Featured Article

Immediate Gene Expression Changes After the First Course of Neoadjuvant Chemotherapy in Patients with Primary Breast Cancer Disease

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ABSTRACT

Purpose: **Our goal was to identify genes undergoing expressional changes shortly after the beginning of neoadjuvant chemotherapy for primary breast cancer.**

Experimental Design: **The biopsies were taken from patients with primary breast cancer prior to any treatment and 24 hours after the beginning of the neoadjuvant chemotherapy. Expression analyses from matched pair samples representing 25 patients were carried out with Clontech filter arrays. A subcohort of those 25 paired samples were additionally analyzed with the Affymetrix GeneChip platform. All of the transcripts from both platforms were queried for expressional changes.**

Results: **Performing hierarchical cluster analysis, we clustered pre- and posttreatment samples from individual patients more closely to each other than the samples taken from different patients. This reflects the rather low number of transcripts responding directly to the drugs used. Although transcriptional drug response occurring during therapy differed between individual patients, two genes (*p21$^{WAF1/CIP1}$* and *MIC-1*) were up-regulated in posttreatment samples. This could be validated by semiquantitative and real-time reverse transcription-PCR. Partial least-discriminant analysis based on approximately 25 genes independently identified by either Clontech or Affymetrix platforms could clearly discriminate pre- and posttreatment samples. However, correlation of certain gene expression levels as well as of differential patterns and clusters as determined by a different platform was not always satisfying.**

Conclusions: **This study has demonstrated the potential of monitoring posttreatment changes in gene expression as a measure of the pharmacodynamics of drugs. As a clinical laboratory model, it can be useful to identify patients with sensitive and reactive tumors and to help for optimized choice for sequential therapy and obviously improve relapse-free and overall survival.**

INTRODUCTION

Breast cancer, clinically, is a very heterogeneous disease. The clinical heterogeneity of breast cancers is due to a broad diversity of somatic mutations and epigenetic rearrangements changing the expression of many genes. Although much effort has been made to develop an optimal clinical treatment course for an individual patient with breast cancer, only little progress could be achieved predicting the individual's response to a certain therapy. Such predictions are usually based on standard clinical conditions such as tumor stage and grade, estrogen receptor (ER) and progesterone receptor (PgR) status, growth rate, overexpression of the *HER2/neu* and *p53* oncogenes (1). However, evidence about association of ER and/or PgR gene expression with outcome prediction for adjuvant endocrine chemotherapy are still controversial. Studies have shown that levels of ER and PgR gene expression in breast cancer patients are of prognostic importance independently from a subsequent adjuvant chemotherapy (2–4). Oppositely, a newer study has demonstrated that PgR status is an independent predictive factor that improves outcome prediction for adjuvant endocrine therapy (5). Moreover, elevated ER and PgR levels are known to be significantly associated with a progressively better response to tamoxifen and longer survival in ER(+) metastatic breast cancer (6). From the theoretical point of view, it is unexpected that the therapeutic response in patients with breast cancer might be independent from the ER/PgR status. It is more probable that the prognostic impact of receptors' expression depends on the impact of other variables, *e.g.*, the impact of the ERBB2 receptor (7, 8). When conventional biological techniques are used, finding such factors is problematical because all of these techniques survey one gene at a time.

For a few years, DNA microarray technology has been very useful for quantitative measurements of expression levels of thousands of genes simultaneously in one sample. Thus far, this technology has been used for the classification of cancer tissues, *e.g.*, breast tumors (9–24), prediction of metastasis and patient's outcome (25–38), and tumor response to chemotherapy (39–43).

It is a well-established fact that adjuvant systematic treatment after surgery reduces the risk of disease relapse and

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Note: Supplementary data for this article can be found at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org).

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death in patients with primary operable breast cancer (44). As an alternative therapeutic concept, neoadjuvant or primary systemic therapy (PST) can be offered either to those patients with larger inoperable breast cancers or to patients interested in breast-conserving surgery (45–53). The PST in general do not offer a survival advantage over standard adjuvant treatment, but may identify patients with a pathologically confirmed complete response (45, 46). This clinical response to PST is associated with improved survival (50, 54, 55) and reflects a great benefit to ~14% of the PST-treated patients. Studies elaborating PST have demonstrated that early gene expression changes are significantly associated with clinical response (56, 57).

In general, all patients of a given cohort do receive the same treatment, even though many will fail in treatment success. Biomarkers reflecting the tumor response can function as sensitive short-term surrogates of long-term outcome. The use of such biomarkers will make chemotherapy more effective for the individual patient and will allow the changing of regimens early, in case of nonresponding tumors as far as the level of evidence for biomarker studies is obtained.

With this study, we aimed to identify effects of epirubicin/cyclophosphamide (EC) or epirubicin/taxol (ET) treatment on gene expression in primary breast cancers at 24 hours after the first treatment. Cyclophosphamide and epirubicin are common therapeutics for advanced and metastatic breast cancer (58). Moreover, a therapeutic advantage of epirubicin is the higher cumulative dose at which the anthracycline-induced cardiotoxicity becomes clinically evident in contrast to the more frequently used doxorubicin (Adriamycin). On the other hand, taxanes have quickly been established as important chemotherapeutic agents in the armamentarium of drugs to treat breast cancer (59). Expression profiles of 25 pre- and post-treatment biopsy pairs have been obtained by low-density cDNA array (BD Biosciences Clontech, Heidelberg, Germany) and were subsequently funneled into bioinformatic and statistical analyses. Additionally, five consecutive biopsies have been sampled from the same tumor of one patient (pretreatment, and 4, 8, 18, and 24 hours after the first EC treatment) to profile a time course of gene expression by the use of oligonucleotide microarrays (Affymetrix Inc., Santa Clara, CA).

Analyzing the data for 25 pairs by applying the paired Student's *t* test, we found only two genes, cyclin-dependent kinase inhibitor 1 ($p21^{WAF1/CIP1}$) and prostate differentiation factor (*MIC1*) to be stimulated equivocally in the breast tumors posttreatment. Several more genes were found to be up- and/or down-regulated after 24 hours of treatment in at least some of the patients analyzed. Partial least discriminant analysis (PLS-DA) and a step-down permutation approach were applied to the data sets to develop a discriminative minimal gene set for future classifications. On the basis of approximately 25 transcripts from the entire data set, PLS-DA is able to discriminate pre- and post-treatment samples. Although absolute changes in gene expression vary in the individual paired samples, as well as according to what detection method is used, a group of genes has been identified characterizing major common transcriptional changes exerted by the onset of the first chemotherapy cycle.

