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 ($ERα^+$) 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 $ERα^+$ breast cancer cell lines and $ERα^+$ 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 ($ERα^+$) 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 $ERα^+$

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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

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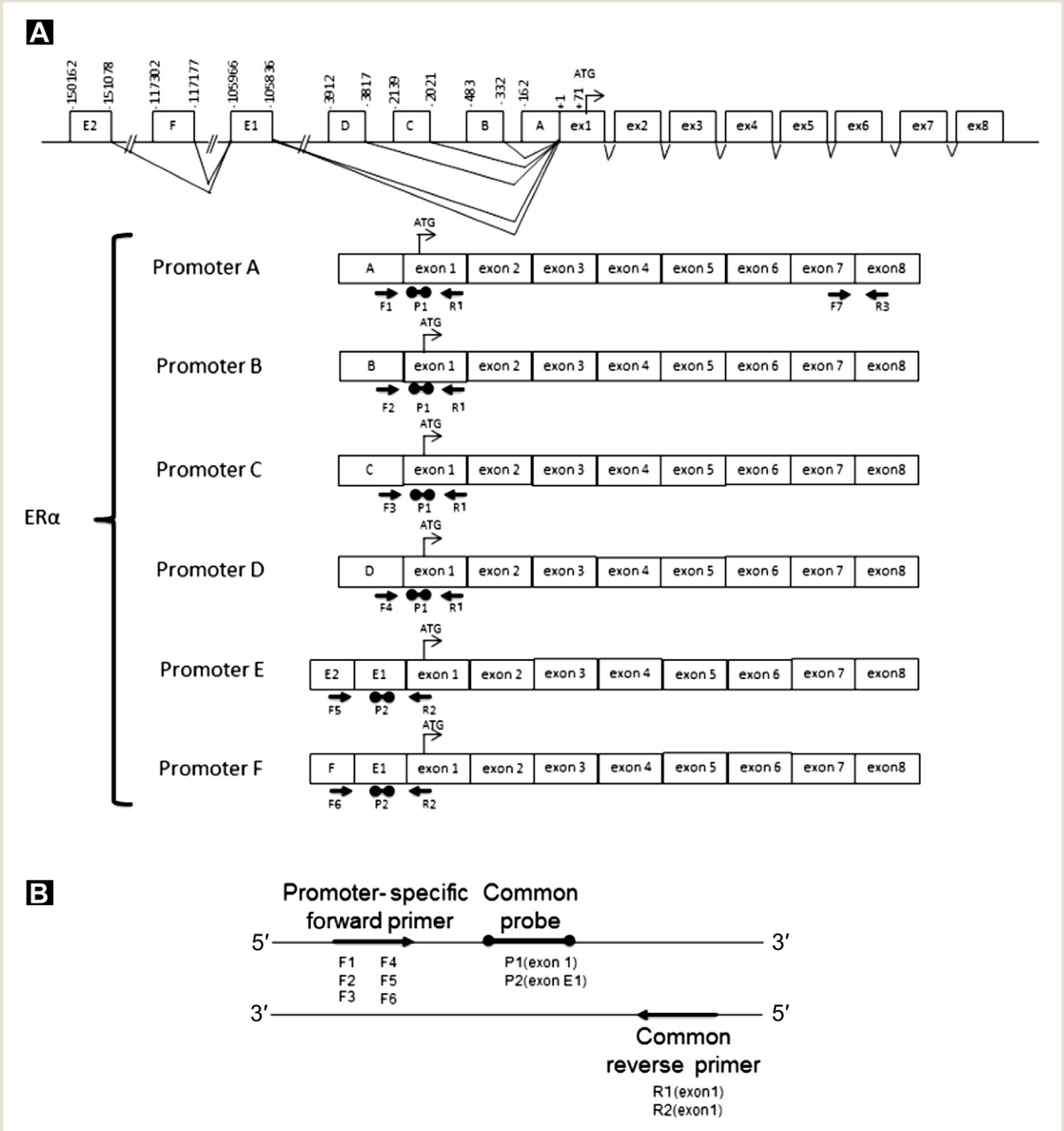
Figure 1 Schematic Study Design and Primer and Probe Design. (A) Exon Structures of Wild-Type *ERα* Primer and Probe Design. The 5′-UTR of Each First Exon was Used to Quantify Messenger RNA (mRNA) Specifically for Each Promoter. Forward *ERα* Primers: F1 ~ 4. Common Reverse Primer (R1) and Probe (P1) 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

