 Tet-Off cells (Inoue *et al.* 2004). EGR3 mRNA levels of these transformants were 57 and 540% in Eg-10 and Eg-11 respectively. As a control, we used Ctl-7, which was stably

transfected with empty vector in the MCF-7 Tet-Off cells (Inoue *et al.* 2004), and the EGR3 mRNA level was negligible ($2.2 \times 10^{-3}\%$). In the immunohistochemistry, EGR3 immunoreactivity was detected in the nuclei of Eg-10 and Eg-11 cells (Fig. 5A), but not in Ctl-7 cells (Fig. 5B). The EGR3 mRNA levels decreased into negligible levels in both Eg-10 ($5.0 \times 10^{-3}\%$) and Eg-11 ($1.4 \times 10^{-2}\%$) cells, when these cells were treated with doxycyclin (50 ng/ml) for 3 days.

Figure 5 (A and B) Immunohistochemistry for EGR3 in *Egr3*-expressing MCF-7 Tet-Off cells. Immunoreactivity of EGR3 was detected in the nuclei of Eg-11 cells (A), but not in Ctl-7 cells (B). Immunohistochemistry was performed using cell blocks from formalin-fixed and paraffin-embedded specimens. Bar = 100 μ m. (C–F) Migration assay (C), invasion assay (D), cell proliferation assay (E), and apoptosis analysis (F) in *Egr3*-expressing cells. Eg-10, Eg-11, and Ctl-7 cells were incubated with 6 h, 24 h, 3 days, and 3 days at 37 °C respectively, without or with the treatment of doxycyclin (50 ng/ml). A total number of cells on the lower surface of membrane of Chemotaxicell was counted in the migration and invasion assays (C and D). While, the cell number and apoptosis index were calculated and evaluated as a ratio (%) compared with that at 0 day after the treatment in the cell proliferation assay and apoptosis analysis (E and F). Data are presented as mean $\pm$ s.d. ($n=4$). An open bar represents the value of cells treated without doxycyclin, and a closed bar shows that under the treatment with doxycyclin. The statistical analyses were performed between the values in the absence and presence of doxycyclin using a one-way ANOVA and Bonferroni test, and $**P<0.01$ and $***P<0.001$ respectively.

Figure 5C shows the result of migration assay in *Egr3*-expressing MCF-7 cells. The number of migrated cells was significantly higher in Eg-10 ($P<0.001$) and Eg-11 ($P<0.01$) when compared with that in the treatment with doxycyclin. However, a number of migrated Ctl-7 cells were not significantly altered between the absence and presence of doxycyclin. Moreover, the number of invaded cells was significantly higher in Eg-10 and Eg-11 ($P<0.001$) than that under the treatment with doxycyclin, and it was 3.3-fold higher in Eg-10 and 3.7-fold in Eg-11 (Fig. 5D). Invasion property was not significantly altered in Ctl-7 cells according to the treatment with doxycyclin.

Cell proliferation (Fig. 5E) and apoptosis index (Fig. 5F) of these three cells were not significantly altered between the absence and presence of doxycyclin for 3 days.

Morphological features of *Egr3*-expressing MCF-7 cells in athymic mice xenograft model

In order to study the biological roles of *EGR3* in breast carcinoma cells *in vivo*, we injected Eg-11 and Ctl-7 cells into female nude mice, and the tumor tissues were resected after 2 months. As shown in Fig. 6A and B,

Ctl-7 showed a well-circumscribed tumor, and tubule formation was remarkable in the carcinoma tissue. Invasion into the surrounding tissue was not detected in any of the cases examined. On the other hand, Eg-11 cells arranged in clusters and trabeculae with focal glandular formation, and irregularly invaded into the adjacent peritumoral tissue such as adipose tissue in all of the cases examined (Fig. 6C and D). As shown in Table 4, Eg-11 carcinoma tissues significantly demonstrated invasion ($P=0.01$) and less tubule formation ($P=0.03$) when compared with the Ctl-7 carcinoma tissues. However, tumor volume, largest dimension histologically determined, and Ki-67 LI of the tumor were not significantly different between these two types of carcinoma. The tumor volumes of Eg-11 and Ctl-7 were monitored weekly, but no significant changes were detected when compared with the original volume (data not shown).

