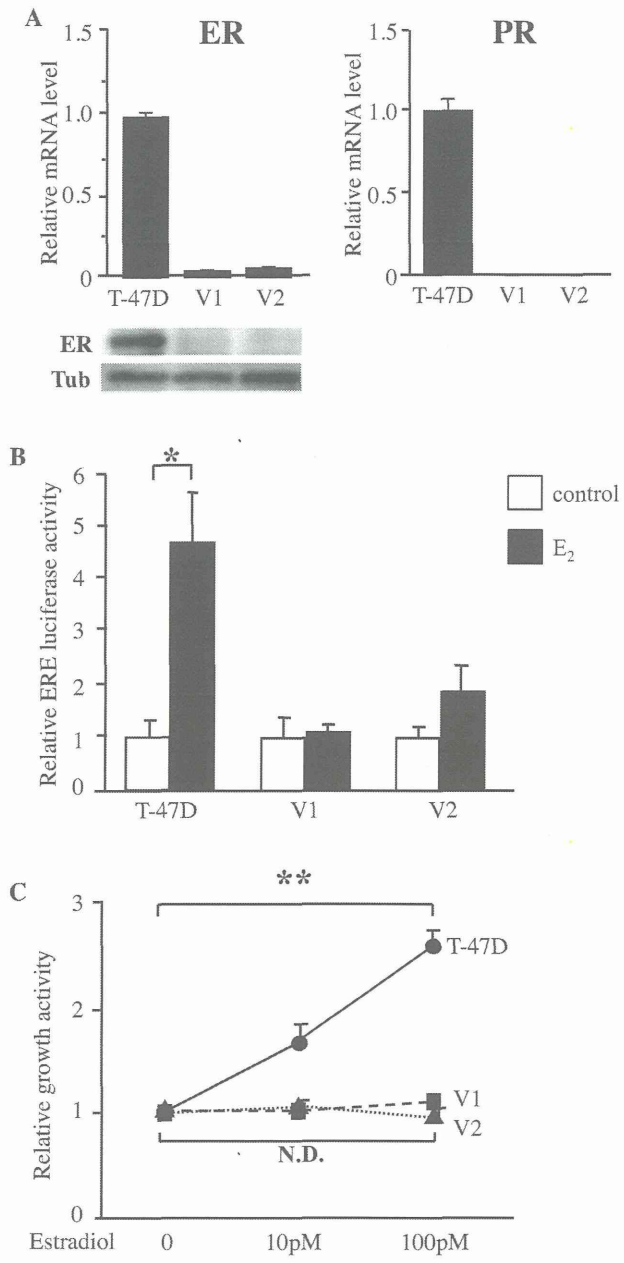

Fig. 2. ER function in AI-resistant breast carcinoma cells. A: ER (left upper panel) and PR (right upper panel) mRNA expression in V1, V2, and T-47D cells as determined by real-time PCR. Relative expression levels are expressed as a ratio compared to those in T-47D cells (left bar). Data are presented as mean ($n=2$). Lower panel demonstrates ER immunoreactivity in cells by immunoblotting. β-tubulin (Tub) immunoreactivity is shown as an internal control. B: ER activity in these cells by ERE- luciferase reporter assays. The cells were treated with 100 pM estradiol (closed bar) or vehicle control (ethanol; open bar) for 2 days. The values relative to vehicle control are shown as mean ± S.D. ($n=3$). C: Estrogen-mediated cell proliferation. V1, V2, and T-47D cells were treated with the indicated concentrations of estradiol or vehicle control (ethanol) for 4 days. The relative proliferative activity is the ratio compared to the vehicle control, presented as mean ± S.D. ($n=3$). $^{*}P<0.05$; $^{**}P<0.01$.

bicalutamide for 24 h. Subsequently, total RNA was extracted, amplified, and labeled using the Low Input Quick Amp Labeling Kit (Agilent Technologies). The relative levels of gene expression were calculated by global normalization, and scatter plot analysis of the microarray data was performed using GeneSpring 12.5 (Agilent Technologies). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under the accession number EMBL: E-MTAB-1933. The biological functions and interactions of each gene were identified by ingenuity pathway analysis (Ingenuity® Systems, www.ingenuity.com).

2.10. Statistical analysis

The Wilcoxon signed rank test and Student's *t*-test were used in the immunohistochemical analyses and *in vitro* experiments, respectively, and $P<0.05$ was considered significant.

3. Results

3.1. Immunohistochemical features in recurrent breast carcinoma during AI treatment

We first compared the immunohistochemical features of recurrent breast carcinoma lesions during AI treatment with those of the corresponding primary lesions. As shown in Fig. 1 and Table S2, LIs of the ER (Fig. 1A) and PR (Fig. 1B) were significantly lower in the recurrent lesions ($P=0.040$ and $P=0.020$, respectively). In the recurrent lesions, 2 out of 21 cases had lost the ER expression completely. In contrast, the AR LI was higher in recurrent lesions than primary lesions from the same patients in 13 out of 21 cases (62%) (Fig. 1C), although this difference did not reach a significance ($P=0.22$). Immunohistochemical analysis of primary lesions for the androgen-induced protein PSA [21] revealed only one positive case, while recurrent lesions were positive in 7 out of 21 cases. This result indicates that the PSA status was markedly increased in patients with AI-resistant recurrence ($P=0.014$) (Fig. 1D). The Ki-67 LI was also significantly higher in the recurrent lesions ($P=0.011$) (Fig. 1E), but that of HER2 was not significantly different between the primary and the recurrent lesions ($P=0.50$).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2014.08.019.

