3-Dimensional Microarray Analysis of Estrogen Signal-related Genes in Breast Cancer Tissues

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Abstract. *Background: Expression analysis of estrogen response genes (ERGs) may help to predict the effectiveness of endocrine therapies in breast cancer patients. We produced a custom-made, 3-dimensional microarray system (3DMS), using previously identified ERGs, and analyzed expression of ERGs on breast cancer tissues. Materials and Methods: aRNA was synthesized from 27 breast cancer tumors. The aRNAs were applied to the infrastructure of a 3DMS with spotted cDNA probes to 36 ERGs. Data were analyzed by cluster analysis. Results: All 27 specimens were classified clearly into one of two clusters based on whether the ERGs were up or down regulated. These groups correlated with high expression of ER (P<0.05) and Her2 (P<0.05). In some cases, ERG expressions were low even though the tumor was ER positive. Conclusion: 3DMS may be useful for accurately predicting prognosis and whether endocrine therapies targeting the ER would be effective in an individual breast cancer patient.*

Estrogen plays an important role in the carcinogenesis, growth and development of breast cancer. Thus, interception of estrogen signals is a standard strategy for endocrine therapy of breast cancer patients (1). The four currently recognized classes of endocrine-therapeutic agents available for treatment are antiestrogens, aromatase inhibitors (AI), luteinizing hormone-releasing hormone (LH-RH) agonists and progestins (2). Because the expression of the estrogen receptor (ER) in patients with primary breast cancer is one of the most powerful indicators of response to adjuvant hormonal therapy, the effects of endocrine-therapeutic agents strongly depend on whether tumor cell growth remains estrogen responsive (3).

The ER is activated by estrogen binding and also by phosphorylation through the PI3K/Akt pathway (4). Activated ER binds to estrogen-responsive elements (EREs), which are located in the promoter regions of various target genes. It activates transcription and contributes to proliferation, antiapoptosis, and metastasis of tumor cells (5). Many investigators consider the expression of estrogen-responsive genes (ERGs) to be evidence of an activated, functional ER (6, 7).

ER-positive breast cancer patients have a 47% reduction in recurrence risk following five years of treatment with adjuvant tamoxifen (TAM). TAM, however, does not appreciably reduce recurrence in most patients with ER-negative tumors (3). However, adjuvant TAM is effective in some ER-negative breast cancer patients who express progesterone receptor (PgR), an ERG.

Examination of the expression levels of various ERGs, by alluding to the presence of an activated ER, may identify individual patients who might benefit from treatments targeting the ER. However, application of a conventional microarray is limited given the assay's operative complexity and low sensitivity. We selected the ERGs for this study from our previously developed custom-made breast cancer cell cDNA glass array (6).

Recently, PamGene International B.V. and Olympus Corporation developed the new, unique, PamChip and FD10 microarray system. The PamChip for kinetic hybridization reactions is a three-dimensional flow-through platform that has long branching capillaries that bind probe DNA (8). This enables the system to sensitively detect signals from as little as 20 ng aRNA. Moreover, the FD10 microarray system automates

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and integrates pumping of the sample solution for hybridization, solution-driven incubation, and image acquisition and analysis functions (8). Hybridization time in this system is 2 hours, an improvement over the 16 hours required with a standard glass array. This system has already been used successfully in several studies for gene expression and mutation analysis (8, 9). Here we investigated whether a custom 3D microarray of 36 ERGs could classify a set of breast cancer tumors.