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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

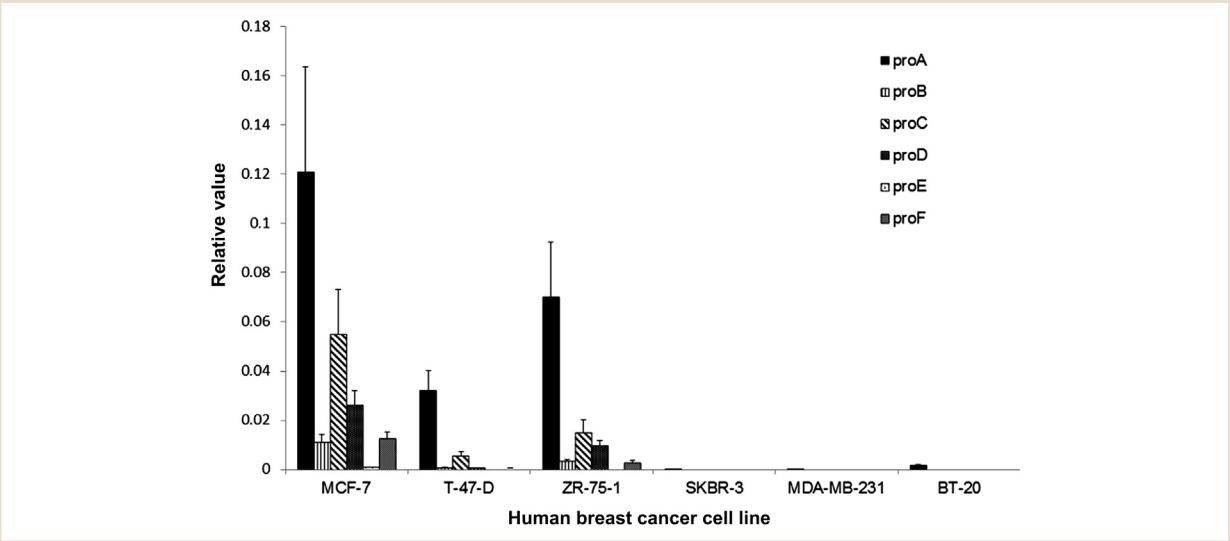
In ERα$^+$ breast cancer cell lines MCF-7, T-47D and ZR-75-1 the greatest amount of transcripts were specific to promoter A followed by those specific to promoter C (Fig. 2), those from promoter D were relatively few, and those from promoters B, E, and F were extremely few; transcripts from all promoters 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.

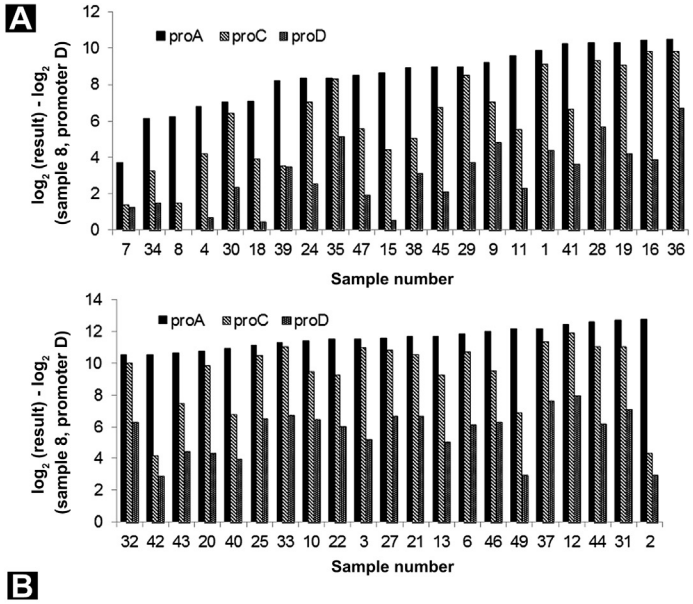
To estimate this result statistically, we analyzed the correlations among transcripts from promoters A, C, and D and that of total *ERα*. Results showed that transcripts from promoter A, C, and D were significantly correlated with each other and to total *ERα*

