Chemosensitivity-related Genes of Breast Cancer Detected by DNA Microarray

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Abstract. *Background: The feasibility of a preoperative docetaxel/5'-deoxy-5-fluorouridine (5'-DFUR) regimen for breast cancer patients was examined and the genes related to the response to it was investigated. Patients and Methods: Women with advanced breast cancer were treated with docetaxel (60 mg/m^2, day 1) and 5'-DFUR (800 mg/day, on days 1-14) q3 weeks by 4 cycles. Microarray analysis was carried out using preoperative core biopsy samples. Based on the mRNA expression levels, genes related to clinical and pathological responses were selected. Results: The docetaxel/5'-DFUR regimen showed a 86% clinical response rate including 42% complete response, one pathological complete response and one ductal carcinoma in situ component. In microarray analysis, we identified 6 genes, including IGF-1, and derived a predictive formula with 67% accuracy. In addition, χ^2 analysis revealed a tendency for good response in ER-negative and Her2/neu-positive cases. Conclusion: Microarray analysis enabled us to predict the pathological response to docetaxel/5'-DFUR chemotherapy.*

In terms of disease-free and overall survival rates, postoperative (adjuvant) and preoperative (neoadjuvant) chemotherapy are equally effective in early breast cancer (1). However, neoadjuvant chemotherapy has led to an increase in breast-conserving surgery, as well as information on sensitivity to chemotherapy (2). In addition, tumor response to this therapy correlates with patient outcome and could be a surrogate for evaluating the effect of chemotherapy on micrometastasis (3). Thus, it contributes to the elucidation of predictive factors of chemotherapeutic agents. The mechanisms of cytotoxic agents are complicated and, therefore, none of the predictive factors has yet been established as a single factor for a particular chemotherapeutic agent.

The cDNA microarray technique can analyze thousands of genes at one time leading to a comprehensive analysis of the mechanism of action of a chemotherapeutic agent and there have been many reports on the results of cDNA microarray analyses (4-8). Chemotherapeutic agents are usually not used as a single agent but in combination and it is necessary to perform cDNA analysis on each regimen to clarify predictive factors. The combination of 5-fluorouracil (5-FU) and taxanes, including docetaxel, is widely used for breast cancer patients and shows good clinical and pathological response. It has been reported that docetaxel with capecitabine (N4-pentoxylcarbonyl-5'-deoxy-5-fluorocytidine) is a highly active regimen in the neoadjuvant setting with 31% complete response (pCR) (9). In this study, we performed cDNA microarray analysis to obtain predictive factors of a regimen of docetaxel with 5'-deoxy-5-fluorouridine (5'-DFUR), an intermediate of capecitabine with a proven effect in combination *in vivo* (10).

Patients and Methods

Patient eligibility criteria. Women with newly diagnosed locally advanced breast cancer (tumor size >3 cm) selected to participate in this study met the following eligibility criteria: no previous treatment, Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, age over 20 years, adequate organ function and no severe complications. Written informed consent was obtained from all patients and the trial was approved by the ethical committees of the institutions taking part. Physical examinations, diagnostic imaging and a core needle biopsy providing material for microarray analysis, as well as histopathological diagnosis, were performed prior to the preoperative systemic chemotherapy.

Treatment. A combination regimen for the primary systemic chemotherapy consisted of docetaxel and 5'-DFUR. 5'-DFUR was administered orally at a dose of 800 mg/day on days 1 to 14, and docetaxel was administered intravenously at a dose of 60 mg/m^2 on day 8 and subsequently every 3 weeks for 4 cycles.

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Efficacy and toxicity assessment. Clinical responses of the primary tumors to the chemotherapy regimen were evaluated according to the criteria established by the Japanese Breast Cancer Society (11), which are essentially the same as those of the World Health Organization (12). Pathological response was also evaluated according to the Japanese Breast Cancer Society classification criteria (11) of Grade 0 (no histological change in the cancer cells), Grade 1a (mild cellular injury in a proportion of the cancer cells, or severe cellular injury or replacement of less than 1/3 of the cancer cells by fibroblasts, histiocytes, or fibrosis), Grade 1b (severe cellular injury or replacement of 1/3 to 2/3 of the cancer cells), Grade 2 (severe cellular injury or replacement of 2/3 or more of the cancer cells) and Grade 3 (no cancer cells, or necrotic or non-viable residual cancer cells).