Discussion

In this study, *EGR3* immunoreactivity was closely correlated with the mRNA level, and was significantly associated with the ER α status, but not with ER β , in the breast carcinoma tissues. In the previous studies using cDNA microarray, *EGR3* mRNA was significantly

Figure 6 Histological features of *Egr3*-expressing MCF-7 cells in athymic mouse xenograft model. (A and B) Tubule formation was remarkable in the Ctl-7 carcinoma and no invasive components were detected. (C and D) Eg-11 carcinoma cells mainly arranged in clusters and trabeculae, and frequently invaded into a tissue which surrounded the primary carcinoma lesion (arrows). (D) shows invasive components of Eg-11 cells in the adipose tissue adjacent to the primary carcinoma site. Hematoxylin and eosin stain. Tumor tissues were resected at 2 months after the injection, and were fixed in 10% formalin and embedded in paraffin wax. Bar = 100 μ m.

Table 4 Histological features of Early growth responsive gene 3 (*Egr3*)-expressing MCF-7 cells injected into athymic mice

	Eg-11 (n=4)	Ctl-7 (n=4)	P value
Tumor volume ^a (mm ³)	399 ± 101	357 ± 74	0.75
Histologically determined largest dimension ^a (mm)	4.8 ± 0.6	5.1 ± 0.4	0.61
Tubule formation			
1 (>75%)	0 (7%)	1 (13%)	0.03
2 (10–75%)	0 (11%)	3 (15%)	
3 (<10%)	4 (31%)	0 (23%)	
Invasive lesions			
Present	4 (100%)	0 (0%)	0.01
Absent	0 (0%)	4 (100%)	
Ki-67 LI of the tumor ^a (%)	54.5 ± 4.6	49.5 ± 7.1	0.58

Tumor tissues were resected at 2 months after the injection, and were subsequently fixed in 10% formalin and embedded in paraffin wax. Tumor volume was evaluated by a formula for a semiellipsoid ($4/3\pi r^3/2$). *P* values <0.05 were considered significant and described as boldface.

^aData are presented as mean ± s.d. All other values represent the number of cases and percentage.

(the relative value of more than 2.0) induced by estradiol in various carcinoma cell lines derived from breast (MCF-7 and MCF-7 c9), endometrium (Ishikawa), ovary (SK-OV-3), and stomach (MKN-28; Inoue *et al.* 2002, 2004, Hayashi *et al.* 2003). Induction of EGR3 mRNA was detected at 6 h after estradiol treatment (10 nM) and reached the maximal level at 24–72 h in MCF-7 cells by northern blot analysis (Inoue *et al.* 2004), and ERE sequence was identified at 2.3 kb from the most upstream mRNA 5' end of *Egr3* (Bourdeau *et al.* 2004). The biological estrogenic actions are mainly mediated through ER α (Korach 1994, Hayashi *et al.* 2003), and MCF-7 cells highly express ER α but low level of ER β (Vladusic *et al.* 2000). Therefore, results from our present study suggest that EGR3 is expressed in the breast carcinoma cells, mainly through ER α , as a result of estrogenic action.

We also found that 21 out of 55 cases were immunopositive for EGR3 in breast carcinoma tissues negative for ER α (LI of <10%). This is partly because EGR3 expression was induced by a low or undetectable level of ER α . However, EGR3 was also reported to be induced by various factors, including mitogenic stimulation (Patwardhan *et al.* 1991, O'Donovan *et al.* 1998, Mercier *et al.* 2001, Jouvert *et al.* 2002). Therefore, factors other than estrogen may also be partly involved in the regulation of EGR3 expression in some breast carcinomas.

In our study, EGR3 immunoreactivity was inversely associated with tubule formation, and positively correlated with metastatic lesions of lymph nodes or other organs in the breast carcinomas. Moreover, overexpression of *Egr3* significantly enhanced invasion properties in MCF-7

cells in both *in vitro* study and nude mouse xenograft model. Therefore, EGR3 is postulated to play a pivotal role in carcinoma cell invasion mediated by estrogens in breast carcinomas. Metastasis is the major cause of treatment failure and death of carcinoma patients, and it is a multi-step process that involves not only invasion of carcinoma cells but also lymphogenous and/or hematogenous spread and cell proliferation in the metastatic sites. In our present study, EGR3 immunoreactivity was not associated with tumor size or Ki-67 LI in the breast carcinoma tissues, and overexpression of *Egr3* was not necessarily involved in the cell proliferation or apoptosis status in MCF-7 cells. Therefore, co-operation with EGR3 and other factors may be required for the metastasis of ER-positive breast carcinoma. It awaits further examinations for the detailed clarification of estrogen-mediated metastatic process, because biological function of a great majority of estrogen-responsive genes currently remains unclear. However, for instance, cyclin D (Steege & Zhou 1998) and estrogen-responsive finger protein (Efp; Urano *et al.* 2002, Suzuki *et al.* 2005b) were shown to induce the estrogen-mediated proliferation in breast carcinoma cells, and histone deacetylase (HDAC) 6 were reported as a regulator of cell motility in ER-positive breast carcinoma cells (Saji *et al.* 2005).