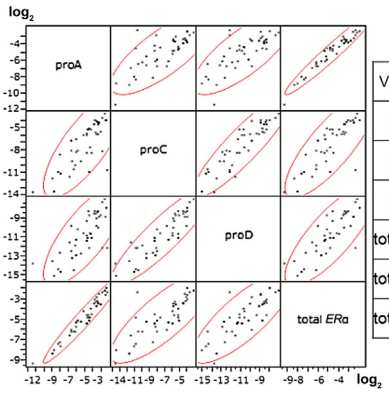
Figure 2 Expressions of Promoter-Specific Messenger RNA in *ERα* of Breast Cancer Cell Lines. Results From Human Breast Cancer Cell Lines MCF-7, T-47D, ZR-75-1, SK-BR-3, MDA-MB-231, and BT-20 are Shown. The Vertical Axis Indicates the Relative Levels of the Transcripts Originating From Each Promoter,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

Figure 3 Real-Time Polymerase Chain Reaction of Messenger RNA in Individual Breast Cancers and Statistical Analyses. (A) Results of Real-Time Polymerase Chain Reaction (PCR) Assays of Individual Breast Cancers. The Vertical Axis Indicates the Quantity Obtained From This Formula "log2 (result) - log2 (sample 8, promoter D)". In Other Words, This Result From That Formula Indicates the Difference of the Result From the Smallest Quantity, Promoter D of Sample 8, in This Real-Time PCR Assay. The Results in That Formula Were Obtained in Real-Time PCR. The Result of Real-Time PCR Were Normalized to β-Actin and Were then Converted to Logarithmic Values (Base 2). These Result Were Listed From the Left End in the Increasing Order in the Result of Promoter A Obtained From That Formula. The Horizontal Axis Indicates Identification Number of Tissue Sample. (B) The Correlation Coefficient of Promoter-Specific *ERα* Messenger RNA (mRNA). A Matrix of Paired Correlation Coefficients With dot Maps is Presented. Oval: 95% of Examined Data Exist. Correlation Coefficients Were Estimated With $P < .05$ Significance. The Horizontal and Vertical Axes Indicate the Amount of Transcripts Specific to Each Promoter, Converted to Logarithm of Promoter-Specific mRNA Normalized to β-Actin (Base 2). (C) The Associations Among Promoter-Specific ERα mRNA and Clinicopathological Factors (age, Status of Menopause, ER Immunohistochemistry [IHC] and HER2 IHC). The Horizontal Axes Indicate age in Years, Menopausal State (Postmenopausal [post] and Premenopausal [pre]), Allred Score in ER IHC and HER2 Status in HER2 IHC. The Vertical Axes Indicate the Levels of Promoter-Specific mRNA Normalized to β-actin, Converted to Logarithmic Values (Base 2). Age was Tested by Single Regression Analysis and Regression Line is Indicated in This Figure. Menopause, ER IHC, and HER2 IHC Were Tested by the Student t Test and the Analysis of Variance (ANOVA). All Values Were Converted to the Logarithm (base 2) of Promoter-Specific mRNA Normalized to β-actin





Variable	Variable	Correction Coefficient	Number	95% CI	*P* value
proC	proA	0.8034	43	0.6108-0.8693	<.0001
proD	proA	0.7846	43	0.6056-0.8672	<.0001
proD	proC	0.9216	43	0.8189-0.9440	<.0001
total *ERα*	proA	0.9731	41	0.8450-0.9843	<.0001
total *ERα*	proC	0.8269	41	0.6854-0.9006	<.0001
total *ERα*	proD	0.8391	41	0.6912-0.9026	<.0001

Abbreviations: proA = promoter A; proC = promoter C; proD = promoter D.

(Fig. 3B), which suggested that *ERα* transcripts in ERα$^+$ breast cancer tissues had the same promoter usage.