Evaluation of gene expression. After 4 cycles of the preoperative systemic chemotherapy and curative surgery, which provided a resected sample specimen, DNA microarray analysis was performed. Total RNA was extracted from the fresh frozen needle biopsy tissues of breast carcinoma before administration of treatment with docetaxel and 5'-DFUR. The RNA was reverse-transcribed to cDNA using T7-(dT)24 primer (Operon, Tokyo, Japan). Biotin-labeled cRNA was synthesized from cDNA using a MEGAscript In Vitro Transcript Kit (Ambion; Austin, TX, USA), and then hybridized to human U95Av2 GeneChip® (Affymetrix; Santa Clara, CA, USA). The hybridized oligonucleotide microarrays were scanned using a confocal scanner (Affymetrix) and analyzed using Affymetrix software (LIMS 5.0).

For elucidation of docetaxel/5'-DFUR treatment on gene expression, 17 genes were selected according to the criteria of >2 or $<1/2$ of mean signal ratio between pre and post samples and $p<0.05$ (Welch's *t*-test). The relationship between gene expression levels and the ratio of reduced tumor size was calculated using Pearson's correlation formula. Genes with an absolute R value >0.7 and $p<0.01$ were considered to be significant. The data from the 22 genes found to match the criteria were applied to Eisen software (13) for supervised hierarchical clustering and visualized by TreeView software (13).

Support Vector Machine. Microarray data from 19 patients were randomly divided into learning (13 patients) and validation (6 patients) sets. Log-transformed learning data were used as a training set to establish a gene expression profile that would predict pathological grades (pGr) of 3 or 2 (effective), or 1 or 0 (ineffective). The Support Vector Machine (SVM) algorithm (14) using a linear kernel was applied in combination with recursive feature elimination (15) to build a predictive model. The linear kernel SVM algorithm elements are weight (w) and b applied to the linear predictor formula of $f(x) = wx + b$ where x is the input vector of a gene expression measurement of a tumor sample, f(x) is a continuous predictive score and b is the intercept value of the Y axis. In order to determine the accuracy of this formula, another set of microarray data (6 patients) were investigated for cross-validation.

Statistics. Statistical analysis was performed using Microsoft Excel software and StatView 5.0 (SAS Institute Inc., Cary, NC, USA) on a Windows PC. A *p*-value below 0.05 was considered to be of statistical significance.

Table I. *Patient characteristics and histological type of tumor.*

	Total
Age (range; years)	52 (30-68)
Menopausal status	
Pre	6
Post	13
ER	
Positive	13
Negative	6
PR	
Positive	4
Negative	15
Her2	
IHC3+	6
IHC2+	2
Negative	11
Tumor size before treatment (cm)	5.4
Clinical response	
Complete	9
Partial	7
Stable disease	3
Progressive disease	0
Surgical method	
Total mastectomy	12
Breast conserving	7
Pathological response	
Grade 2, 3	10
Grade 0, 1	9

Results

Nineteen patients were enrolled in this trial. Patient characteristics are described in Table I. The median age was 52 years old and mean tumor size before chemotherapy was 5.4 cm. The clinical response rate was 86%, with 42% complete response. Pathological complete response was seen in one patient and another patient revealed only ductal carcinoma *in situ* (DCIS) component.

Gene expression patterns of core biopsies and surgical specimens. Gene expression patterns of the surgical specimens showed no correlation with the core biopsy specimens (Figure 1). The dentrogram shows that preoperative samples and postoperative samples both tended to cluster. ER-positive samples showed a representative tendency of this pattern (four out of six). The location on the dentrogram of two sample sets (3, 13) shows positions nearest each other but there was no similarity in clinical response or pathological response in these two patients. It was not possible to obtain surgical specimens and thus no comparison of the DNA signature between the core biopsy sample and the surgical specimen in cases with