MATERIALS AND METHODS

Patients and Clinical Specimens. From September 1999 to June 2001, patients with primary breast cancer were enrolled with informed consent for this study with neoadjuvant EC or ET treatment (epirubicin, 90 mg/m^2, and cyclophosphamide, 600 mg/m^2, or Taxol, 175 mg/m^2) as the first therapeutic intervention prior to surgery (Table 1). Tumor samples were taken according to institutional review board guidelines. Serial core biopsies of the primary tumor were performed before treatment and 24 hours after the initiation of the first course of the chemotherapy from a locally anesthetized region with Bard MAGNUM Biopsy Instrument (C.R. Bard, Inc., Covington, GA) with Bard Magnum biopsy needles (BIP GmbH, Tuerkenfeld, Germany). Serial biopsies were taken from a distinct area of the tumor with a 90-degree angle and an additional skin entry site. This approach was used to avoid the detection of gene expression unrelated to treatment, such as tissue injury-related processes. All biopsy samples were snap-frozen in liquid nitrogen and stored at −80°C until further processing. Hematoxylin and eosin-stained sections from tumor specimens were examined to assess the relative amounts of tumor cells, benign epithelium, stroma, and lymphocytes. Standard clinical factors such as ER, PgR, Ki-67, p53, cerbB2/HER2neu have been routinely defined[4] in our laboratory.

Total RNA Isolation, cDNA Probe Synthesis, and Atlas Array Data Analysis. Total RNA from tissue specimens was extracted according to the protocol recommended for the Atlas Pure Total RNA labeling system (BD Biosciences Clontech). The amount and quality of RNA were evaluated with UV spectrophotometry (Photometer ECOM 6122, Eppendorf AG, Hamburg, Germany), agarose gel electrophoresis, and Agilent 2100 Bioanalyzer RNA 6000 LabChip kit (Agilent Technologies GmbH, Boeblingen, Germany) following the manufacturer's instructions. α^{32}P-labeled cDNA probes were prepared from 5–10 μg of total RNA with gene-specific primer mix (BD Biosciences Clontech). Hybridization steps were performed according the manufacturer's instructions with filter arrays Atlas Human Cancer 1.2 with 1185 genes. Duplicate, parallel, independent hybridization experiments were performed for all of the samples to prove the reproducibility of the results. The results have shown good reproducibility (correlation coefficients, 0.9 to 0.98).

Primary data collection and analysis were carried out with phosphoimager (BAS-1500, Fuji, Raytest) and TINA software (Fuji). Areas of arrays with obviously overlapping signals and artifacts were manually excluded from the additional analysis. Normalization of signal intensities obtained from different hybridization experiments was based on the sum of background-subtracted signal data of all expressed genes (60) with Excel 97 (Microsoft). The resulting data table is available from our website.[4]

Oligonucleotide Array Hybridization and Analysis. Total RNA of 10 paired pre- and posttreatment tumor biopsies were subjected to further analysis with the Affymetrix Human Genome U133A GeneChip (22,283 probe sets; Table 1). For the time course study, biopsies have been taken from the same tumor of one patient before the first treatment with EC chemotherapy and after different lengths of time, namely, 4, 8, 18,

[4] Internet address of authors' website: http://www.cancertoday.com.

Table 1 Patients' demographics

Case ID	Age	ER status*	PgR status*	HER2 status†	*p53*‡	Ki67%	Node	Grading	Therapy	Histology	Tumor size cm × cm
13	65	0	0	2	0	50	NA	3	EC	Invasive metaplastic	4 × 5
14	50	0	0	2	1	28	0	3	EC	Invasive multifocal	3 × 3
20	45	1	1	2	0	6	2	2	ET	Invasive ductal + intraductal	7 × 9
25	63	0	0	45	0	17	0	2	EC	Invasive lobular	6 × 6
28	48	1	1	2	0	10	0	2	ET	Invasive lobular	5 × 5
29	71	1	0	2	0	10	1	2	EC	Invasive ductal + lobular	2.5 × 1
33	60	1	1	2	0	1	0	1	EC	Invasive lobular + tubular	2.2 × 2
34A	59	0	0	15	0	15	0	2	EC	Invasive lobular + ductal	5.5 × 2.5
35A	44	1	1	2	0	2	0	2	EC	Invasive intraductal	2.5 × 2
37A	58	1	1	2	0	15	0	2	EC	Invasive ductal	3.3 × 3
39A	36	1	1	2	0	30	1	2	EC	Invasive ductal	4 × 2
40	50	1	1	2	0	3	0	2	EC	Invasive lobular	4 × 1.9
42A	40	1	1	2	0	28	0	2	EC	Invasive ductal	5 × 4
47	46	0	0	2	1	26	2	2	ET	Invasive intraductal	5 × 5
49A	38	0	0	2	0	35	0	3	EC	Medullary + intraductal	3.3 × 3
52A	68	1	1	2	0	6	1	1	EC	Invasive lobular + tubular	5 × 4
53A	65	1	0	10	0	16	0	2	ET	Invasive ductal + intraductal	2.7 × 2
56	60	0	0	2	0	25	2	2	EC	Invasive ductal	3 × 3
58	51	0	0	2	1	26	1	2	ET	Invasive ductal	2.5 × 2
66A	61	1	1	2	0	3	1	2	EC	Invasive lobular + tubular	4.3 × 2
68	40	1	1	2	0	6	0	1	ET	Invasive lobular + tubular	2.5 × 1.9
72	60	1	1	2	0	6	0	1	ET	Invasive lobular + tubular	3 × 3
73	64	1	1	11	0	18	0	2	ET	Invasive ductal + intraductal	4 × 4
76	47	1	1	2	0	12	1	2	EC	Invasive ductal	6 × 4
78A	61	0	0	4	1	30	0	3	ET	Invasive ductal	3.5 × 1.6

Note. Case identification number followed by an A indicates that the tumor samples were used with the Affymetrix platform.
Abbreviations: ID, identification; NA, not available.
* ER and PgR status were determined by immunohistochemistry; 1, positive (>7 fmol/mg protein); 0, negative (<7 fmol/mg protein).
† HER2/neu status is presented as copies of *HER2/neu* gene.
‡ Status of *p53* oncogene: 0, <180 score; 1, >180 score.

and 24 hours, after the therapy onset. We used the Affymetrix HG-95A microarray consisting of 12,626 probe sets.