3.2. ER-dependent cell proliferation is absent in variant cell lines established to model AI resistance

To facilitate further studies of androgen activity in recurrent tumors, we next established AI- resistance breast carcinoma cell lines from T-47D cells that expressed both the ER and AR [22]. We cultured T-47D cells stably transfected with ERE-GFP for 3 months in an androgen-supplemented steroid-depleted medium, thus reflecting the hormonal conditions under AI treatment (Supplementary Fig. S1). The cells gradually lost GFP fluorescence with the progression of passages under these conditions. Finally, none of them had vivid fluorescence. We picked two colonies as variant cell lines, temporarily named them V1 and V2 and cultured them under the same conditions (androgen-supplemented steroid-depleted medium). Disappearance of ERE–GFP fluorescence indicated a loss of the ER activity in variant cell lines. As shown in Fig. 2A, the ER mRNA expression level was much lower in variant cell lines (0.048-fold in V1 and 0.07-fold in V2) compared to parental T-47D cells (upper left panel), and the ER immunoreactivity was undetectable in these cells (lower panel). The expression of PR mRNA, encoding a known estrogen-induced protein [23], was undetectable in V1 and V2 cells (upper right panel). The ERE–luciferase reporter assays revealed that the ER transcriptional activity was not significantly changed by an estradiol treatment in V1 or V2 cells, differing from that of the T-47D cells (Fig. 2B). The proliferation of T-47D cells was significantly induced by estradiol in a dose-dependent manner but V1 and V2 cells did not proliferate in response to estradiol

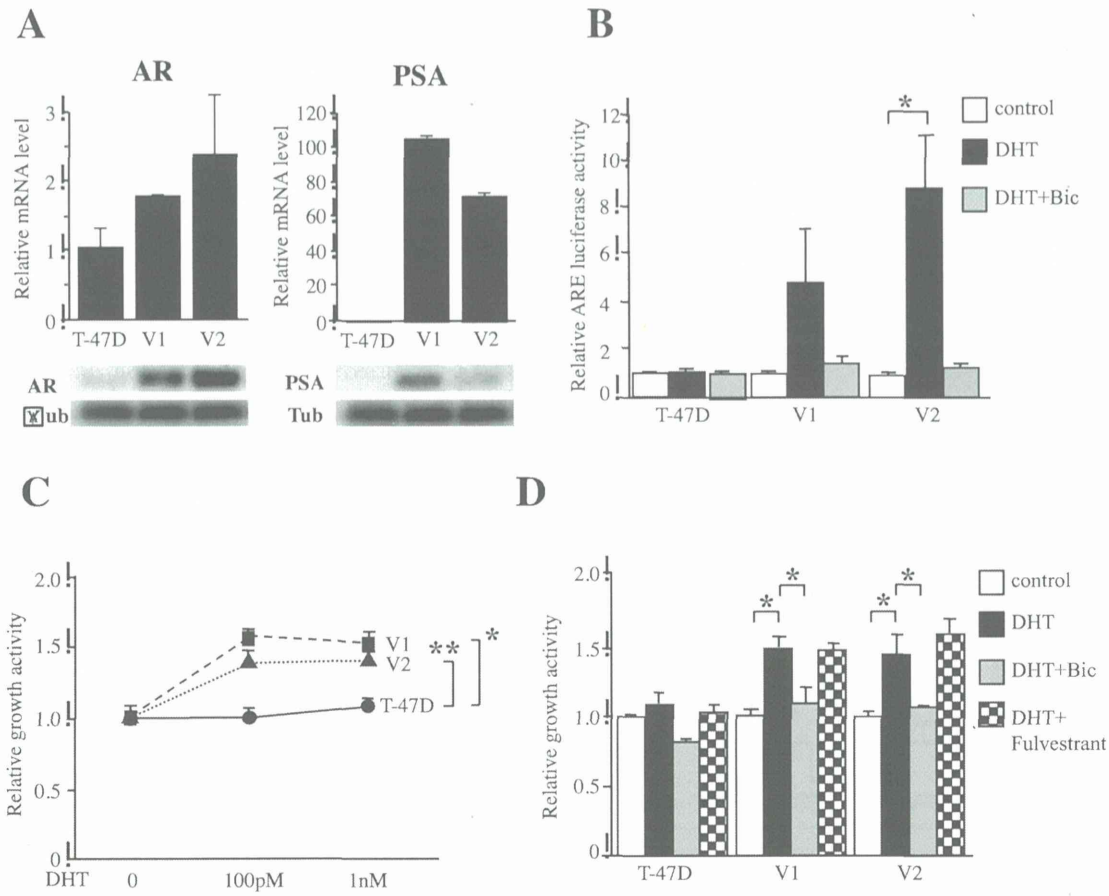

Fig. 3. AR function in AI-resistant breast carcinoma cells. A: AR (upper left panel) and PSA (upper right panel) mRNA expression in V1, V2, and T-47D cells as determined by real-time PCR. The relative expression level is the ratio compared to that of T-47D cells (left bar), and is presented as mean ($n = 2$). Lower panel summarizes immunoblotting for AR (lower left panel) and PSA (lower right panel) in these cells. β-tubulin (Tub) immunoreactivity is shown as an internal control. B: AR activity by ARE-luciferase reporter assays. Cells were treated with 1 nM DHT alone (closed bar), 1 nM DHT and 10 μM bicalutamide (gray bar), or vehicle control (ethanol; open bar) for 2 days. The values relative to the vehicle control are shown and data are presented as mean ± S.D. ($n = 3$). C: Androgen-mediated cell proliferation. Cells were treated with the indicated concentrations of DHT or vehicle control (ethanol) for 4 days. The relative proliferation activity is the ratio compared to the vehicle control and are presented as mean ± S.D. ($n = 3$). D: Effects of the AR and ER antagonists on androgen-meditated cell proliferation. V1, V2, and T-47D cells were treated with 1 nM DHT alone (closed bar), DHT with 10 μM bicalutamide (gray bar), or 100 nM fulvestrant (checkered bar) for 4 days. The relative proliferation activity is the ratio compared to the vehicle control (ethanol alone; open bar), and data are presented as mean ± S.D. ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$.

(Fig. 2C). These results suggest that the variant cells do not depend on estrogen-mediated signals to proliferate after long-term exposure to estrogen-depleted and androgen-supplemented conditions.

3.3. Variant cell lines exhibit androgen receptor-mediated proliferation

We next examined the response of variant cell lines to androgen. As shown in Fig. 3A, AR mRNA expression was higher (1.7-fold in V1 and 2.3-fold in V2) in the variant cell lines than the T-47D (left upper panel), and the level of AR protein was markedly higher in variant cell lines (left lower panel). The PSA mRNA expression was higher in V1 (114-fold) and V2 (78-fold) than T-47D (upper right panel), and similar results were observed for protein expression (lower right panel). ARE–luciferase reporter assays revealed that the level of AR transcription induced by DHT was higher in the variant cell lines (V1, 4.7-fold, $P = 0.07$; V2, 8.7-fold, $P < 0.05$) than the T-47D cells (1.0-fold; $P = 0.95$); AR transcription was potently inhibited by the addition of the AR-antagonist bicalutamide (Fig. 3B).