To investigate variations in *ERα* promoter choice by another method, we quantified promoter-specific transcripts and that of total ERα mRNA according to clinicopathological factor. The statistically significant ($P < .05$) result of this analysis came from 4 factors: patient's age, status of menopause, ER status, and human epidermal growth factor receptor 2 (HER2) status (Fig. 3C). ER

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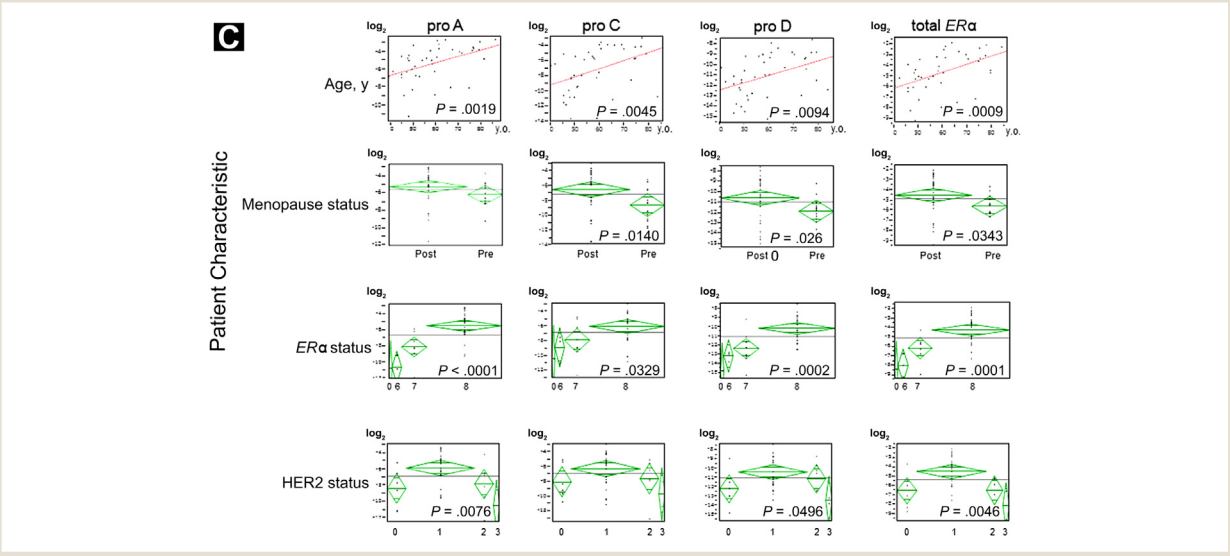
Figure 3 continued



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and HER2 status were ascertained using immunohistochemistry. Transcripts of each promoter and total ERα increased similarly with age. Transcripts for promoters C and D and for total ERα were larger in postmenopausal breast cancers than in premenopausal cancers. Transcripts for each promoter and for total ERα were positively related with patients' ER-Allred scores,[23] but inversely related to HER2 scores except for score 0. These results showed that the association of each promoter with clinicopathological factors was the same as that of total ERα, which suggests that *ERα* transcripts in ERα$^+$ breast cancer have the same promoter usage.

Regression Analyses of ERα Transcription by 3 Promoters

As transcripts from 3 promoters were quantified, we used single and multiple regression analyses of associations among promoters and total *ERα* mRNA expression. Single regression analyses positively related transcripts from all 3 promoters to that of total ERα (Fig. 4A). Although our multiple regression analysis posited transcripts from the 3 promoters as independent variables, we considered that these variables examined for total ERα might influence each other, thus biasing this analysis. To overcome this problem, we calculated a variance inflation factor (VIF). For a VIF < 10, this influence could be generally excluded. As the VIF was < 10 for this study, we felt multiple regression analysis could account for total *ERα* mRNA. Only the coefficient of promoter A was significant ($P < .05$) in this analysis (Fig. 4B).

Discussion

In our previous study of an estrogen response element reporter gene assay for promoter-specific activity, a very high level of ER activity by promoter A and a moderate level of activity by promoter D were observed in ERα$^+$ breast cancer cell lines.[9] Though the results of this study differ from those of the previous report in the strict sense, they agree with the pattern of high luciferase activity for promoter A and moderate activity for promoter D in ERα$^+$ cell lines. Whereas promoter C luciferase activity was low in the previous study, promoter C transcripts significantly ($P < .05$) correlated with ER status assessed by EIA.[8] Promoter C transcripts have also been significantly ($P < .05$) associated with poor prognosis in breast cancer tissue.[22] Low luciferase activity for promoter C in the previous study might have been because the length of the sequence inserted to reporter plasmid was approximately 1.5k base pairs (bp) and the long insert might have included an unknown silencer for transcriptional activity in breast cancer cell lines.