For hybridization, 5 μg of total RNA was used as starting material. The whole experimental procedure was performed according to the manufacturers protocols, and hybridizations were carried out at the laboratories of Bayer Diagnostic Research, Bayer Healthcare AG, Leverkusen, Germany. The raw expression value for each gene was calculated with Affymetrix Microarray Suite 5.0 Software.

Hierarchical Clustering. Genes and probe sets with low signal intensities have been excluded form the additional analysis. Expression data of 249 genes from the Clontech platform and 6,980 probe sets from Affymetrix HG-133A were log-transformed, and data for each gene were median-centered and normalized. Data for each sample were also median-centered. The hierarchical clustering program (Version 3.1) developed by Eisen *et al.* (61) was applied. Results were displayed with the TreeView program Version 1.6.6 (ref. 61; Figs. 1 and 2). *Red* reflects expression levels higher than the median value of the normalized data sets; *blue* reflects expression levels lower than the median.

Semiquantitative and TaqMan Real-time Reverse Transcription-PCR. Primer sequences are shown in Supplemental Materials Table 1.[5] Expression of the gene for β-*actin* served for the standardization of the individual PCR reactions. The number of cycles in PCR reactions was optimized to ensure product intensity within the linear phase of amplification. Additionally, real-time PCR analyses with ABI PRISM 7900 (Applied Biosystems, Foster City, CA) for $p21^{WAF1/CIP1}$, interferon-induced transmembrane protein 9-27, and β-*glucoronidase* (as housekeeping gene) have been done with Assays-on-Demand according to the manufacturer's protocol. For the quantification, the ΔΔ cycle threshold (C_t) method was used (62).

Identification of Epirubicin/Cyclophosphamide-Regulated Genes in Time Course Study in a Single Patient. To identify genes, which are differentially expressed after chemotherapy, we calculated a confidence score for each gene at each point of time after the first onset of chemotherapy treatment. We used the method reported by Jelinsky *et al.* (63) with some modification. The confidence score (*CS*) was defined as the sum of individual scores given for fold change (*FC*), expression level (*EL*), and present calls (*PC*) as defined by Affymetrix software. The maximum confidence score for any gene could be 35 based on this scoring. We considered a gene to be regulated under the chemotherapy treatment if the confidence score (*CS*) was ≥27 (see Materials and Methods on the www.cancertoday.com website).[4]

Principal Components Analysis and Partial Least Squares Discriminant Analysis of Array Data. Principal component analysis and partial least squares discriminant analysis (PLS-DA) were performed with SIMCA-P software (Umet-

[5] Internet address for Supplementary data for this article: http://clincancerres.aacrjournals.org.

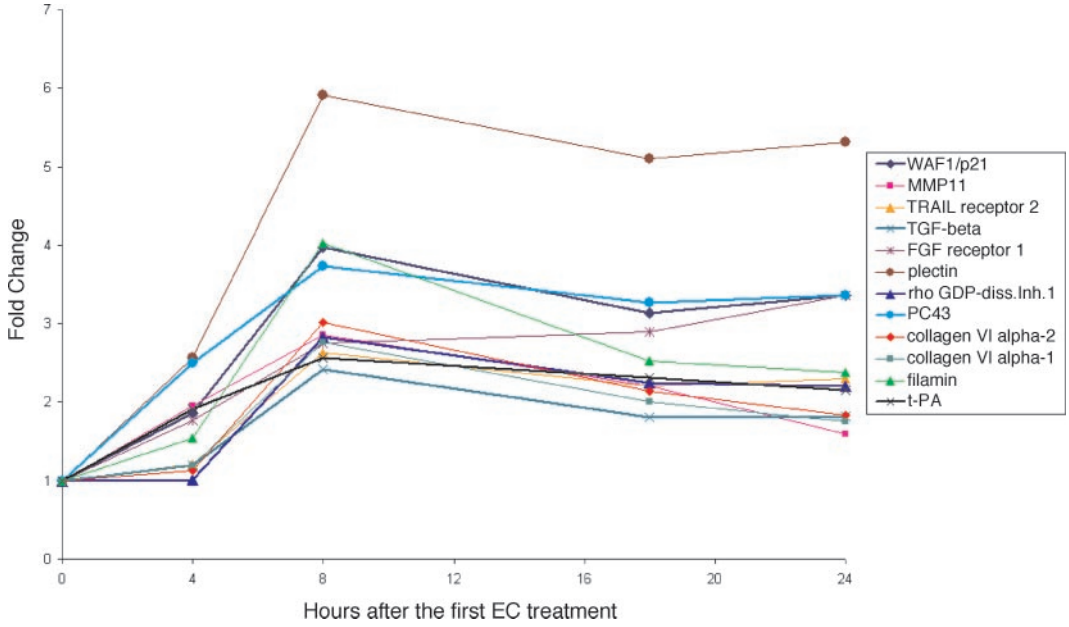

Fig. 1 Microarray data, over the 24-hour period after EC treatment, for 12 genes with stimulated expression and highly coregulated with cyclin-dependent kinase inhibitor 1A ($p21^{WAF1/CIP1}$). *k*, clustering was performed using Spotfire software.

rics AB, Umea, Sweden) (64) as described by Pérez-Enciso and Tenenhaus (65). Model validation was carried out via permutation. Q^2 is a measurement of the predictive ability of the model, and R^2 is related to the goodness of fit of the model.