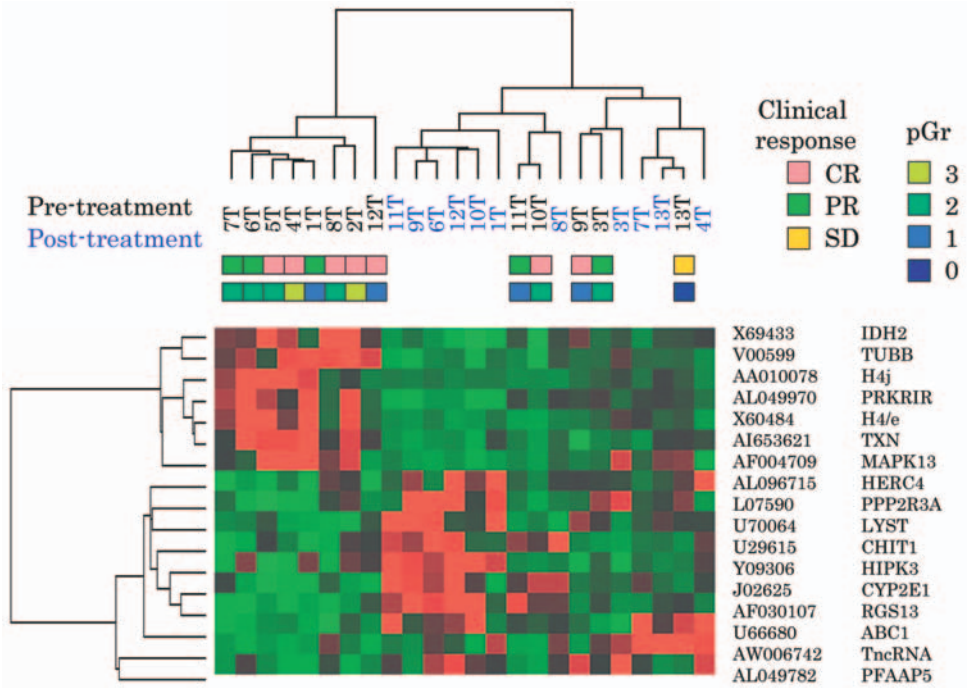


Figure 1. *Comparison of gene expression profiles of docetaxel/5'-DFUR pre- and post-treatment samples. Supervised hierarchical clustering analysis was applied to 17 genes selected.*

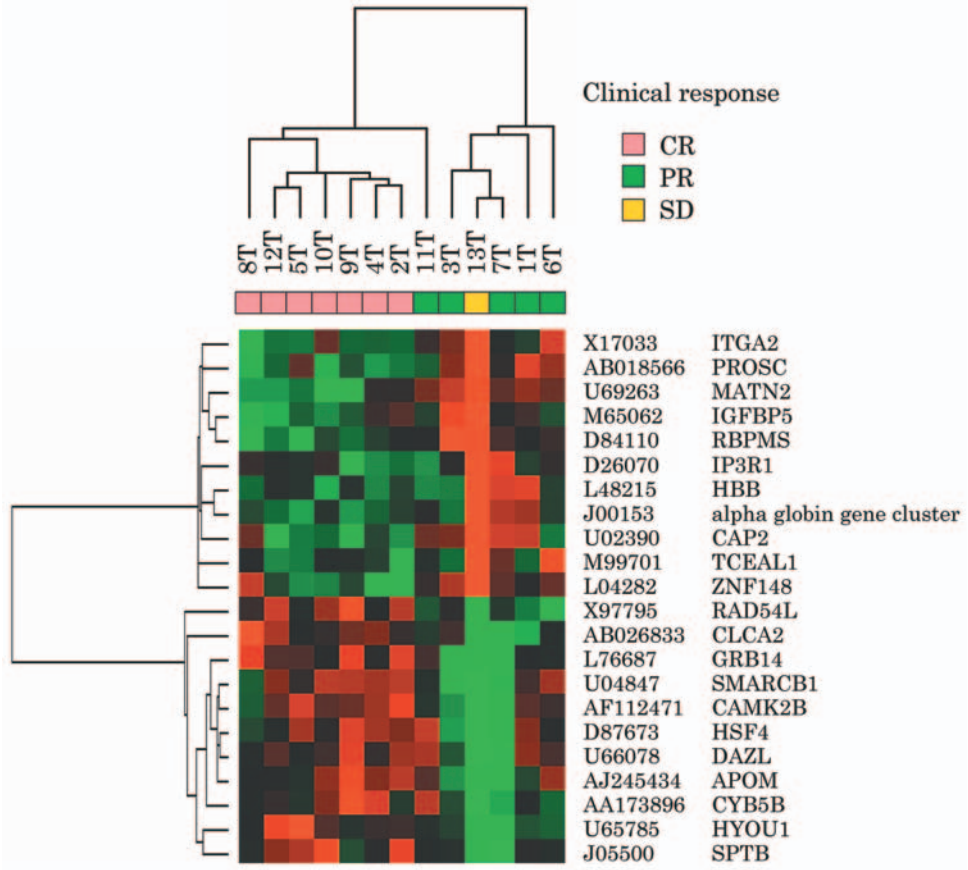


Figure 2. *Gene expression profiles based on pathological grade. A total of 22 genes with a correlation of absolute R value >0.7 and p<0.01 were selected.*