Another of our previous studies reported that ER status in breast cancer tissues (per EIA) was significantly ($P < .05$) correlated with transcripts from promoter C rather than promoter A.[8] Results from this study also differed from those of our previous study about the correlation of promoter A transcripts, but this may have been affected by the stability of mRNA. The half-life of promoter A transcripts was much shorter than that of promoter C (promoter A: 2.85 h, promoter C: 7.42 h),[22] which implies that the instability of promoter A–specific mRNA might affect the associations of promoter A transcripts compared with those of total ERα in the previous study. Moreover, an RNA storage reagent was used to prevent total RNA degradation in this study, and efficiency of RNA collection in this study was thought to be improved over the previous study, allowing more precise measurement of promoter A transcript in this study. In any case, we are convinced that the result of this study did not negate the findings of our previous studies.

We analyzed *ERα* promoter usage by correlating promoter-specific transcripts with those of total *ERα*, and these transcripts with clinicopathological factors. These results suggest that ERα transcripts in ERα$^+$ breast cancer had the same usage of promoters. Alteration of promoter usage in *ERα* was reported previously in analyses of non–breast cancer cell lines[9,17] and normal human and rat tissues,[17,24-26] which suggested that tissue type drove the choice of promoters in *ERα* transcription. We therefore speculated that promoter usage was important to regulate expression of ERα in

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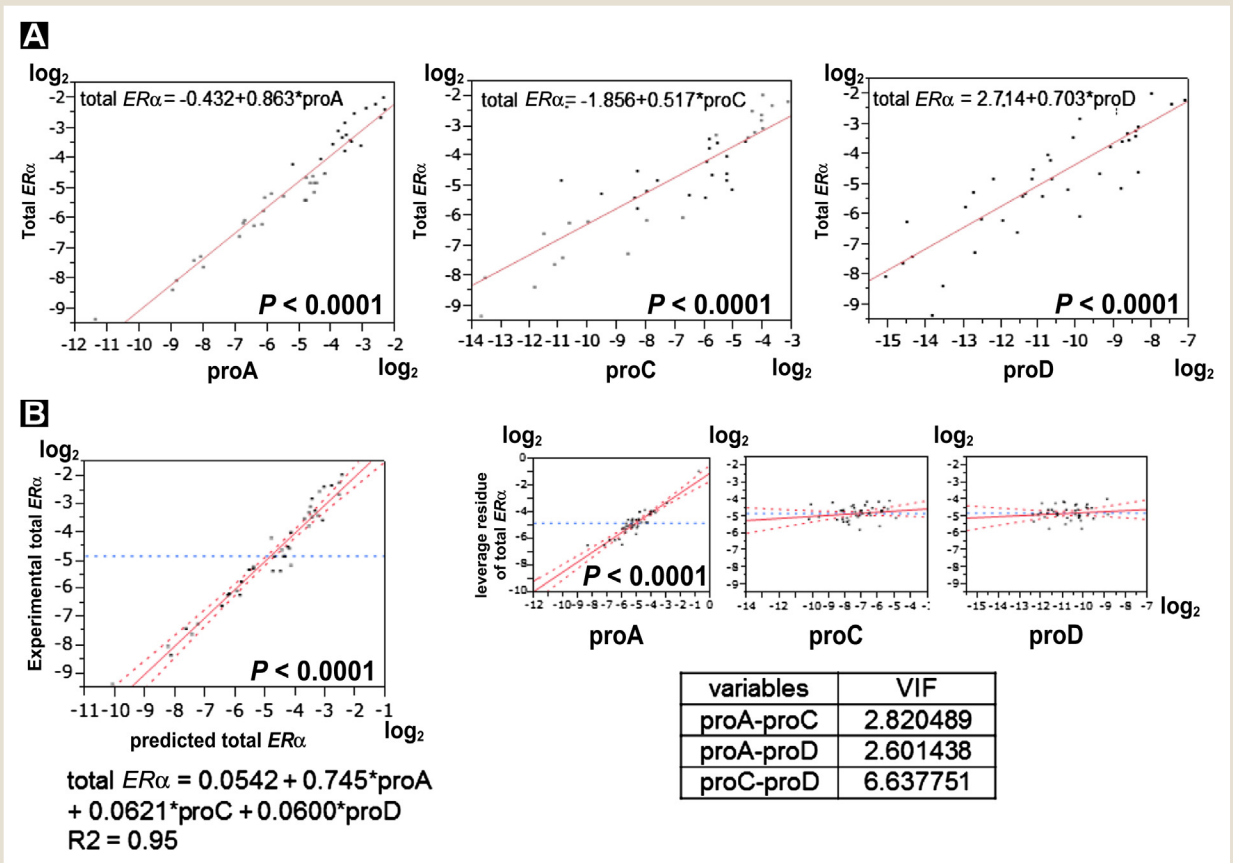
Figure 4 Regression Analyses of Promoter-Specific Messenger RNA. (A) The Single Regression of Promoter-Specific Results for Total *ERα* Messenger RNA (mRNA), Shown With the *P* Value of the Analysis of Variance (ANOVA). The Horizontal and Vertical Axes Indicate the Amount of Transcripts Specific to Each Promoter and Total *ERα*. The Values of the Results Obtained in Real-Time Polymerase Chain Reaction Were Normalized to β-Actin and Were Converted to Logarithmic Values (Base 2). (B) The Multiple Regression Analysis of Promoter-Specific mRNA. Left: A dot Plot of Predicted Experimental Data; Regression Equation Shown With the ANOVA *P* Value. Right: Figures of Leverage Residue Plot Shown With the *P* Value and Variance Inflation Factor (VIF). Horizontal Dotted Line: Average Value. Solid Line: Approximate Line of Dots Intersected by Leverage of Promoter-Specific mRNA and Leverage Residue of Predicted Total *ERα*; Dotted Curves: 95% CI. The Horizontal Axis Indicates the Leverage Residues of Promoter-Specific Transcripts, and the Vertical Axis Indicates That of the Total *ERα* Transcripts. The Unit of the Vertical Axis is Logarithm of Promoter-Specific Total ERα mRNA Normalized to β-Actin