RESULTS

Time Course Patterns of Gene Regulation under Epirubicin/Cyclophosphamide Therapy in a Single Patient. To improve the time choice for the posttreatment biopsy, we used global gene expression profiling by Affymetrix GeneChip array analysis for the characterization of those genes the expression of which would be regulated posttreatment in a single patient with a primary breast cancer disease. To examine the time-dependent regulation of gene expression in a treated breast tumor *in vivo*, biopsies have been taken from the same tumor of one patient before the first treatment with EC chemotherapy and after different intervals, namely 4, 8, 18, and 24 hours after the first therapy onset. We used a defined score value (*CS*; see Materials and Methods on www.cancertoday.com)[4] to determine a given gene as regulated by chemotherapy treatment. By use of the confidence score, we took into account the fold change for each gene as compared with control, the expression level, and the present or absent calls as determined by the Affymetrix software. By setting the confidence score cutoff at 27 or greater, we identified 479 genes that were expressed at detectable levels and showed a robust pattern of regulation (fold change, ≥2.0) after the first chemotherapy treatment of the breast tumor.

Then, we subdivided all of the regulated genes into three groups: (*a*) regulated early only (≥2-fold at 4 and/or 8 hours only); (*b*) regulated early and late (≥2-fold at 4 and/or 8 hours and at 18 and/or 24 hours); and (*c*) regulated late only (≥2-fold at 18 and/or 24 hours only). For up-regulated genes, we observed the following numbers of genes in each of the three groups (first group, $n = 22$; second group, $n = 303$; third group, $n = 8$). For down-regulated genes, the number of genes in each group is as follows: first, $n = 20$; second, $n = 118$; third, $n = 8$. Therefore, the up- or down-regulation of the majority of the genes after the treatment was at the early and late time points.

Of 479 genes that were found to be up-regulated in posttreatment samples, the major functional categories included a cytoskeleton/structural category, including collagens, elastin, filamin, fibroblast factors and receptors, and cell adhesion/extracellular matrix, as well as several growth-arrest and DNA-damage-inducible proteins (*e.g.*, GAS, GADD45). The inhibited genes could be subdivided into several major groups such as a group of ribosomal proteins, nucleotide and protein synthesis and processing, transcription factors, as well as some oncogenes (*e.g.*, c-*myc*, K-*ras*, *bcl*-2).

Cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ was stimulated in this tumor. We performed *k*-means clustering analysis and, with Spotfire software (Spotfire Inc., Somerville, MA), found genes with expression profiles similar to that of $p21^{WAF1/CIP1}$. Twelve genes the expression profiles of which are highly correlated to the profile of p21, are shown on Fig. 1.

Analysis of Expression Profiles by Using Clontech and Affymetrix Arrays. The expression profiles have been obtained for pre- and post-treatment breast cancer biopsy samples of 25 patients with Clontech Atlas Human Cancer 1.2 low-density cDNA array.

We used a hierarchical clustering algorithm (61) to group 50 biopsy samples on the basis of their similarities measured over 249 genes, scored as expressed at the average level in at least 6 of the 50 samples. On the basis of these analyses, the following observations could be made. First, in more than 90% of the cases, the pre- and post-therapy biopsies were clustered