Variant cell lines demonstrated dose-dependent DHT-mediated cell proliferation that was significantly higher in V1 (1.4-fold; $P < 0.05$) and V2 (1.3-fold; $P < 0.05$) cells than T-47D cells under treatment with 1 nM DHT (Fig. 3C). DHT-mediated proliferation of variant cells was significantly inhibited by bicalutamide but was not affected by the ER-antagonist fulvestrant (Fig. 3D). These results suggest that the variant cell lines acquired an androgen receptor-mediated proliferation activity.

3.4. Expression profile of androgen-induced genes differs between variant and parental cell lines

To further examine the molecular effects of androgens on variant cells, gene expression profiles of V1 and T-47D cells were assessed by microarray analysis. We defined "androgen-induced genes" as those demonstrating greater than 2.5-fold higher expression in cells treated with DHT alone compared to those treated with DHT plus bicalutamide. A total of 390 androgen-induced genes were identified (Fig. 4A). Of these, 116 (30%) were induced only in V1 cells and 262 (67%) were induced only in T-47D cells. Only 12 genes (3%) were induced in both the cell lines. Comparison of expression levels of the 390 androgen-induced genes in cells treated with DHT alone by scatter plot revealed that 100 genes (26%) were predominantly expressed in V1 cells (Group A; V1/T-47D ratio >2.0), while 185 genes (47%) were predominantly expressed in T-47D cells (Group B; ratio <0.5)

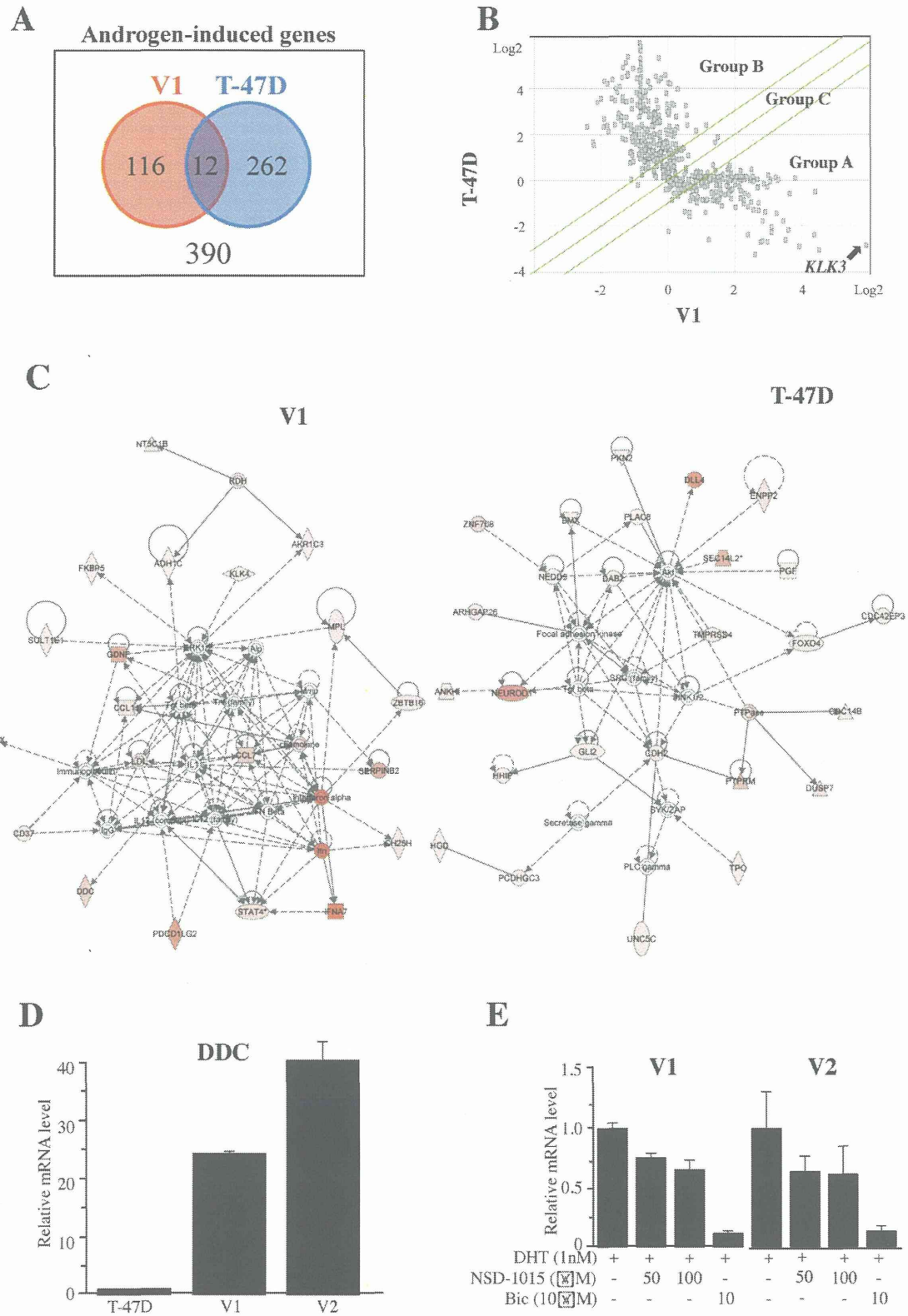

Fig. 4. Expression profile of androgen-induced genes in AI-resistant cells. A: Venn diagrams showing the numbers of androgen-induced genes identified in V1 and/or T-47D cells using microarray analysis. B: Scatter plot analysis of microarray data for 390 DHT-induced genes in V1 and T-47D cells. The position of each dot corresponds to the normalized average signal intensity (log_2 scale) of a single gene. The middle line indicates values that represent the V1/T-47D ratio of 1.0, and the outer lines represent the V1/T-47D ratio of 2.0 (lower line) and 0.5 (upper line). Genes were classified by V1/T-47D ratio as follows: with a ratio of >2.0, group A; <0.5, group B; and 0.5–2.0, group C. The location of KLK3 (PSA) is marked. C: Networks of top-ranked androgen-induced genes in V1 (left panel) and T-47D (right panel) cells. The intensity of the grey node indicates the degree of up-regulation. D: Expression of DDC mRNA in V1, V2, and T-47D cells as determined by real-time PCR. Relative expression levels are the ratios in V1 and V2 cells compared to those in T-47D cells (left bar) and are presented as mean ($n = 2$). E: Effects of NSD-1015 on androgen-induced PSA mRNA expression in V1 and V2 cells. Cells were treated with 1 nM DHT and the indicated concentration of NSD-1015 or bicalutamide (Bic) for 24 h. The relative expression level is the ratio compared to that of cells treated with DHT alone (left bar), and are presented as mean ($n = 2$).