Materials and Methods

Tissue samples. For the microarray analysis, we surgically obtained 27 breast cancer tissue samples from patients undergoing treatment at the Saitama Cancer Center Hospital (Saitama, Japan) from 25/8/2003 - 6/11/2003. All patients provided informed consent. The clinicopathological classifications of the 27 patients are summarized in Table I. We confirmed ER and PgR status with immunohistochemistry. The ER was detected with a monoclonal ERa antibody 1D5 (M7047; DakoCytomation, Glostrup, Denmark); PgR was detected with a monoclonal PgR antibody PgR 636 (M3569; DakoCytomation). Immunointensity was graded based on Allred scoring (10). Her2 positivity was also assessed using the Hercep Test (DakoCytomation) and the results scored as 0, 1, 2, and 3, according to the manufacturer's instructions. Specimens with a score of 2 or 3 were classed as Her2 positive and those with scores of 0 or 1+ were Her2 negative. Histological grading was evaluated according to the modified and simplified Bloom-Richardson grading scheme (11). Fixation, serial, sectioning paraffin embedding, H&E staining and immunohistochemical staining were carried out as described previously 12).

Production of the custom-made aRNA microarray chip. To screen for estrogen response activity of breast cancer tissues, a total of 36 genes were selected from a set showing up- or down-regulation following estrogen stimulation in a previous study (6) (Table II). The 36 genes contained estrogen metabolism enzyme-related genes, ER transcriptional activity-related genes and 11 genes that were able to divide ER-positive patients into two groups in our previous study (13). The PamChip (Pamgene, the Netherlands) was used as the infrastructure of the custom-made microarray in this study. Sixty-mer single-strand oligoDNA probes for the ERGs were spotted onto the PamChips (Olympus Corporation, Tokyo, Japan).

Preparation of aRNA. Tissue samples were immersed in liquid nitrogen then ground, while frozen, with a pestle into fine granules. Total RNA was extracted from the samples using an ISOGEN kit (Invitrogen, USA) according to the manufacturer's instructions. FITC-labeled aRNA was synthesized using MessageAmp aRNA kit (Ambion Inc., TX, USA). Briefly, for the first-strand cDNA synthesis, 2 μg of total RNA isolated from the breast cancer tissues were reverse-transcribed in the presence of a T7 Oligo(dT) primer and multiple copies of aRNA were synthesized in the presence of FITC-UTP.

Hybridization. Microarray hybridization against the ERG set was performed using the 3D microarray system FD10 (Olympus Corporation), according to the manufacturer's instructions. Briefly, 5 μg of FITC-labeled aRNA were fragmented at 70˚C for 15 min in Fragmentation Reagent (Ambion Inc.). To concentrate the RNA and remove the fragmentation reagent, the aRNA solution was applied to a Microcon YM-30 column (Millipore, MA, USA), 37.5 μl distilled water added, and the aRNA was denatured by treatment at 95˚C for 5 min followed by cooling on ice. Hybridization buffer (7.5 μl of 3xSSPE and 5 μl of 1% SDS) was added to the aRNA solution and the aRNA solution hybridized to a PamChip containing the estrogen response gene cDNA spots. Hybridization was performed at 42˚C for 150 cycles in the FD10. The hybridized PamChip was then washed three times with wash buffer (3xSSPE, 0.5% L-sarcosine) at 37˚C, followed by incubation with blocking buffer (BSA) for 5 min at 37˚C in the FD10.

Scanning and data analysis. The scanning and quantitative values analyses of the fluorescent signals were performed with the FD10 according to the manufacturer's instructions. Data were processed with Microsoft Excel software (Microsoft, Redmond, WA, USA). We normalized each fluorescent signal value using the median of RPS7, the internal control, and transformed the values to log2. Spots with a low fluorescence signal (lower than 100) were omitted.

The processed data were clustered using average-linkage hierarchical clustering with the CLUSTER program (14) and the results visualized with the TREEVIEW program (14).

Statistical analyses of data were carried out using the Mann-Whitney U test for comparison of two independent groups with StatFlex V5.0 software (Arttech, Inc., Osaka, Japan). We considered a p-value of 0.05 to be statistically significant.

Results

To determine individual differences in estrogen response of breast tumors, we examined the expression levels of 36 estrogen-regulated or -related genes identified in previous studies (6, 13). As shown in Figure I, the cluster analysis clearly classified all 27 breast cancer patients into one of two distinct groups based on ERG expression patterns.