Table II. *List of genes correlated with tumor reduction.*

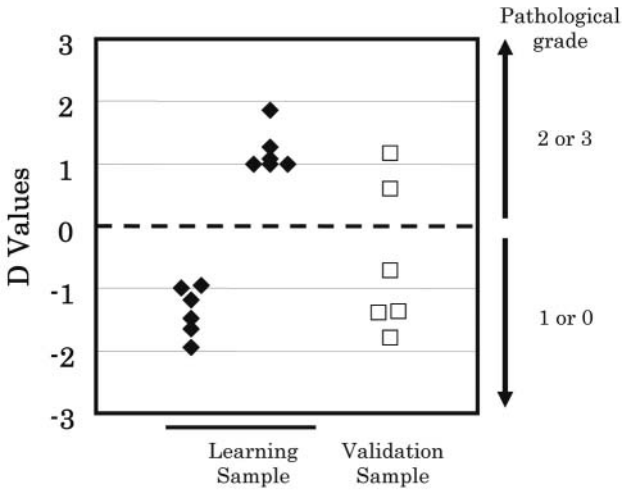
GenBank #	Symbol	Gene name	R	*P*-value
AF112471	CAMK2B	Calcium/calmodulin-dependent protein kinase II beta	0.8319	0.0015
AB026833	CLCA2	Chloride channel, calcium activated, family member 2	0.8175	0.0021
L76687	GRB14	Growth factor receptor-bound protein 14	0.7973	0.0033
J05500	SPTB	Spectrin, beta, erythrocytic	0.7750	0.0051
U04847	SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	0.7743	0.0051
AJ245434	APOM	Apolipoprotein M	0.7673	0.0058
U65785	HYOU1	Hypoxia up-regulated 1	0.7662	0.0060
AA173896	CYB5B	Cytochrome b5 outer mitochondrial membrane precursor	0.7643	0.0062
U66078	DAZL	Deleted in azoospermia-like	0.7632	0.0063
X97795	RAD54L	RAD54-like (*S. cerevisiae*)	0.7581	0.0069
D87673	HSF4	Heat shock transcription factor 4	0.7457	0.0084
M65062	IGFBP5	Insulin-like growth factor binding protein 5	–0.7389	0.0094
M99701	TCEAL1	Transcription elongation factor A (SII)-like 1	–0.7505	0.0078
D84110	RBPMS	RNA binding protein with multiple splicing	–0.7577	0.0069
L04282	ZNF148	Zinc finger protein 148 (pHZ-52)	–0.7808	0.0046
D26070	IP3R1	Inositol 1,4,5-triphosphate receptor, type 1	–0.7811	0.0045
X17033	ITGA2	Integrin, alpha 2	–0.8039	0.0029
L48215	HBB	Beta-globin	–0.8126	0.0024
U69263	MATN2	Matrilin 2	–0.8127	0.0024
AB018566	PROSC	Proline synthetase co-transcribed homolog (bacterial)	–0.8136	0.0023
U02390	CAP2	CAP, adenylate cyclase-associated protein, 2	–0.8172	0.0021
J00153		Alpha globin gene cluster on chromosome 16- zeta gene	–0.9007	0.0002

Table III. *Genes to predict pathological response identified by Support Vector Machine (SVM).*

GeneBank#	Gene name	D' value
X57025	IGF-I	0.850
AI535828	Jumping translocation breakpoint	0.572
AF004230	Leukocyte immunoglobulin-like receptor	0.548
L48215	Beta globin (HBB)	–0.505
	Chorionic Somatomammotropin Hormone Cs-5	–0.679
AI953789	Matrix Gla protein	–0.841

pathological complete response was made. Clustering analysis showed there are two types of genes, up-regulated and down-regulated, after neoadjuvant chemotherapy.