Table 2
List of genes showed more than 10-fold expression ratio in V1 compared to T-47D cells.

Fold Change	Entrez gene ID	Gene symbol	Official full name
438.2	354	KLK3 (PSA)	Kallikrein-related peptidase 3
113.4	1734	DIO2	Deiodinase, iodothyronine, type II
107.3	1644	DDC	Dopa decarboxylase
68.7	4604	MYBPC1	Myosin binding protein C, slow type
57	54498	SMOX	Spermine oxidase
55.5	4837	NNMT	Nicotinamide *N*-methyltransferase
51.2	214	ALCAM	Activated leukocyte cell adhesion molecule
45.2	83539	CHST9	Carbohydrate (*N*-acetylgalactosamine 4-0) sulfotransferase 9
44	3248	HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)
40.7	23767	FLRT3	Fibronectin leucine rich transmembrane protein 3
37.1	9073	CLDN8	Claudin 8
35.2	7704	ZBTB16	Zinc finger and BTB domain containing 16
29.8	9172	MYOM2	Myomesin (M-protein) 2
27	963	CD53	CD53 molecule
25.3	10720	UGT2B11	UDP glucoronosyltransferase 2 family, polypeptide B11
24	91464	ISX	Intestine-specific homeobox
19.5	23498	HAAO	3-Hydroxyanthranilate 3,4-dioxygenase
18.4	2668	GDNF	Glial cell derived neurotrophic factor
16.4	7364	UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7
14.2	7365	UGT2B10	UDP glucuronosyltransferase 2 family, polypeptide B10
14	8644	AKRIC3	Aldo-keto reductase family 1, member C3
13	2554	GABRA1	Gamma-aminobutyric acid (GABA) A receptor, alpha 1
12.8	8529	CYP4F2	Cytochrome P450, family 4, subfamily F, polypeptide 2
11.6	126	ADH1C	Alcohol dehydrogenase 1C (class I), gamma polypeptide
10.7	11283	CYP4F8	Cytochrome P450, family 4, subfamily F, polypeptide 8

Gene performed real-time PCR is noted in boldface.

(Fig. 4B). Only 105 genes (27%) showed a similar level of expression in V1 and T-47D cells (Group C; ratio 0.5–2.0).

Networks of top-ranked androgen-induced genes in V1 and T-47D were determined by Ingenuity Pathway Analysis (Fig. 4C). Gene networks in V1 contained genes associated with "cell-to-cell signaling and interaction" (*CCL7, CCL11, GDNF, IL8, PDC1LG2*), "cell cycle" (*CD37, GDNF, STAT4, ZBTB16*), and "cellular development" (*GDNF, IFNA7, MPL, ZBTB16*) (left panel), while those in T-47D cells were associated with "the nervous system development and functions" (*CDH2, GLI2, NEUROD1, UNC5C*), "tissue development" (*ARHGAP26, BMX, CDH2, DLL4, GLI2, NEDD9, NEUROD1, PCDHGC3, PGF, UNC5C*), and "cell-to-cell signaling and interaction" (*ARHGAP26, BMX, CDC42EP3, CDH2, DLL4, FNPP2, GLI2, NEDD9, PGF, PTPRM, TPO, UNC5C*) (right panel). These results indicate that the expression profile of androgen-induced genes differed markedly between the variant and parental cell lines.

Of the genes classified into Group A in Fig. 4B, 25 genes showed more than a 10-fold difference in expression between V1 and T-47D cells (Table 2), with *KLK3* (PSA) exhibiting the greatest difference (438-fold). The third highest, *DDC* (L-DOPA decarboxylase; 107-fold), has been reported as an AR coactivator in prostate carcinoma [24,25], but no information is currently available regarding its involvement in breast carcinoma. DDC expression levels were validated by real-time PCR. DDC mRNA expression was 22-fold and 36-fold higher in V1 and V2 cells, respectively, than in the T-47D cells (Fig. 4D). Moreover, the DDC-inhibitor NSD-1015 decreased the DHT-induced PSA mRNA expression in variant cell lines in a dose-dependent manner (Fig. 4E). These results suggest that DDC plays an important role in increasing the AR activity in variant cell lines.

4. Discussion

This is the first report to evaluate AR activity in AI-resistant recurrent breast carcinoma. All the recurrent samples examined in this study were regarded as AI-resistant carcinoma because all the cases relapsed during the adjuvant AI therapy. In this study, PSA expression was frequently detected in recurrent breast carcinoma. The stable variant cell lines established as AI-resistance models demonstrated AR-dependent cell proliferation and overexpressed PSA. These data suggest that increased AR activity has an oncogenic role in AI-resistant breast carcinoma.

PSA expression was significantly higher in recurrent tumor tissues than in the corresponding primary lesions. PSA expression was also significantly higher in the V1 and V2 variant cell lines than in the parental T-47D cells. PSA was originally believed to be a tissue-specific protein produced by epithelial cells of the prostate gland. Several androgen-responsive elements (AREs) have been identified in the PSA promoter region [21]. PSA expression is markedly induced by DHT in AR-positive breast carcinoma cell lines, and it is now recognized as a potent androgen-induced protein in breast carcinoma cells as well as prostate carcinoma cells [26]. PSA immunolocalization has been reported in female breast carcinoma [17,27–29], and Kraus et al. [17] recently showed that PSA immunopositivity was 4% (2 of 56 cases), which is in good agreement with our data (5%). Altogether, our data and previous reports suggest that the AR activity is increased in AI-resistant recurrent lesions.