Next, we completed a statistical analysis of the clinicopathological variables (age, menopausal status, tumor diameter, ER, PgR, Her2, stage, histology, grade, and nodal status) between the high ERG expression and low ERG expression groups. As shown in Table II, three patients (4, 13 and 20) had confirmed recurrence and metastasis within three years of surgery. ER-positive cases abounded in the high ERG expression group as compared with ER-negative cases ($p<0.05$, Mann-Whitney U-test). Cluster analysis classified nearly all of the Her2-negative cases into the low ERG expression group ($p<0.05$, Mann-Whitney U-test). Furthermore, stage was significantly related to ERG expression by the Mann-Whitney U-test ($p<0.05$). Age, menopausal status, tumor diameter, PgR, stage, histology, grade, and nodal status were not related to ERG expression. This result showed that not only the presence of the ER but also the presence of the Her2 were important in the expression of ERGs.

Three out of 27 patients (case no. 4, 13, and 29) had a documented relapse during the study period. Cases no. 4 and 13 had recurrence about three years after surgery in the contralateral breast and as a metastasis to the liver, respectively. Case no. 29 died from lung metastasis that

Table I. *Correlations between clinical data and expression of ERGs.*

	Expression of ERGs		Mann-Whitney *U*-test (*p*-value)
	High	low	
Total patient number	12	15 (3*)	
Age (years)			
<50	2	5	0.250
>50	10	10	
Menopausal state			
Post	10	9	0.195
Pre	2	6	
Tumor diameter			
<2 cm	9	6	0.743
>2 cm	3	9	
Nodal status			
Negative	6	7	0.956
Positive	6	8	
ER Score			
0	1	4 (2*)	**0.012**
5	0	1	
6	1	2 (1*)	
7	1	5	
8	9	3	
PgR Score			
0	3	4	0.548
4	0	2	
5	2	2	
6	3	4	
7	4	2	
8	0	1	
Her2 Score			
0	1	8 (1*)	**0.044**
1	7	5 (1*)	
2	1	0	
3	2	2 (1*)	
Unknown	1	0	
Stage			
0	1	0	**0.034**
I	7	4	
II	4	11 (3*)	
Histological grade			
1	4	5	0.629
2	1	2	
3	7	6	
Unknown	0	2	

*Number of patients with recurrence or metastasis.

occurred two years after surgery. Patients with breast cancer that is ER negative by immunopathology generally have a poor prognosis. Two patients with recurrences (case no. 4 and 29) had ER-negative tumors and the expressions of the ERGs were low in these cases. Tumor in one patient (case no. 13) was ER positive; however, expression of ERGs was low (Table I). Therefore, these results may suggest that activation of the ER is more important than its overall abundance in terms of its effect on prognosis.

Table II. Estrogen response genes.

	Definition (ERE responsive genes)	Accession no.
1	Amplified in breast cancer	AF012108
2	Prostate differentiation factor	NM_004864
3	Cathepsin D	X05344
4	Estrogen receptor beta	NM_001437
5	Neuropeptide Y receptor Y1	NM_000909
6	Histone deacetylase 6	NM_006044
7	Fas ligand	NM_000639
8	IGF 1R	NM_000875
9	Phorbol-12-myristate-13-acetate-induced protein 1	BC032663
10	SRC-1	NM_147223
11	TGFβ-inducible early growth response	NM_005655
12	17β-HSD type 2	NM_002153
13	Progesterone receptor	NM_000926
14	Cyclin D	NM_053056
15	MYC promoter-binding protein 1	NM_005848
16	Insulin-like growth factor-binding protein 5	NM_000599
17	17β-HSD type1	NM_000413
18	Early growth response 3	NM_004430
19	Solute carrier family 26 member3	NM_000111
20	Nuclear receptor interacting protein 1	NM_003489
21	Estrogen receptor alpha	NM_000125
22	Estrogen sulfotransferase	NM_005420
23	NGFI-A binding protein 2	NM_005967
24	Erb B2	NM_004448
25	Estrogen finger protein	NM_005082
26	Estrogen sulfatase	NM_000351
27	Retinoblastoma-binding protein 8	NM_002894
28	ACTB	NM_001101
29	Fos-like antigen 2	NM_005253
30	Insulin-like growth factor-binding protein 4	NM_001552
31	Cyclin A1	NM_003914
32	Vascular endothelial growth factor	AF022375
33	Heat-shock 70 kDa protein 5	NM_005347
34	Inhibitor of DNA binding 4	NM_001546
35	Trefoil factor 1	NM_003225
36	RPS7	NM_001011