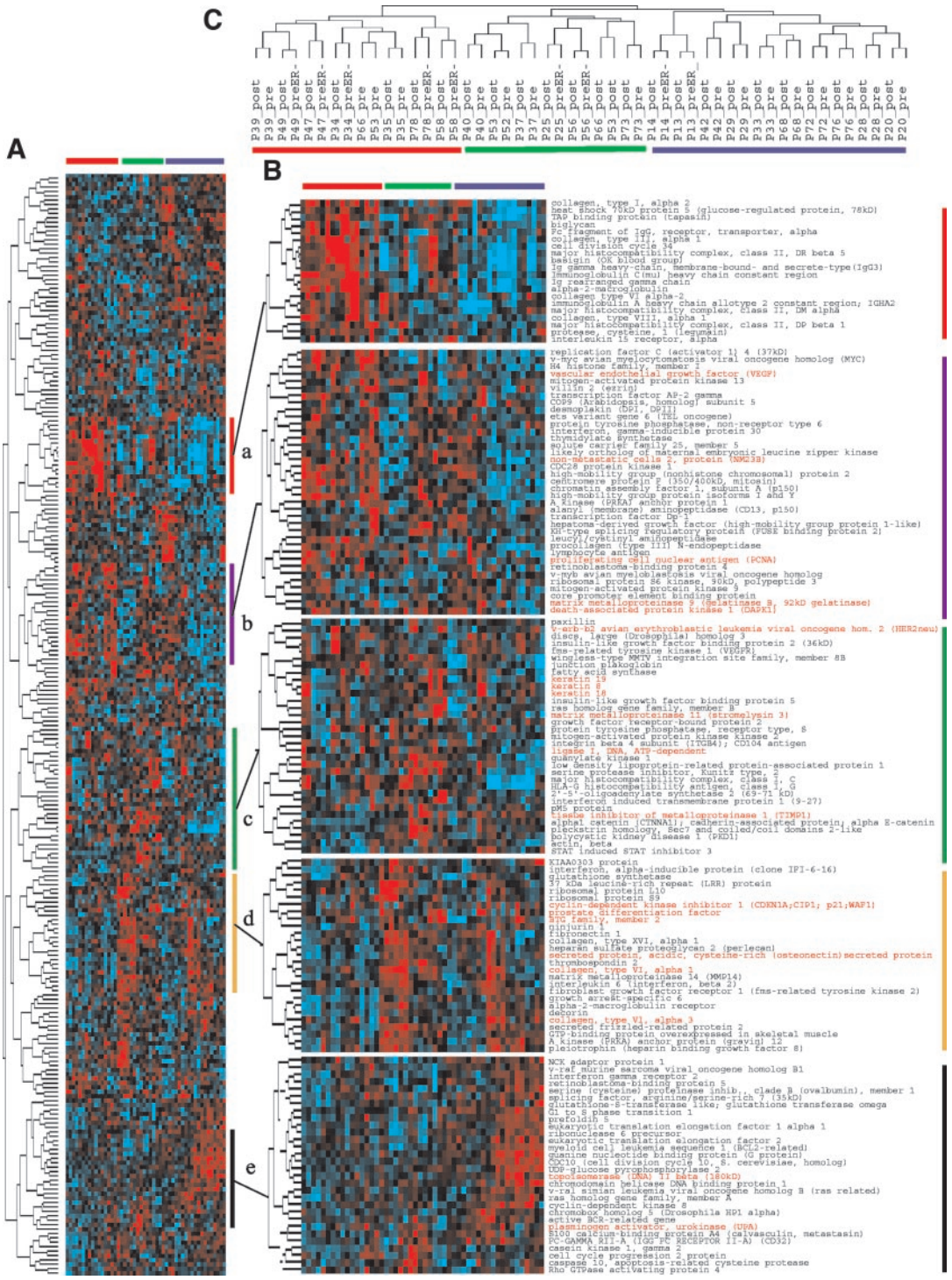

Fig. 2 A two-way clustering analysis of breast cancer biopsies. Lined up on the *horizontal axis*, 50 pre- and posttreatment biopsy samples; on the *vertical axis*, 249 genes prefiltered from 1185 genes listed on Clontech Atlas Human Cancer 1.2 cDNA array (see Materials and Methods). Each cell in the color matrix represents the relative expression level of a single gene transcript in a single sample. Expression level was normalized per gene, and the value relative to the mean among 50 arrays is shown by color: *red*, transcript levels above the mean for the given gene across the samples; *blue*, transcript levels below the mean for the given gene across the samples; *color saturation*, the magnitude of deviation from the mean value. *A*, matrix representation of expression levels and the dendrogram of samples. *On the right side, black bars from a to e*, the locations of gene clusters of interest. *B*, expanded view of several gene clusters of interest named from top to bottom: *a*, group contains genes expressed in the endothelial cells and B lymphocytes; *b*, group includes many oncogenes and growth factors; *c*, group contains *v-erb-b2/HER2*, *keratin 19*, *keratin 18*, and *keratin 8*, *MMP11*, *DNA ligase I*, *TIMP1*, and *SATA-induced STAT inhibitor 3*; *d*, cluster includes a group of genes with expression correlated with *WAF1/p21*; *e*, cluster includes genes for cell cycle control and proliferation. *C*, the tumor samples are clustered according to their global expression profiles on the horizontal axis.

together, reflecting that expression patterns of tissue specimens obtained from the same tumor show a more conserved expression pattern than one can observe attributable to the interindividual variance from another patient (Fig. 2). Only two pretreatment samples of patients 53 and 66 were clustered separately from their post-treatment biopsies. Second, expression patterns varied significantly among different tumors. Third, tumor samples subdivide by hierarchical clustering into three major subclasses independent of their ER status presented in Table 1.

Expression data, as analyzed by two-dimensional gene clustering algorithms, are presented in Fig. 1, in which those genes with similar expression levels are grouped together on the vertical axis (Fig. 2*A*), and the tumor samples are clustered according to their global expression profiles on the horizontal axis (Fig. 2*C*).

The 249 quality-checked and pre-selected genes form discrete clusters of correlated gene expression. Some of these clusters are shown at higher magnification in Fig. 2*B*. Genes in the cluster "a" reflect the expression of genes represented in endothelial cells and B lymphocytes, indicating possible lymphocytic infiltration into the tumor tissue. The second gene cluster, "b" consists of oncogenes and growth factors such as *MYC, VEGF,* and *MYB*. Cluster "c" contains the genes *v-erb-b2/HER2, keratin 19,* and *keratin 18,* also known to be discretely expressed in certain breast tumors. The gene cluster "d" includes a group of genes with a correlated expression with $p21^{WAF1/CIP1}$ and *MIC-1* (prostate differentiation factor). The fifth highlighted gene cluster "e" harbors genes involved in cell cycle control and cellular proliferation (details on gene composition of individual clusters is indicated in Fig. 2*B*).

We additionally analyzed the gene expression of 10 of the 25 paired breast cancer samples with hybridization of the extracted RNA to Affymetrix oligonucleotide microarray with 22,283 probe sets. Aliquots of the same total RNA, which have been previously used in experiments with Clontech arrays were analyzed with Affymetrix arrays. Expression of 4,906 transcripts hybridizing to the chip surface was reliably detected in all biopsy samples. A total of 15,122 transcripts could be detected in at least 1 of 10 pairs, whereas 7,161 transcripts could not be detected according to the manufacturers analysis software MAS 5.0 and the quality control signal "absolute call" in any of the paired samples.

An unsupervised analysis of gene expression in the 10 pre-/posttreatment paired cancer biopsies based on a quality-checked gene set of 6,980 transcripts of the total HG-133A GeneChip content was performed. The quality control and gene selection was based on signal intensity levels and reliable detection on the individual array (see Materials and Methods on www.cancertoday.com).[4] The hierarchical cluster algorithm ordered the pre- and posttreatment biopsies of all 10 patients together without the previously observed exceptions (Supplemental Materials, Fig. 1).[5] The higher coverage of genes and, putatively, of cellular mechanisms and pathways on Affymetrix platform does give the higher rate of coclustered sample pairs.

Of 1,185 genes listed on Human Cancer 1.2 Clontech array, 1,164 could be identified on the Affymetrix HG-133A list. Taking into account 19 genes that were flagged from the Clontech data, we could list 1,145 cDNAs for both platforms. By comparison of signals intensity data between two platforms, we found 414 genes, which were detected as "present" by absolute call of presence on the Affymetrix platform, which expression could not be detected by use of the Clontech platform. Expression levels of about 100 genes have been detected on the Affymetrix platform at significantly lower levels as compared with Clontech arrays. Therefore, there is a limited agreement for gene expression levels measured with the two platforms used.

Identification of Differentially Expressed Genes. A paired *t* test was applied to identify transcripts that were differentially expressed in pre- and posttreatment samples. After selection of 249 expressed genes, a group of 19 genes that frequently showed increased or decreased expression under EC or ET therapy were identified (Student's *t* test $P < 0.05$). There were some differences in frequently regulated genes between therapies (EC against ET; data not shown). Table 2 lists genes significantly regulated in a number of paired samples within the first 24 hours of chemotherapy treatment with ratios >1.8 or <0.6 for both therapy arms. The range of fold changes for the regulated transcripts was broad, presumably reflecting variability among patients. Two genes, the cyclin-dependent kinase inhibitor 1 ($p21^{WAF1/CIP1}$) and prostate differentiation factor (*MIC-1*), were up-regulated in more than 95% of all samples posttreatment.