Androgenic activity is characterized by the functions of androgen-induced genes. Thus, examination of the expression profiles of androgen-induced genes in variant and T-47D cell lines was important to obtain a better understanding of androgen activity in AI-resistant cells. Interestingly, of the 390 androgen-induced genes identified by microarray analysis, only 12 (3%) were common to V1 and T-47D cells (Fig. 4A). Kabos et al. [30] reported that the patient-derived luminal breast cancer xenografts had diverse responses to endocrine therapy and had different tumor-specific ER transcriptomes. Similarly, our results showed that the V1 and T-47D cells had different AR-transcriptomes. Therefore, the molecular functions of the AR in AI-resistant recurrent lesions may be markedly different from their primary lesions. Moreover, we found that 25 androgen-induced genes whose expression was more than 10-fold higher in V1 than in T-47D cells. These genes included *DDC* (L-DOPA decarboxylase) (107-fold), known as an AR-coactivator [24], and *AKR1C5* (14-fold), which synthesizes testosterone from androstenedione [31]

(Table 2). In the present study, the growth of parental T-47D cells was slightly increased by DHT (Fig. 3C), although the proliferation effect was smaller than that mediated by estrogen (Fig. 2C). Although the T-47D cells certainly depend on ER signals for the most part, they may also have an AR-mediated proliferative pathway. In the process of adapting to estrogen-depleted and androgen- supplemented conditions, variant cell lines lost their ER and depended on the AR-mediated pathway for proliferation. In addition to increased AR expression, altered expression profiles of the androgen-induced genes may partly contribute to an increased AR activity in AI-resistant cell lines.

In our study, ER and PR LIs were significantly lower in the recurrent breast carcinoma tissues, with similar findings for variant cell lines. The PR expression is considered to indicate an intact estrogen-signaling pathway [32] and is often used as an indicator of a response to endocrine therapy for breast carcinoma [33]. A fraction of the ER-positive breast carcinomas lose the ER immunoreactivity until recurrence [34]. Of the patients who received adjuvant hormonal treatment, 9 of 49 (18%) converted from ER-positive in the primary tumor to ER-negative in the recurrent lesions [35], which is consistent with our present results.

We have established several AI-resistant cell lines that depend on an ER-mediated pathway from MCF-7 cells [36,37]. Moreover, there have been many reports that the LTED (long-term estradiol deprivation) cells overexpressed ER [5,6]. However, in this study, established cell lines from T- 47D lost the ER expression. In addition, a previous study has reported that the T-47D cell line lost the ER under long-term estrogen- deprived conditions [38]. The T-47D cells may easily develop other proliferative pathways without the ER in an estrogen-free environment. These AI-resistant cell lines indicate that the character of parental cell lines determines the distinct types of resistance mechanisms.

Possible interaction between the ER and AR function has been proposed by several groups. Panet- Raymond et al. [39] reported that co-expression of the ER and AR decreased the AR trans-activation, and Takagi et al. [16] suggested that the androgen activity is suppressed in breast carcinoma by predominant estrogen activity. Therefore, these observations suggest that increased AR activity in recurrent lesions is, at least in a part, caused by decreased estrogen activity due to AI treatment.

In our study, Ki-67 LI was significantly higher in recurrent tissues, and the results of *in vitro* experiments revealed that the DHT significantly increased proliferation in variant cell lines. AR is generally considered to exert anti-proliferative effects in ER-positive breast carcinoma cells [40], but some divergent findings have been reported [41]. Recent studies suggest that the AR activity might differ with breast carcinoma subtype [14]. ER-negative breast carcinoma cell lines frequently exhibit AR- mediated cell proliferation, similar to prostate carcinoma; cross-talk between the AR and HER2 signaling pathway has been suggested [12,13]. Harvell et al. [42] demonstrated that the AR level in breast carcinoma after neoadjuvant AI therapy increased in AI non-responders, but decreased in responders, suggesting that the AR may play an important role in resistance to endocrine therapy in ER-positive breast carcinoma. Foekens et al. [43] reported that the PSA level in breast tumor cytosol correlated with poor response to tamoxifen therapy in the recurrent disease. Altogether with these reports, our results indicate that the AR activity may be more oncogenic in the AI-resistant breast carcinoma.

PSA expression was not always observed in the recurrent tissues in our study, suggesting that the induction of an AR-mediated proliferation pathway is one of the AI-resistance mechanisms.

Kabos et al. [30] suggested that the acquired resistance mechanisms differ in each individual tumor, which may explain our findings. We are currently establishing several breast carcinoma cell lines to use as AI-resistance models under estrogen-depleted and androgen-supplemented conditions. Androgen metabolite-dependent and estrogen-depletion-resistant cells derived from the MCF-7 cells were recently reported to show dose-dependent activation of ER functions by the estrogenic androgen 5α-androstane-3β,17β-diol (3β-diol) and markedly decreased AR expression [36]. In addition, several ER-independent proliferative pathways have been reported as AI-resistance mechanisms, including up-regulation of the ER-mediated pathway [5,6], the growth factor receptor- mediated pathways [7,8], mitogen-activated protein kinase (MAPK), and phosphatidylinositide 3- kinase (PI3K)/Akt [6,9,37]. Therefore, acquired AI-resistance mechanisms are suggested to be diverse and appropriately targeted therapies, according to case are required.

The AR was recently reported to have therapeutic potential in some breast carcinomas. A phase II trial of bicalutamide for ER-negative/AR-positive metastatic breast carcinoma showed the efficacy of AR-blockade in these patients [44], and a phase II trial evaluating the efficacy of the AR-inhibitor enzalutamide in combination with exemestane in advanced ER-positive breast cancer patients is ongoing (NCT02007512). The results of our present study suggest that AR inhibitors may be effective in a select group of AI-resistant breast carcinoma patients, and the PSA status may be a useful indicator of the response. Further investigations are needed to clarify the clinical significance of AR inhibitors in AI-resistant breast carcinoma.

In summary, examination of the immunohistochemical features of 21 AI-resistant recurrent breast carcinomas demonstrated that the PSA expression and the Ki-67 LI were increased and ER LI and PR LI were decreased in the recurrent lesions compared to the corresponding primary lesions. Moreover, we established two AI-resistant breast carcinoma cell lines and demonstrated that both PSA expression and AR-mediated cell proliferation were increased in these cell lines compared to a parental cell line. The expression profiles of androgen-induced genes in AI-resistant cells differed markedly from parental cells. These results suggest that the increased oncogenic AR activity in recurrent breast carcinoma is a mechanism of acquired AI-resistance, and the AR inhibitors may be effective in a select group of patients.

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Original Study

Variation in Use of Estrogen Receptor-α Gene Promoters in Breast Cancer Compared by Quantification of Promoter-Specific Messenger RNA

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Abstract

Estrogen receptor (ER)-α has multiple promoters upstream of the transcriptional start points in its gene. We examined the promoter usage of 43 ERα-positive breast cancer tissue samples and found the promoters to be used at similar ratios. The usage of ERα promoters may be important for development, differentiation, or carcinogenesis.