Discussion

Recently, several technologies such as cDNA and oligonucleotide arrays and multiplex PCR have been developed to screen for the expression of multiple genes simultaneously. Gene expression analysis has the potential to identify targets to which specific treatments may be tailored. Microarray analysis is particularly useful since the expression of thousands of genes can be investigated simultaneously. Microarray analysis has not been widely used clinically because a large quantity of high quality RNA is required and the experimental procedures are extremely complex. However, the application of microarray analysis to the design of highly individualized cancer treatments is not out of the realm of possibility. OLYMPUS

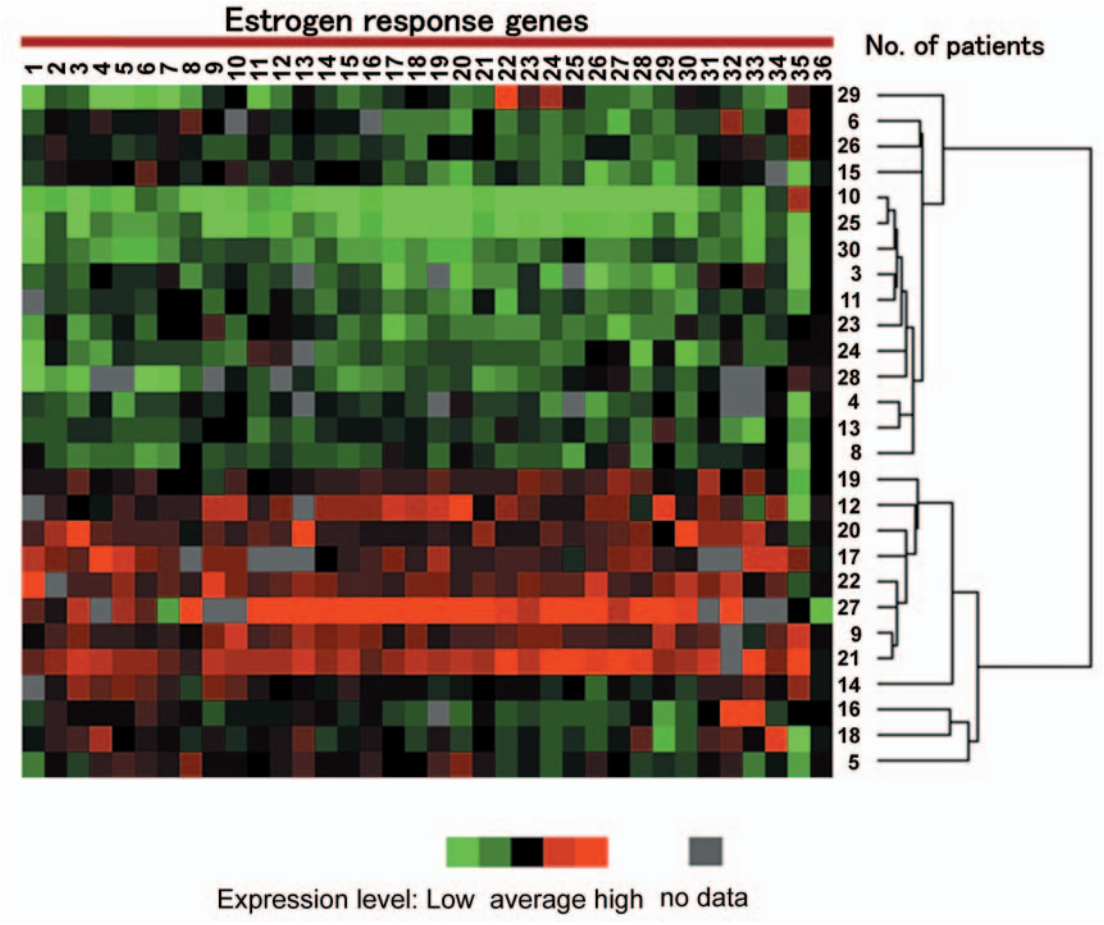

Figure 1. *Cluster analyses of the estrogen response gene (ERG) expression levels in 27 breast cancer patients. Expression analysis of 36 ERGs in 27 breast cancer tissue samples performed using the FD10 microarray system. Microarray data were analyzed using average-linkage hierarchical clustering with the CLUSTER program and visualized the results using the TREEVIEW program. Patient characteristics and the 36 ERGs are listed in Tables I and II, respectively.*

has developed a 3D microarray system with convenient handling and operation that has improved sensitivity over conventional arrays (8, 9).

Our previous study examining the expression of 11 ERGs with real-time RT-PCR of ER-positive tumors classified the tumors into two distinct clusters (13). Therefore, in the current study, we focused on cases in which the expression levels of the ERGs were low even though ER expression was positive. ER protein expression level is one of the most important factors for treatment selection as patients with ER-positive breast cancer receive endocrine therapy. Adjuvant TAM has only reduced the recurrence risk in ER-positive patients by about 50% (13, 15). Interestingly, the ERG expression analysis in this study classified 48% of patients with ER-positive tumor into the high expression group, which means that only about 50% of patients with ER-positive tumor are estrogen sensitive. This system might be able to select the patients who will benefit from endocrine therapy.