Both uni- and multivariate analyses in our study have demonstrated that EGR3 immunoreactivity is a potent prognostic factor for both the recurrence and overall survival in breast carcinoma patients, and similar tendency was also detected in the patients who received tamoxifen therapy. Estradiol is well known to be locally produced and act in breast carcinomas

regardless of the menopausal status (Suzuki et al. 2005a). In the present *in vitro* experiments, tamoxifen suppressed estradiol-mediated expression of EGR3 mRNA in a dose-dependent manner, but the EGR3 mRNA level in MCF-7 cells treated with estradiol and 10 μ M tamoxifen was significantly higher than the control level. Optimal concentrations of tamoxifen were generally considered 10 nM to 10 μ M in *in vitro* studies (Vendrell et al. 2005), and serum concentration of tamoxifen was reported at 1.8 μ M in patients who received high-dose tamoxifen (320 mg), nevertheless 20 mg tamoxifen is usually administered in breast carcinoma patients. Therefore, tamoxifen may not completely block the estradiol-mediated EGR3 expression in the breast carcinoma patients.

Regarding the molecular mechanism leading to tamoxifen resistance, recent studies demonstrated that breast carcinoma cells adapt by changing their response to estradiol and developing an increased sensitivity to the growth-stimulating action (Martin et al. 2003, Berstein et al. 2004, Santen et al. 2004). These processes are called 'hypersensitivity to estradiol', and the potential association with increased concentrations of ER α and ER-mediated events is proposed (Santen et al. 2001, Chan et al. 2002, Vendrell et al. 2005). In this study, EGR3 mRNA level in LY-2 cells was 5.5-fold higher than that in MCF-7 cells in the absence of exogenous estradiol, but it was dose-dependently decreased by ICI 182 780. In addition, LY-2 cells showed marked amplitude of estradiol-mediated EGR3 mRNA expression when compared with MCF-7 cells. Therefore, it is suggested that EGR3 expression is mainly mediated through ER in LY-2 cells, and these findings of our present study are possibly explained by the hypersensitivity to estradiol in tamoxifen-resistant state of MCF-7 cells. Considering that the EGR3 mRNA level in LY-2 cells treated with estradiol and 10 μ M tamoxifen was 2.8-fold higher than that in MCF-7 cells treated with estradiol alone, EGR3 may play an important role also in the tamoxifen-resistant breast carcinoma patients. Therefore, residual carcinoma cells following surgical treatment in EGR3-positive breast carcinomas could rapidly invade in the presence of local estrogens regardless of the tamoxifen therapy, thereby resulting in an increased recurrence and poor prognosis in these patients.

In summary, EGR3 immunoreactivity was detected in carcinoma cells in 52% of breast carcinoma tissues in this study, and it was associated with its mRNA level. EGR3 immunoreactivity was positively associated with lymph node status, distant metastasis into

other organs, ER α , or EGR3 immunoreactivity in the recurrent lesions, and negatively correlated with tubule formation. EGR3 immunoreactivity was significantly associated with an increased risk of recurrence or worse prognosis, regardless of the tamoxifen therapy. Estradiol significantly induced EGR3 mRNA expression in a dose-dependent manner in MCF-7 cells, which was markedly amplified in a tamoxifen-resistant MCF-7 cell variant (LY-2). Tamoxifen suppressed the estradiol-mediated induction of EGR3 mRNA in a dose-dependent manner in these cells, but tamoxifen could not inhibit its expression completely. *Egr3*-expressing MCF-7 cells significantly increased the invasion property, but not cell proliferation, both *in vitro* and *in vivo* experiments. These results from our present study suggest that EGR3 plays an important role in estrogen-mediated invasion and is a potent prognostic factor in human breast carcinoma.

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