Performing the same analysis with the expression data obtained with the Affymetrix GeneChip system to identify the treatment effects on gene expression, we have identified a group of 37 frequently regulated genes (Table 3). As also seen with the data obtained from hybridization to filter arrays, the genes $p21^{WAF1/CIP1}$ and *MIC-1* were up-regulated most prominently in the posttreatment biopsy samples. However, the relative signal intensities detected for *MIC-1* were lower with the Affymetrix system in comparison with the Clontech arrays.

Genes encoding for immune system proteins were found to be frequently down-regulated during the chemotherapy treatment. As an additional notable observation, the expression levels of matrix metalloproteinase 9 (*MMP9*) and tissue inhibitor of metalloproteinase-3 (*TIMP3*) genes were often decreased in posttreatment samples. According to Clontech data, both *MMP9* and *TIMP3* were often down-regulated in the posttreatment samples. However, their *P* values of statistical significance were higher than 0.05.

Obviously, the direct comparison of genes significantly regulated after the treatment according to Clontech arrays with *t* test *P*-values from the Affymetrix data set revealed a lack of agreement between the two platforms. To illustrate this discrepancy, Affymetrix data have been added to those genes reaching significance on the Clontech arrays (Table 2).

Principal Component Analysis and PLS-DA: Advanced Data Analysis Algorithms. As one of the statistical methods for the estimation and selection of discriminative gene sets, we used PLS-DA to analyze expression data obtained by Clontech arrays. This method is superior when a much larger number of variables (genes) than observations (samples) has to be taken into consideration. We evaluate the discriminative ability of PLS questioning for small discrete gene sets to separate pre- and postchemotherapy samples. In addition we challenged a classical principal component analysis algorithm with the identification of the major components separating the sample pairs and the two treatment conditions.

Table 2 A list of the top 19 genes associated with EC/ET exposure

Gene name	GenBank accession no.	*t* test paired Clontech	*t* test paired Affymetrix
Cyclin-dependent kinase inhibitor 1 (*CDKN1A*; *WAF1*; *p21*)	U09579	5.578E-07 ↑ *	0.0243
Prostate differentiation factor	AF019770	0.00014 ↑	0.0216
Desmoplakin (*DPI, DPII*)	M77830	0.00022 ↑	0.0322
Growth arrest-specific 6 (*GAS*)	L13720	0.00063 ↑	0.6152
BTG family, member 2	U72649	0.00154 ↑	0.877
UDP-glucose pyrophosphorylase 2	U27460	0.00217 ↑	No expression
Nth (*Escherichia coli endonuclease III*)*-like 1*	U79718	0.00514 ↑	0.1426
Ribosomal protein L13a	X56932	0.00592 ↑	0.0913
IMP (*inosine monophosphate*) *dehydrogenase 2*	L33842	0.00621 ↑	0.1151
Interferon, gamma-inducible protein 30	J03909	0.00795 ↑	0.088
Ligase I, DNA, ATP-dependent	M36067	0.01041 ↑	0.77
Thymidylate synthetase	X02308	0.01228 ↑	0.641
Basigin (*OK blood group*)	L20471	0.01287 ↓ (↑)	0.1
DNA repair protein XRCC9	U70310	0.01666 ↑	No expression
Protein tyrosine phosphatase type IVA, member 1	U48296	0.0208 ↑	0.217
Major histocompatibility complex, class II, DR β *5*	M20430	0.02697 ↓ (↑)	0.687
Solute carrier family 25 (*adenine nucleotide translocator*), *member 5*	J02683	0.03112 ↓ (↑)	0.675
v-jun avian sarcoma virus 17 oncogene homolog	J04111	0.03133 ↑	No expression
Collagen, type VI, α *1*	X15879	0.04988 ↑	0.5

NOTE. The table presents genes that show statistically significant pre-post chemotherapy differences in expression levels for 25 breast cancer patients. (cDNA array data was obtained with Clontech filters).

* ↑, up-regulation of a gene under the chemotherapy; ↑ (↓), up- or down-regulation of a gene in different tumors.

We used the expression data of all 25 tumor samples in the principal component analysis and PLS-DA analyses, which were carried out in the first iterative level with all 249 reliably expressed genes previously used in the hierarchical clustering analysis (see Materials and Methods; hierarchical clustering). During the course of analysis, we selected those genes satisfying the cutoff criterion of having the variable importance in the projection (*VIP*) more than 1.8 with both PLS components. In a second iterative PLS-DA performed with the SIMCA-P analysis software, only the genes ($n = 25$) with a VIP above a threshold of 1.8 were reanalyzed separately to avoid running an overfitted system (see genes listed in Table 4).

The previously described statistical selection of genes with a simple *t* test is appropriate because the problems of defining classes are limited to two (66). Nevertheless, the *VIP* criterion used for PLS-DA is more robust and discriminative. PLS-DA is not hampered by the problem of data that is not distributed per normality; an assumption that has to be made for the standard parametric *t* test. This is very important when working with relatively small numbers of samples as in the present study.

Results of principal component analysis and PLS-DA analyzing the expression levels of the 249 genes used are depicted in Figs. 3*A* and *B*. Whereas all samples before and after chemotherapy are mixedly scattered in principal component analysis (Fig. 3*A*), they are grouped into distinct areas by the PLS-DA (Fig. 3*B*). Furthermore, a permutation test was carried out to test for robustness (data not shown). Fig. 3*C* shows the PLS-DA analysis with the reduced data set containing 25 genes. Apparently, the pre- and postbiopsy samples are better discriminated compared with the PLS-DA with all 249 genes. However, there are some false classified samples, *e.g.*, postbiopsy samples 35, 53, 66, and 47. We performed an additional permutation analysis for the new model covering only 25 genes, which revealed that the current model was the one with the highest predictive power (data not shown). Thus, by use of PLS analysis it was possible to identify changes exerted by the advent of chemotherapy within each individual sample pair (Table 4). However, it is obvious that the absolute values vary from patient to patient. The *VIP* values (Table 4) correspond to the model with selected 25 genes and display lower values than obtained in the model with all 249 genes, a phenomenon that has been reported previously (67, 68).