Abbreviations: proA = promoter A; proC = promoter C; proD = promoter D.

normal development and differentiation or carcinogenesis of breast cancer. Furthermore, the investigation of another cancer tissue with ERα expression (eg, endometrium) could confirm the biological significance of promoter choice.

These results also suggested that *ERα* transcription in breast cancer tissue mainly originated from the most proximal promoter and that more distal promoters were additionally utilized. However, the distance from the most proximal promoter to the most distal one is about 4 kbp; only the mechanism by which identical trans-factors were used in proportion to distance for initiation of *ERα* transcription from each promoter could not account for *ERα* transcription in ER^+ breast cancer tissues. Therefore, epigenetic dynamics might be associated with *ERα* transcription in breast cancer tissues. Because $ERα^+$ cell lines (MCF-7, T-47D, and ZR-75-1) showed very similar promoter choices for the ERα gene (Fig. 2), we analyzed the methylation status of CpG islands in regions from promoter A to promoter C in $ERα^+$ breast cancer cell lines, using the direct sequence method. The methylation status of CpG islands in these promoter regions was found to be different among these cell lines (data not shown), implying that methylation of CpG islands in the promoter regions of ERα gene could not fully account for the promoter use of *ERα*. Histone modulation might be associated with *ERα* transcription in breast cancer tissues, but this hypothesis needs further study.

ERα has at least 2 variants, the 46-kDa ERα (ERα46)[27] and the 36-kDa ERα (ERα36),[28] and these variants are prognostic factors.[29,30] We analyzed the transcripts of ERα46 because its mRNA had the same 5′-UTR of transcripts from promoter E and F and lacked only exon 1 among normal ERα exons (see Supplemental Fig. 1A in the online version at http://dx.doi.org/10.1016/j.clbc.2013.10.015). Our results indicated that the transcripts of ERα46, originating from both promoter E and F, were negligible in

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Table 1 Patients' Clinicopathological Information