Introduction: Estrogen receptor (ER)-α expression offers a critical characterization of breast cancer, but risk of recurrence is difficult to predict using only ERα status. The *ERα* gene has at least 6 transcription start sites, 6 distinct first exons, and probably 6 promoters. To examine whether these promoters have differential effects in breast cancer, we quantified expression of promoter-specific *ERα* messenger RNA (mRNA), using real-time polymerase chain reaction (PCR) and statistical assessment. **Patients and Methods:** We examined variations in the use of breast cancer cell lines and 43 ERα positive ($ER\alpha^+$) breast cancer tissue samples by quantifying promoter-specific mRNA of *ERα* with real-time PCR analysis using primers and probes specially designed for this study. Moreover, we correlated the results of quantified the promoter-specific mRNA with mRNA of total *ERα* and related them to clinicopathological factors statistically. We also examined multiregression analyses for promoter-specific mRNAs of *ERα*. **Result:** We found the promoters to be used at almost similar ratios among $ER\alpha^+$ breast cancer cell lines and $ER\alpha^+$ breast cancer tissues. Clinicopathological variations were associated with identical *ERα* promoter choices. When we examined the contribution of mRNA from 3 promoters in breast cancer tissues to total *ERα* using multiple regression analysis, we found that only promoter A showed a significant ($P < .05$) transcript coefficient. **Conclusion:** Our findings imply that the use of *ERα* promoters as prognostic biomarkers is unfeasible. However, our results suggest that promoter usage of *ERα* may contribute to its expression in normal development and differentiation of individual or carcinogenesis of breast cancer.

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Introduction

About 70% of all breast cancers express estrogen receptor alpha (ERα). Treatment of ERα-positive ($ER\alpha^+$) breast cancer by selective estrogen receptor modulators (SERMs) has brought about better prognosis than has treatment by surgery alone,[1] whereas treatment with aromatase inhibitors for postmenopausal $ER\alpha^+$

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breast cancer shows better prognosis than does SERM therapy.[2-4] However, some ERα$^+$ breast cancers recur, and current predictive biomarkers for such cancers are clinically insufficient; therefore, we have been prospecting for important biomarkers. We previously reported that ERα transcriptional activity was inversely related to Ki-67 expression,[5] which implied that ERα activity could be a biomarker for recurrence.

In looking for a new biomarker to assess recurrence risk in breast cancer, we investigated transcriptional regulation of ERα,[6-9] as have other groups.[10-13] We discovered a specific transcriptional enhancer for promoter C,[6] and we found this promoter to be transcriptionally regulated by methylation in ZR-75-1 cells.[7] We also found that transcripts from promoter C significantly ($P < .05$) correlated with ERα expression assessed by enzyme immunoassay (EIA).[8] Furthermore, typical tissue promoter use in cell lines was found, using an estrogen response element luciferase assay.[9] These previous works, especially those correlating promoter-specific transcripts with total *ERα* mRNA, suggested the possibility of using ERα promoter transcripts as biomarkers for recurrence risk.

The *ERα* gene (*ESR1*) is located on chromosome arm 6q subband 25.1.[14] *ESR1* has at least 6 transcription start sites and 6 distinct first exons.[15-18] It also probably has 6 promoters, which is unusual for functionally discovered nuclear receptors,[19,20] but the biological meaning of the promoters is unclear. The use of > 3 *ERα* promoters in cell lines[9,17,21] and the use of promoters A and C in breast cancer tissues have been reported.[8,22] However, the use of 3 *ERα* promoters, promoters A, C, and D simultaneously in the same breast cancer tissues has not been reported previously. Furthermore, reports indicate that the ERα status determined by EIA was significantly related to the transcripts from promoter C ($P < .05$), but not to those from promoter A,[8] and the ERα-positive breast cancer cases with relatively more transcripts from promoter C showed poorer prognoses than those with fewer transcripts from the same promoter.[22] These reports suggest that the transcription initiated by specific promoters might differentially influence the ERα activity as well as the prognosis of ERα$^+$ breast cancer. In addition, there is no study about associations among the choice of *ERα* promoter and clinicopathological factors. We therefore reinvestigated *ERα* promoter usage in individual breast cancers using new methods and examined the association between variations in the use of *ERα* gene promoters and the clinicopathological factors of ERα$^+$ breast cancers.

Notably, we first evaluated *ERα* promoter choice in breast cancer cell lines and breast cancer tissues by quantifying 3 messenger RNAs (mRNAs) that were different for each first exon but translated into identical proteins, using primers and probes specially designed for this study. By correlating expressions of mRNA for 3 promoters with mRNA expression of total ERα, and promoter choice with clinicopathological factors, we examined whether *ERα* promoter choice differed in breast cancer tissues, with an eye toward using *ERα* promoters as clinical biomarkers.

Patients and Methods

Cell Lines and Breast Cancer Specimens

Human breast cancer cell lines, including MCF-7, T-47D, ZR-75-1, SK-BR-3, MDA-MB-231, and BT-20, were cultured in triplicate in 6-cm dishes with Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, St Louis, MO) at 37°C with 5% CO_2 concentration. These cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). Forty three patients of ERα + breast cancer who underwent breast cancer surgery in Gunma University Hospital from May 2010 to May 2011 provided to this study breast cancer tissues samples, which were obtained in surgery, and immediately absorbed in RNAlater (Sigma-Aldrich) to prevent total RNA degradation. All these patients agreed to the use of their mRNA for our research in a comprehensive agreement about research use. This study was conducted in conformity with Helsinki Declaration.