Furthermore, we applied PLS-DA to the 6980 reliably expressed transcripts, as detected by the Affymetrix platform. After the analysis with two PLS components had been performed, we retained 24 transcripts with *VIP* values >2 for the second iterative PLS analysis run. Fig. 4 shows the PLS-DA scatter plot with one component subdividing pre- and posttreatment samples. Genes with corresponding *VIP* criteria are listed in Table 5. An independent permutation test showed the robustness and precision of this model (data not shown).

Semiquantitative and TaqMan Real-time Quantitative Reverse Transcription-PCR Validation. To confirm the array data for gene expression levels, we performed semiquantitative reverse transcription-PCR (sqRT-PCR) for six genes with aliquots of the same RNA samples. Although the extent of measured values detected by the two methods varied, direct comparison of sqRT-PCR and Clontech and Affymetrix ratios of differential gene expression for all chosen genes compared between pre- and posttreatment samples showed an overall qualitative concordance with cDNA array experiments (*i.e.*, same trend of induction or no change detected by both methods for each target; Supplemental Materials Fig. 2).[5] PCR reactions were settled and samples were taken at alternative cycle numbers between 17 and 33 to ensure that the sqRT-PCR reaction products were in a linear range of accumulation. For all sqRT-PCR, primers specific to β-*actin* were used as a control to normalize each experiment.

Table 3 A list of the top 37 genes associated with EC/ET exposure

Gene name	GenBank accession no.	*P* value *t* test paired
Tissue inhibitor of metalloproteinase-3, complete cds.	U67195	0.0046 ↓ *
40S ribosomal protein S27 isoform	NM_015920	0.0055 ↑
Cytochrome b-245, α polypeptide (*CYBA*)	NM_000101	0.0074 ↓
Immunoglobulin kappa-chain VK-1 (*IgK*)	M85256	0.0081 ↓
Consensus incl.: Homo sapiens mRNA for immunoglobulin heavy chain variable region	AJ239383	0.0110 ↓
Human (*clone HSY3RR*) *neuropeptide Y receptor* (*NPYR*)	L01639	0.0115 ↓
Consensus incl.:Homo sapiens partial mRNA for human Igλ light chain variable region, clone MB91 (*331 bp*)	AJ249377	0.0121 ↓
Consensus incl.:Human rearranged immunoglobulin heavy chain	AB035175	0.0125 ↓
Consensus incl.: transmembrane 4 superfamily member 1	AI346835	0.0126 ↑
Consensus incl.: colony stimulating factor 2	BF002659	0.0130 ↓
FK506 binding protein precursor (*LOC51303*)	NM_016594	0.0134 ↓
Damage-specific DNA binding protein 2 (*48kD*) (*DDB2*)	NM_000107	0.0138 ↑
Human immunoglobulin rearranged mu-chain gene VH3-D2110-JH2	L34164	0.0143 ↓
SH3 domain-binding protein SNP70	AF118023	0.0184 ↓
Tropomyosin 2 (β) (*TPM2*)	NM_003289	0.0203 ↑
Consensus incl.:Human rearranged immunoglobulin heavy chain	BG340548	0.0212 ↓
Prostate differentiation factor (*MIC1*)	AF003934	0.0216 ↑
Cyclin-dependent kinase inhibitor 1A (*p21, Cip1*) (*CDKN1A*)	NM_000389	0.0243 ↑
Consensus incl.: human HLA class II associated protein I	BE560202	0.0266 ↓
Consensus incl.: NS1-associated protein 1	AI472757	0.0279 ↓
T cell-specific protein (*RANTES*)	M21121	0.0283 ↓
Ribonucleotide reductase M1 polypeptide (*RRM1*)	NM_001033	0.0331 ↑
Hemoglobin, α 1 (*HBA1*)	NM_000558	0.0344 ↑
Consensus incl.:H.sapiens cDNA FLJ13302 fis	AI189753	0.0346 ↑
Consensus incl.: insulin-like growth factor binding protein 3	BF340228	0.0349 ↓
Matrix metalloproteinase 9 (*gelatinase B, MMP9*)	NM_004994	0.0352 ↓
Hemoglobin, α 2, MGC:14541	BC005931	0.0355 ↑
Consensus incl.:rearranged immunoglobulin heavy chain A1VH3 gene	U92706	0.0357 ↓
β chemokine Exodus-3 mRNA	U88321	0.0374 ↓
Cluster incl. U88964:Human HEM45	U88964	0.0390 ↓
Forkhead box M1 (*FOXM1*)	NM_021953	0.0392 ↑
Ferredoxin reductase (*FDXR*), *transcript variant 2*	NM_004110	0.0398 ↑
Consensus incl.: partial IGKV gene for immunoglobulin kappa chain variable region, clone 30	AW404894	0.0423 ↓
Consensus incl.: Human V108 gene encoding an immunoglobulin kappa orphan	X51887	0.0429 ↓
Ubiquitin carrier protein (*E2-EPF*)	NM_014501	0.0429 ↓
Consensus incl.:protein phosphatase 3, (*calcineurin A α*)	AA911231	0.0447 ↓
Adipose differentiation-related protein, clone MGC:10598	BC005127	0.0478 ↑

NOTE. Array data were obtained with Affymetrix GeneChip HG-133A for 10 breast cancer patients.
Abbreviations: cds., coding sequence; *incl*., including.
* ↓ (↑), down- (up-)regulation of a gene under the chemotherapy.

Array hybridization results were validated additionally by TaqMan real-time RT-PCR analysis of $p21^{WAF1/CIP1}$ and *interferon-induced transmembrane protein 9-27* in a subset of breast tumors. As shown on Fig. 5, quantification with both methods resulted in similar ranges on expression although the absolute expression levels detected varied by methods. Clontech array showed false differential expression for *interferon-induced transmembrane protein 9-27* in the sample "p53" (probably a hot spot because of hybridization with radioactively labeled probe). Notably, Affymetrix results for the differential expression of $p21^{WAF1/CIP1}$ were constantly lower for some tumors than it was in both Clontech and real-time data. This may follow from the probe design or hybridization conditions with the GeneChip system.