Her2 is a member of the ErbB family and activated-ErbB stimulates the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)-Akt pathways (16). Not only estrogen stimulation but also protein phosphorylation by kinases activates the ER (4). The phosphorylation in these pathways from Her2 may enhance the activity of the ER. We believe that ER activation is more important than the absolute ER protein expression level in predicting the effect of hormone therapy and prognosis. For example, if a tumor has abundant ERs, but none of the ERs are activated, then endocrine therapies such as selective estrogen receptor modulators (SERMs) and aromatase inhibitor (AI) will be ineffective. Conversely, if activated ERs are present even at a very low level, endocrine therapy may be effective.

Here we showed that a 3D microarray system was able to analyze the expression of ERGs as a surrogate measure of ER activation. Thus, the 12 patients with high expression

levels of ERGs regardless of ER status likely would have benefitted from endocrine therapies targeting the ER. In contrast, those with low ERG expression levels may have not obtained significant endocrine therapeutic benefit. This 3D microarray analysis of ERG expression may be used as a strategic model for treatment screening of the individual patient with breast cancer. We have already reported that the individual measurement of expression levels of 4 ERG proteins (insulin-like growth factor-binding protein 4 (13, 17), insulin-like growth factor-binding protein 5 (17), histone deacetylase 6 (13) and early growth response protein 3 (18)) may be used to predict prognosis.

Patients with ER-positive breast cancer generally have a better prognosis than these with ER-negative cancer. Indeed, 2 out of the 3 patients with recurrence in this study had ER-negative tumors (Table I). However, ERG expression analysis classified all 3 of these patients into the low ERG expression level group (Table I). Thus, this analysis not only assesses the benefit from endocrine therapy, but may also predict the likelihood of breast cancer recurrence. A larger study evaluating new patients, with additional follow-up of those included in this study, may contribute to the development of a new method of characterizing breast cancer tumors that will not only facilitate the individualization of chemotherapy, but also improve the overall understanding of breast cancer pathophysiology.

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