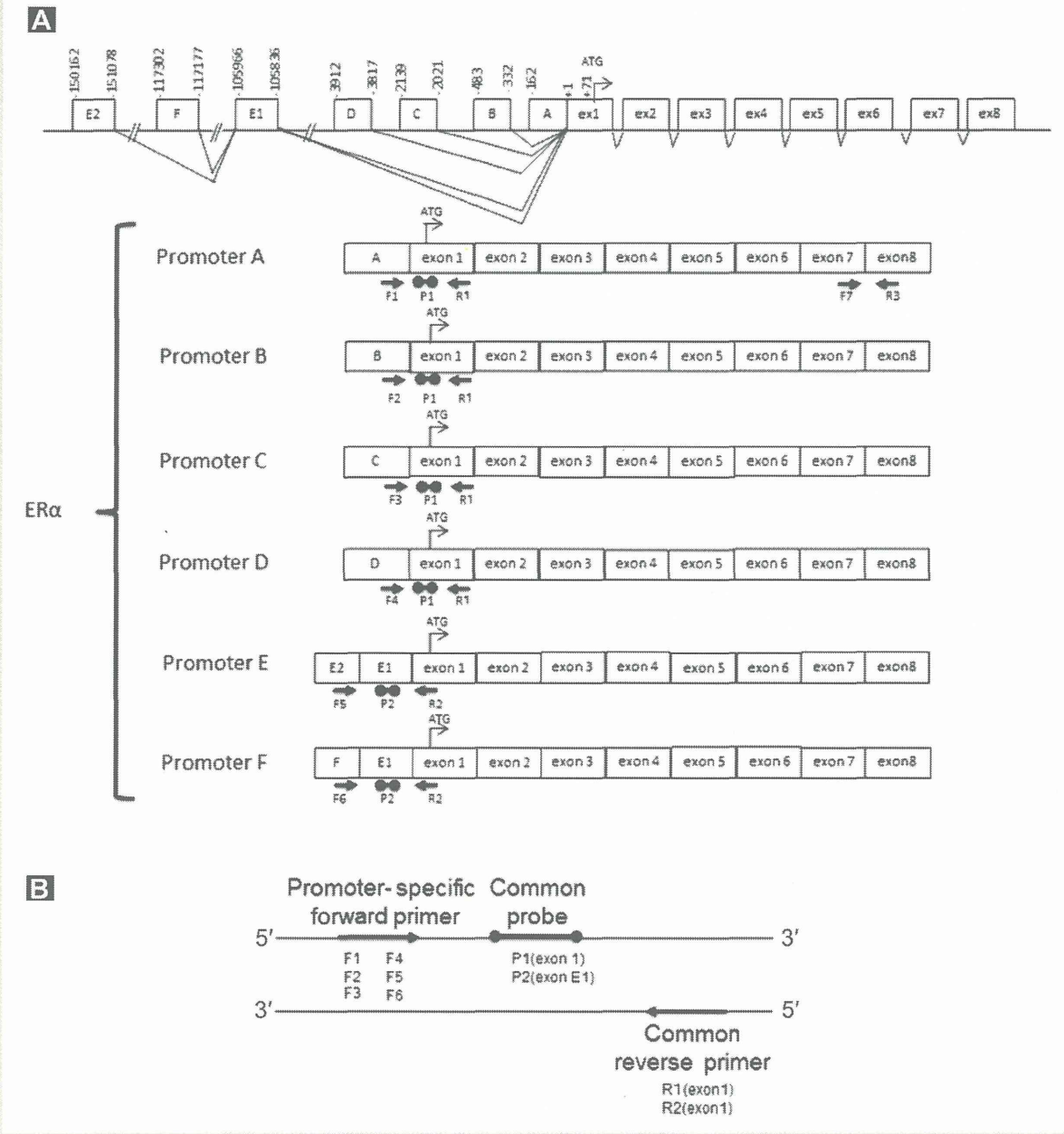
Primer Design

We referred mainly to mRNA sequences from the database of GenBank (promoter A: NM_000125.3; promoter B: NM_001122740.1; promoter C: NM_001122741.1, promoter D: NM_001122742.1; promoter E: AJ002561.1; promoter F: AJ002562.1). We designed forward primers (F1, F2, F3, and F4) for the first exon specific for the transcript from each *ERα* promoter. The common reverse primer (R1) and the probe (P1) for promoters A, B, C, and D were also designed on exon 1 (Fig. 1A). By using the same reverse primer and probe for promoter-specific mRNA from promoters A, B, C, and D and setting the probe on the sense strand following the promoter-specific forward primers (Fig. 1B), we decreased the specific bias in real-time polymerase chain reaction (PCR) assays, adjusting the rising cycles of the standard curve and amplification efficacy at almost the same level in different real-time PCR assays. Forward primers specific to promoters E (F5) and F (F6) were designed on exons E and F, respectively. The same probe for promoters E and F (P2) was designed on exon E1; their common reverse primer (R2) was set on exon 1 for the reason described previously. Forward and reverse primers for mRNA expression of total *ERα* estimation were designed on exons 7 and 8, respectively. Because primers for total *ERα* were designed for a distant position, total *ERα* transcripts could be independently measured at a point apart from the region of interest.

Reverse Transcriptase PCR and Real-Time PCR

Total RNA from cells cultured to about 70% confluence was extracted by the acid guanidinium phenol chloroform method with ISOGEN (Nippon Gene, Toyama, Japan) as the protein denaturant; that of breast cancer tissues was extracted by QIAGEN RNeasy mini kit (Qiagen, Mississauga, Ontario, Canada), both according to manufacturers' protocols. We produced complementary DNA (cDNA) from 1 μg RNA using a QIAGEN Quantitect RT-PCR Kit (Qiagen) according to manufacturer's protocol. All transcripts were measured by a Step One Real-Time PCR System (Applied Biosystems Inc, Foster City, CA). For the probes, 10ml of Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies, Inc, Santa Clara, CA) was used in total 20 μL mix per well for real-time PCR. The SYBR green method used Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) in the same quantity as with the probes. Concentrations for primers, probes, and reference dye were 500 nM, 200 nM, and 300 nM, respectively. The quantity of added cDNA sample in the total volume was 2μL. The PCR protocol was 95°C

Figure 1 Schematic Study Design and Primer and Probe Design. (A) Exon Structures of Wild-Type *ERα* Primer and Probe Design. The 5′-UTR of Each First Exon was Used to Quantify Messenger RNA (mRNA) Specifically for Each Promoter. Forward *ERα* Primers: F1 ~ 4. Common Reverse Primer (R1) and Probe (P1) for Promoters A ~ D Were Designed for Their Exon 1. Forward Primers for Promoters E (F5) and F (F6) Were Designed on the 5′-UTR of Their First Exon and Their Probe (P2) was Designed on Their Second Exon; the Common Reverse Primer (R2) for Promoters E and F was Also Based on Their Exon 1 (not Identical to R1). The Forward (F7) and Reverse Primers (R3) Were Designed on Exons 7 and 8, Respectively. Names of Promoter-Specific mRNA and 5′ UTR of Exons Followed Flouriot et al.[17] Open Boxes Represent Exons Responsible for the Translation of *ERα*; Numbers Above the Open Boxes Represent the Distance (in Base Pairs) to Translational Starting Site. (B) Specific Forward Primers for Each Promoter Were Designed for 5′-UTR; Probes Were set on Sequences of the Sense Strand Following Forward Primers; Reverse Primers Were Designed on the Antisense Strand of Complementary DNA Products