Genes predicting clinical and pathological response. To search for genes associated with clinical responses, Pearson's correlation (R) values were determined between gene expression levels and the ratio of reduced tumor size in patients after treatment with docetaxel and 5'-DFUR; 11 positively correlated genes and 11 negatively correlated genes were selected (Figure 2, Table II). We next examined microarray data (13 patients) to clarify predictive factors for pathological grade using SVM, since it has been reported that pathological responses do not



Validation sample#	Predict values	Patholigical grade	Judge
#1	1.161	3	True
#2	−1.402	2	False
#3	−0.725	0-1	True
#4	−1.803	0-1	True
#5	−1.376	0-1	True
#6	0.590	1	False

Figure 3. *Predictive scores of learning and validation samples using SVM for 6 genes. Positive scored values indicate 'effective (pathological grades 2 or 3)' and negative scored values indicate 'ineffective (pathological grade 1 or 0)'.*

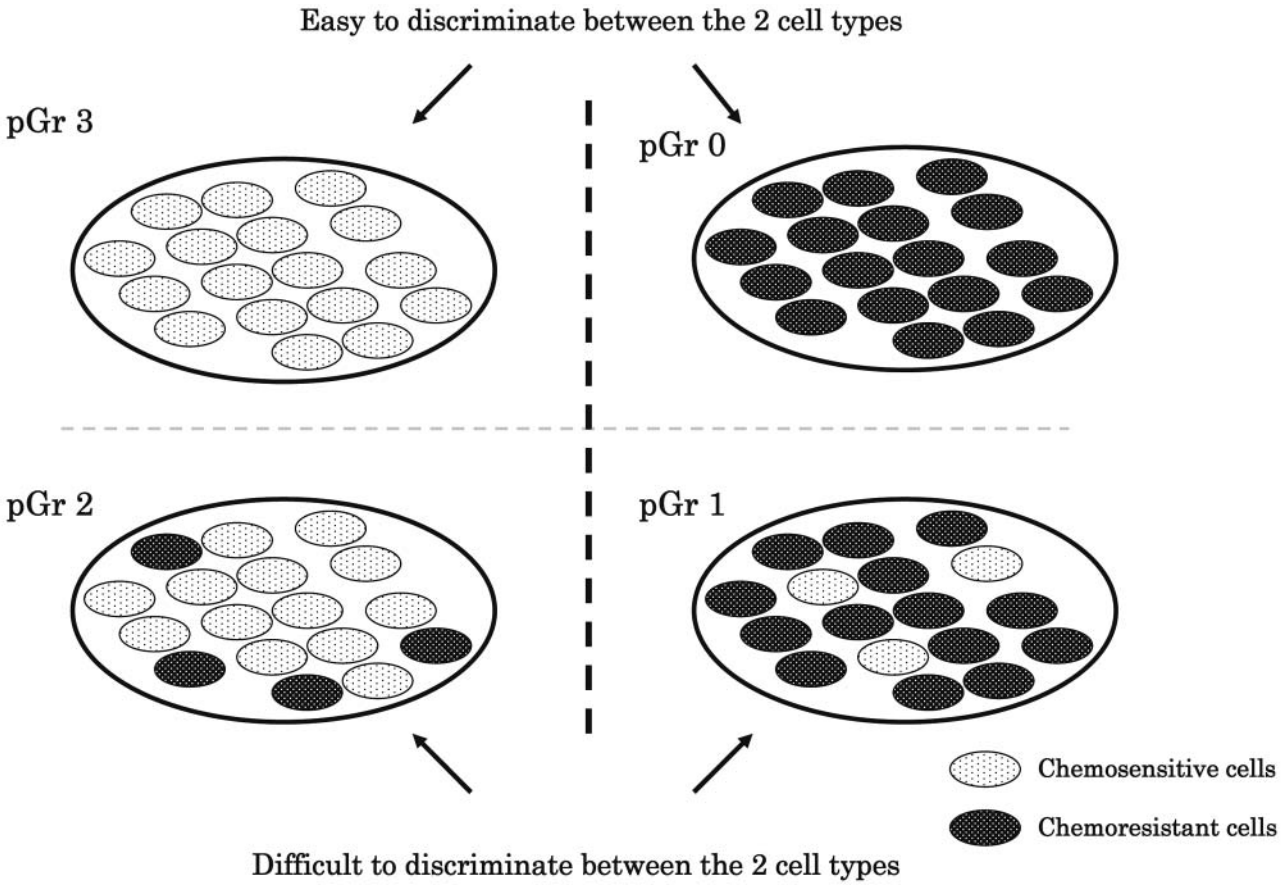


Figure 4. *Hypothesis of discrimination from tumor cell heterogeneity within different pathological grades.*

always correspond to clinical responses (16). We identified 6 genes (Table III) including insulin-like growth factor (IGF) 1 and jumping translocation breakpoint and leukocyte immunoglobulin-like receptors, which positively correlated with pathological grades. In contrast, beta globin, chorionic somatomammotropin hormone Cs-5 and matrix Gla protein negatively correlated with pathological grade. As is commonly found, only beta globin negatively correlated to clinical and pathological response (Tables II and III). Figure 3 shows the definition (D) values of the 6 validation genes categorized into subgroups according to pathological grade.