Age, years	Median	59.4	(40.2-87.2)
Menopause status	post	29	69.0%
	pre	13	31
	no data	1	
Cancer stage	I A	19	44.2%
	I B	1	2.3
	II A	13	30.2
	II B	8	18.6
	III A	2	4.7
ER IHC, Allred score	8	24	66.7%
	7	8	22.2
	6	3	8.3
	0	1	2.8
	no data	7	
PR (Allred)	8	11	30.6%
	7	8	22.2
	6	5	13.9
	5	6	16.7
	3	3	8.3
	0	3	8.3
	no data	7	
HER2 IHC, Allred score	3	2	5.6%
	2	6	16.7
	1	22	61
	0	6	16.7
	no data	7	
Lymph/vascular invasion	ly 2	6	13.9%
	1	18	41.9
	0	19	44.2
	v 2	1	2.3
	1	11	25.6
	0	31	72.1
Nuclear grade	3	18	41.9%
	2	13	30.2
	1	12	27.9
Nuclear Atypia	3	8	18.6%
	2	34	79.1
	1	1	2.3
Mitotic index	3	15	34.9%
	2	16	37.2
	1	12	27.9
Node metastasis	negative	29	69.0%
	positive	13	31
	no data	1	
Histology	papillotubular	11	25.6%
	solid-tubular	5	11.6
	scirrhous	18	41.9
	special type	9	20.9
E2 concentration in plasma, pg/mL	average	39.25	(22.8-208.9)

Estrogen receptor (ER) and progesterone receptor (PR) positivity of patients without ER and PR Allred scores were estimated as strong, moderate, weak, or none. Patients whose ER and PR scores could not be obtained were excluded.

the ERα-positive breast cancers (see Supplemental Fig. 1B in the online version at http://dx.doi.org/10.1016/j.clbc.2013.10.015). When the ERα46 transcripts were compared with those from promoter A, they were at most 1/400 in number of those originating from promoter A. In other words, cycle values exceeding threshhold for ERα46 transcripts were > 33, suggesting that the

amount of ERα46 transcripts was too little to evaluate the correlation with the clinicopathological factors of ERα+ breast cancer.

In this study, we found out that *ERα* transcription used the same promoter choice as promoter A, which was significantly ($P < .05$) associated with mRNA expression of the *ERα* gene in individual breast cancer.

Conclusion

We have investigated the transcriptional regulation of *ERα,* but the mechanism of the regulation remains to be discovered. In this article, we reinvestigated variations in the use of > 3 *ERα* promoters in breast cancer tissues and breast cancer cell lines with an eye toward using *ERα* promoter usage as a new biomarker, and found that the *ERα* promoter usage of ERα$^+$ breast cancer tissues and cell lines were similar, and the similarity was validated by examinations using correlation among transcripts from each promoter and that of total *ERα* and relation to clinicopathological factors. Although the likelihood of using *ERα* promoter usage in breast cancer tissues as a clinical biomarker was small, this article is meaningful in presenting the possibility that *ERα* promoter usage might be important for individual development, differentiation, or carcinogenesis, and that the biological meaning of *ERα* promoter usage could be discovered by comparison of the promoter usage in breast cancer cell lines with the promoter usage of other cancer tissues with ERα positivity.

Clinical Practice Points

- The *ERα* gene has at least 6 transcription start sites and 6 distinct first exons. It also probably has 6 promoters, which is unusual for functionally discovered nuclear receptors.
- Typical tissue promoter usages in cancer cell lines and normal tissues were found, using an ERE luciferase assay and quantification of promoter-specific mRNA of *ERα*.
- In this article, we investigated *ERα* promoter usage in individual breast cancer with an eye toward using *ERα* promoter usage as a new biomarker, using a real-time PCR method with primers and probes designed especially for this assay. We found that the *ERα* promoter usages of ERα$^+$ breast cancer tissues and cell lines were similar, and the similarity was validated by examinations using correlation among transcripts from each promoter and that of total *ERα* and relation to clinicopathological factors.
- Although the likelihood of using *ERα* promoter usage in breast cancer tissues as a clinical biomarker was small, this article is meaningful in presenting the possibility that *ERα* promoter usage might be important for individual development, differentiation, or carcinogenesis.

Disclosure

The authors have stated that they have no conflicts of interest.