for 3 minutes to denature first; 95°C for 5 seconds to denature second; 60°C for 10 seconds to anneal and extend. Second denaturation steps and simultaneous annealing and extension steps were repeated for 40 cycles. A melt curve protocol was added to the SYBR green assay. Cell and tissue results were selected when the standard-curve threshold cycle value of 1pg cDNA was

between 14 and 16 and the correlation coefficient of efficacy quantification was > 0.95. Results were normalized to β-actin transcripts and were then converted to logarithms (base 2). Transcripts of cell lines was examined in triplicate. Primer sequences are shown in Supplemental Table 1 (available in the online version at http://dx.doi.org/10.1016/j.clbc.2013.10.015).

Statistical Analyses

All statistical analyses were conducted on JMP version 9.0.2 (SAS Institute Inc, Cary, NC). In Figures 2, 3, and 4, normalized transcript values are shown with logarithms (base 2) for statistical analysis. Figure 3A shows differences between individual values and the minimum value of the results (ie, promoter D, sample number 8, −15.0878) for simplicity. Correlations of transcripts from promoters A, C, and D of *ERα* with those of total *ERα* were tested by the Pearson correlation coefficient with 5% significance. Transcript averages divided by clinicopathological factors were analyzed by Student *t* test and analysis of variance (ANOVA) test with 5% significance. Associations among investigated mRNA and other clinicopathological factors were tested by single regression analysis with 5% significance. Single and multiple regression analysis of transcripts from the 3 promoters A, C, and D and transcripts of total *ERα* were tested by ANOVA with 5% significance. A *P* value < .05 was considered significant.

Results

Confirmation of Promoter Usage of ERα$^+$ and ERα$^-$ Cell Lines

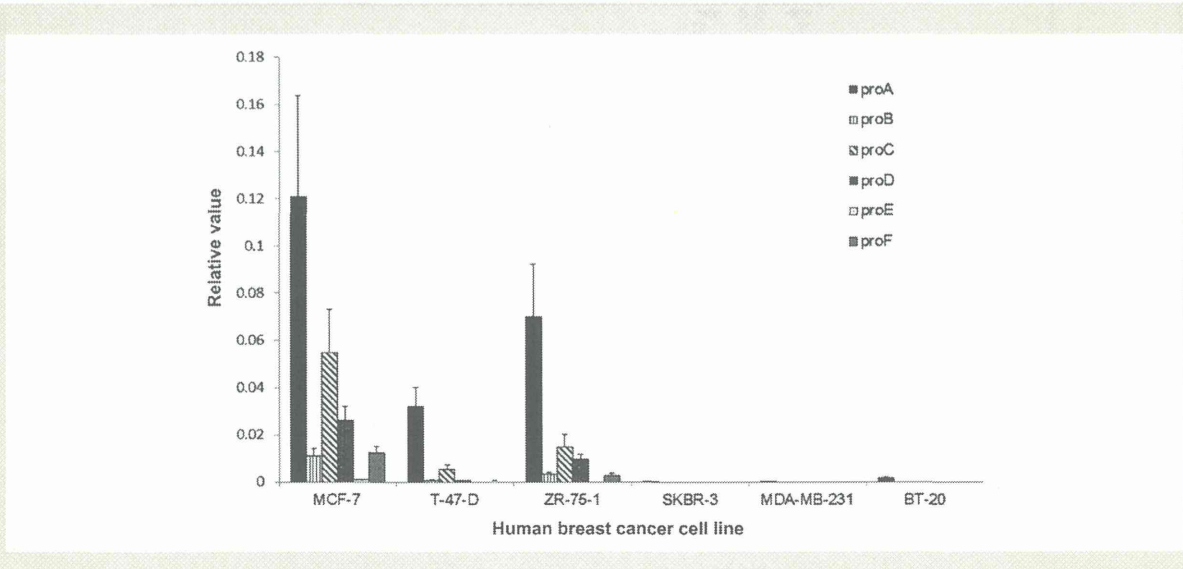
In ERα$^+$ breast cancer cell lines MCF-7, T-47D and ZR-75-1 the greatest amount of transcripts were specific to promoter A followed by those specific to promoter C (Fig. 2), those from promoter D were relatively few, and those from promoters B, E, and F were extremely few; transcripts from all promoters in ERα$^-$ cell lines were also extremely few. This result agreed with the findings of our previous study (which used an estrogen response element luciferase assay), which also showed the greatest and second-greatest activities to lie with promoter A and promoter D, respectively.[9] Because transcripts from promoter C, which was significantly ($P < .05$) correlated with ERα expression assessed by EIA in our previous study,[8] was also correlated with expression of *ERα* mRNA in this study (Fig. 3B), this result did not contradict that of the previous study. In addition, as more transcripts were seen for promoters A, C, and D than for other promoters, these 3 promoters may be more important for *ERα* transcription. We therefore focused on mRNA expression from promoters A, C, and D in the subsequent assays.

ERα Promoter Usage in Breast Cancer Tissues

Clinicopathological factors of breast cancer tissues examined in the following assays are shown in Table 1. The bias of clinicopathological factors in provided specimens was not recognized, and the clinical stage of most of examined patients was under stage II. Most breast cancer tissues showed the same pattern of ERα promoter usage as that of ERα$^+$ breast cancer cell lines (Fig. 3A). Promoter A gave the largest amount of transcript, followed by promoter C and then promoter D.

To estimate this result statistically, we analyzed the correlations among transcripts from promoters A, C, and D and that of total *ERα*. Results showed that transcripts from promoter A, C, and D were significantly correlated with each other and to total *ERα*

Figure 2 **Expressions of Promoter-Specific Messenger RNA in *ERα* of Breast Cancer Cell Lines. Results From Human Breast Cancer Cell Lines MCF-7, T-47D, ZR-75-1, SK-BR-3, MDA-MB-231, and BT-20 are Shown. The Vertical Axis Indicates the Relative Levels of the Transcripts Originating From Each Promoter, Which Were Normalized to β-Actin.**



Abbreviations: proA = promoter A; proB = promoter B; proC = promoter C; proD = promoter D; proE = promoter E; proF promoter F.