Other genes, including ER, PR and Her2/neu, were analyzed according to the pathological response. There was a negative association between pathological response and estrogen receptor ($p=0.031$; χ^2 test). On the other hand, there was a tendency for positive association between pathological response and Her2/neu positivity ($p=0.086$; χ^2 test). Patients who were estrogen receptor-positive, progesterone receptor-positive, and Her2/neu-negative (so-called luminal types) responded less frequently to the chemotherapy (17).

Validation of the formula. To assess the validity of the predictive algorithm, we used independent microarray data (6 patients) and predicted pathological response. In four out of six samples, responses were correctly predicted for an accuracy of 67%. The highest D values were from pCR (Figure 3). Interestingly, the modest score data showed the greatest variance from the predicted values and the highest and lowest score data were most accurately predicted.

Discussion

Although expression patterns of surgical specimens were different from preoperative specimens, some samples from each tended to cluster. This suggests there may be some selection of genes by chemotherapy. We also observed that the dentrogram of ER-positive samples showed a tendency to represent this pattern. Previous microarray analysis has indicated that breast cancer was divided into ER or Her2/neu status (18). Thus, we considered that this dentrogram showed gene expression patterns reflecting both drug treatment and ER/Her2/neu status.

The marked change in the gene signature of pCR cases can be estimated but it is impossible to confirm. By using the predicting formula, predictive accuracy was 67%. One of the reasons why predictive accuracy was not 100% may be the heterogeneity of tumor cells. We hypothesize that predictions of pathological CR (Grade 3) and no effect (Grade 0) are clearer because cell populations in these categories are rather homogenous, in contrast to groups of pathological response (Grades 1 and 2) (Figure 4). Cell populations of Grades 1 and 2 are rather heterogeneous, consisting of a mixture of chemosensitive and resistant cells. There is no basic difference between Grades 1 and 2 except for quantity of cells. The six genes selected in this study are not the same as previously reported. The plausible reasons for this discrepancy are (i) protocol difference, (ii) definition of response, (iii) sample number of response too small compared to the number of genes analyzed, and (iv) methods of statistical analysis were different. Predictive analysis of genes from one chemotherapeutic regimen is relatively simple but chemotherapy protocols used in clinical practice today are usually a combination of cytotoxic agents and the regimen as a whole requires analysis. Compared to previous reports, our accuracy rate may appear to be rather low. However, most studies have used a large number of genes (70 to 92 genes) to predict chemosensitivity (4-8); we consider our prediction analysis using 6 genes as being more practical.

As the predictive factor for pathological response, we selected six genes which included IGF-1, three genes related to membrane integration and another related to extracellular matrix structure. The role(s) of these 6 genes in sensitivity to docetaxel/5'-DFUR remains to be elucidated. Except for beta globin, there are no genes obviously common to clinical and pathological responses. This study reflects the previous observation that pathological responses do not always correspond to clinical responses (16).

We also revealed a relationship between ER expression and the response to a decetaxel/5'-DFUR regimen. ER expression is reported to be correlated to the response to anthracyclines and taxanes (19). In addition, Her2/neu expression is also said to be related to chemotherapy response. There was a tendency for better response in Her2/neu-positive cases in this study. Better response from this regimen shows the universal tendency of good response in cases of ER-negative and/or Her2/neu-positive subgroups. Evidence has suggested potent cross-talk between IGF signaling pathways and ER (20, 21) or Her2/neu signaling pathways (22). Interestingly, formation of Her2/neu/IGF-1R receptor complexes has been observed in breast cancer cells (23). Although their clinical and prognostic significance in this study remains unclear, it might be possible to consider that IGF-1 was selected as a surrogate marker of ER or Her2/neu status. Recently, gene expression patterns have been divided into luminal, basal, erbB2-positive and normal types (18). However basal-like and erbB2-positive subgroups are associated with the highest rates of pathological complete response among these molecular subtypes (24); a predictive marker for chemotherapy of basal-like subgroup remains undetermined. In this regard, we expected that six genes, including IGF-I, could predict the response to docetaxel/5'-DFUR chemotherapy, although this requires further confirmation.

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