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Supplemental Table 1 The Sequence of Primers and Probes Used in This Study

Primer List	Sequence (5'→3')
ERα Promoter A Forward Primer (F1)	CTGTGCTCTTTTTCCAGGTG
ERα Promoter B Forward Primer (F2)	CAGCGACGACAAGTAAAGTG
ERα Promoter C Forward Primer (F3)	GTTCTTGATCCAGCAGGGTG
ERα Promoter D Forward Primer (F4)	CACCTGAGAGAGCCAGTG
ERα Promoter Common Reverse Primer (R1)	AGGGTCATGGTCATGGTC
ERα Promoter E Forward Primer (F5)	ACCAATCCTTTTGATTGTGAA
ERα Promoter F Forward Primer (F6)	GCATAAGAAGACAGTCTCTGAGTGA
ERα Promoter Common Reverse Primer (R2)	GGCAGAAGGCTCAGAAACC
ERα Promoter Common Probe (P1)	CCGGTTTCTGAGCCTTCTGCCC
ERα Promoter Common Probe (P2)	ACATTCTCCGGGACTGCGGTACCA
Total ERα Exon7 Forward Primer (F7)	CTCCCACATCAGGCACAT
Total ERα Exon8 Reverse Primer (R3)	CTCCAGCAGCAGGTCATA

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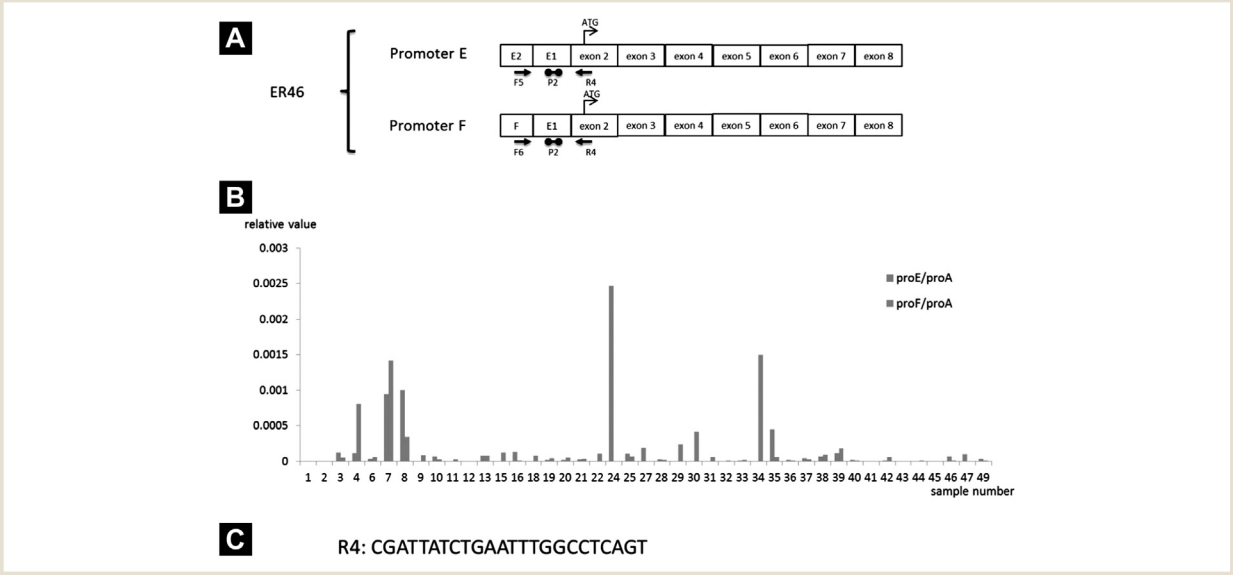
Supplemental Figure 1 **Analyses of ER Alpha Variants With This Promoter-Specific Method. (A) Exon Structure of the ERα Variant ERα46. This Messenger RNA (mRNA) Originates From the Same Promoters, E and F, as Those of Normal *ERα* Gene. Two 5′-UTR Exons of This mRNA Were Directly Spliced to Exon 2, but not to Exon 1. (B) The Real-Time Polymerase Chain Reaction Analysis of *ERα46* Transcripts. The Amount of *ERα46* Transcripts Originating From Both Promoter E and F was Normalized to That of the Transcripts From Promoter A. The Horizontal Axis Indicates the Identification Number of Breast Cancer Samples and Vertical Axis Indicates the Levels of Transcripts Originating From Both Promoter E and F, Relative to Those Originating From Promoter A. (C) The Sequence of the Reverse Primer Designed for the Analysis of *ERα46***



Abbreviations: proA = promoter A; proE = promoter E; proF promoter F.

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