DISCUSSION

We obtained RNA from needle biopsy samples of primary human breast cancers before and 24 hours after the first treatment with EC or ET. Gene expression patterns have been identified with cDNA and oligonucleotide arrays. A cluster analysis that was based on either Clontech or Affymetrix gene expression data sets revealed a wide genetic heterogeneity among individual breast tumors, as reported previously (9–12). Moreover, the pre- and posttherapy samples revealed a much higher degree of similarity concerning their gene expression profiles, compared with samples taken from different individual tumors. These data support the hypothesis that intratumor transcriptional heterogeneity due to sampling of distinct subpopulations of cells is less than the heterogeneity among different tumors.

Unlike earlier publications that dealt with the prediction of patients' outcome we aimed at the identification of genes the expression levels of which will be changed immediately after the treatment. No additional information about the patients' response to treatment have been used. For the present study, we selected expression differences shortly after the initiation of the treatment. Important cellular processes, *e.g.*, proliferation, DNA repair, and apoptosis, often occur within up to 48 hours after chemotherapy exposure (56, 69–73).

Table 4 Genes with expression levels involved in discrimination between before-and-after EC or ET chemotherapy, ordered by *VIP* as defined by SIMCA-P software using Clontech data

GenBank accession no.	Gene name	*VIP*	*P* value (*t* test paired)
U09579	*Cyclin-dependent kinase inhibitor 1* (*CDKN1A*); *p21$^{WAF1/CIP1}$*	2.11	5.5778×10^{-7}
M80563	*S100 calcium-binding protein A4* (*calvasculin, metastasin*)	1.14	0.13178
Y07604	*Non-metastatic cells 4*	1.11	0.03540
X14356	*Fc fragment of IgG, high affinity Ia, receptor for* (*CD64*)	1.09	0.34425
M95712	*v-raf murine sarcoma viral oncogene homolog B1*	1.05	0.12130
L11285	*Mitogen-activated protein kinase kinase 2*	1.04	0.0047
Y09305	*Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 4*	1.03	0.29491
J03909	*Interferon, gamma-inducible protein 30*	1.02	0.00795
U72649	*BTG family, member 2*	1.02	0.00154
M35663	*Protein kinase, interferon-inducible double stranded RNA dependent*	0.96	0.15634
M36067	*Ligase I, DNA, ATP-dependent*	0.95	0.01041
X62055	*Protein tyrosine phosphatase, non-receptor type 6*	0.95	0.00441
L20471	*Basigin* (*OK blood group*)	0.94	0.01290
L13720	*Growth arrest-specific 6*	0.91	0.0006277
X02308	*Thymidylate synthetase*	0.87	0.01228
X57398	*PM5 protein*	0.87	0.01195
Z30183	*Tissue inhibitor of metalloproteinase 3* (*TIMP3*)	0.85	0.05674
L07597	*Ribosomal protein S6 kinase, 90kD, polypeptide 1*	0.83	0.01848
U70310	*DNA repair proteon XRCC9*	0.81	0.01667
X56932	*Ribosomal protein L13a*	0.80	0.00592
M34671	*CD59 antigen p18–20*	0.80	0.00107
D55696	*Protease, cysteine, 1* (*legumain*)	0.78	0.09813
U48296	*Protein tyrosine phosphatase type IVA, member 1*	0.73	0.02080
M15796	*Proliferating cell nuclear antigen* (*PCNA*)	0.72	0.05624
U58048	*Procollagen* (*type III*) *N-endopeptidase*	0.71	0.09210

To our knowledge, this is the second experimental study that assesses direct influence of neoadjuvant chemotherapy on gene expression changes in human breast cancer and the fourth one that studies expression profiles of breast carcinomas under the neoadjuvant chemotherapy treatment. Sotiriou *et al.* (41) evaluated the correlation between expression profiles from fine-needle aspirations performed on breast carcinomas and subsequent clinical responses after the neoadjuvant chemotherapy. Expression profiles of samples before chemotherapy and 21 days after the first cycle of chemotherapy have been compared. The authors discovered that good responders exhibited more changes in their gene expression profiles after the therapy than did the poor responders. After the individual *t* test has been applied, 16 genes were identified of which the change in expression level allowed best differentiation between groups of good and poor responders. Chang *et al.* (42) performed a study on the correlation of gene expression profiles obtained from the pretreatment core biopsies of primary breast cancer with their clinical response after the fourth cycle of docetaxel therapy. They found 92 genes for which the expression profiles correlated with docetaxel response. Buchholz *et al.* (40) tried to analyze gene expression changes in serial tumor core biopsies taken during neoadjuvant chemotherapy of breast cancer at 24 and 48 hours after the chemotherapy onset. Besides the fact that they could not collect enough RNA from all of the serial samples and that they did not obtain all of the samples needed from five studied patients under the same therapy protocol, the difficulty was that no individual gene was up- or down-regulated across the three tumors treated with the same chemotherapy. Briefly, it seems that interindividual genetic heterogeneity could explain a remarkably diverse transcriptional response to chemotherapy. Expression levels of some genes might be significantly changed earlier or later than after 24 hours, and one will not be able to discover them in a consistent manner across every tumor. This statement is supported in the present study by the time course gene expression profiles, which we obtained for five serial biopsies taken from a single tumor. Expression levels of some up- or down-regulated genes achieved maximum or minimum values as early as 8 hours after the treatment and were often insignificantly changed after 24 hours.

Nevertheless, appropriate statistical analyses have identified genes that are differentially expressed between pre- and posttherapy biopsies. In the analysis of pre- and posttreatment expression profiles obtained with both Clontech and Affymetrix platforms, we identified several differentially expressed genes that, together, could separate pre- from posttreatment samples.

Because the major focus of this study was to identify differentially expressed genes, we aimed at independent confirmation of the whole data set. Therefore, the results from the Clontech platform were compared with those of the Affymetrix platform. The direct comparison revealed a limited agreement between the two different array platforms. First of all, the relative intensities of gene expression on the Affymetrix platform were significantly different when compared with the Clontech platform. For example, genes having high signal intensity on the Affymetrix microarray were spread throughout the whole distribution expression intensities on the Clontech arrays. Clontech expression data were confirmed for 3 of 19 genes that were significantly differentially expressed between pre- and posttherapy samples. The lack of agreement between gene expression measurements from different commercial microarray platforms has been reported previously (74–76). Thus, correlation in gene expression levels, as well as the results of a comparison of significant gene expression changes, may show considerable

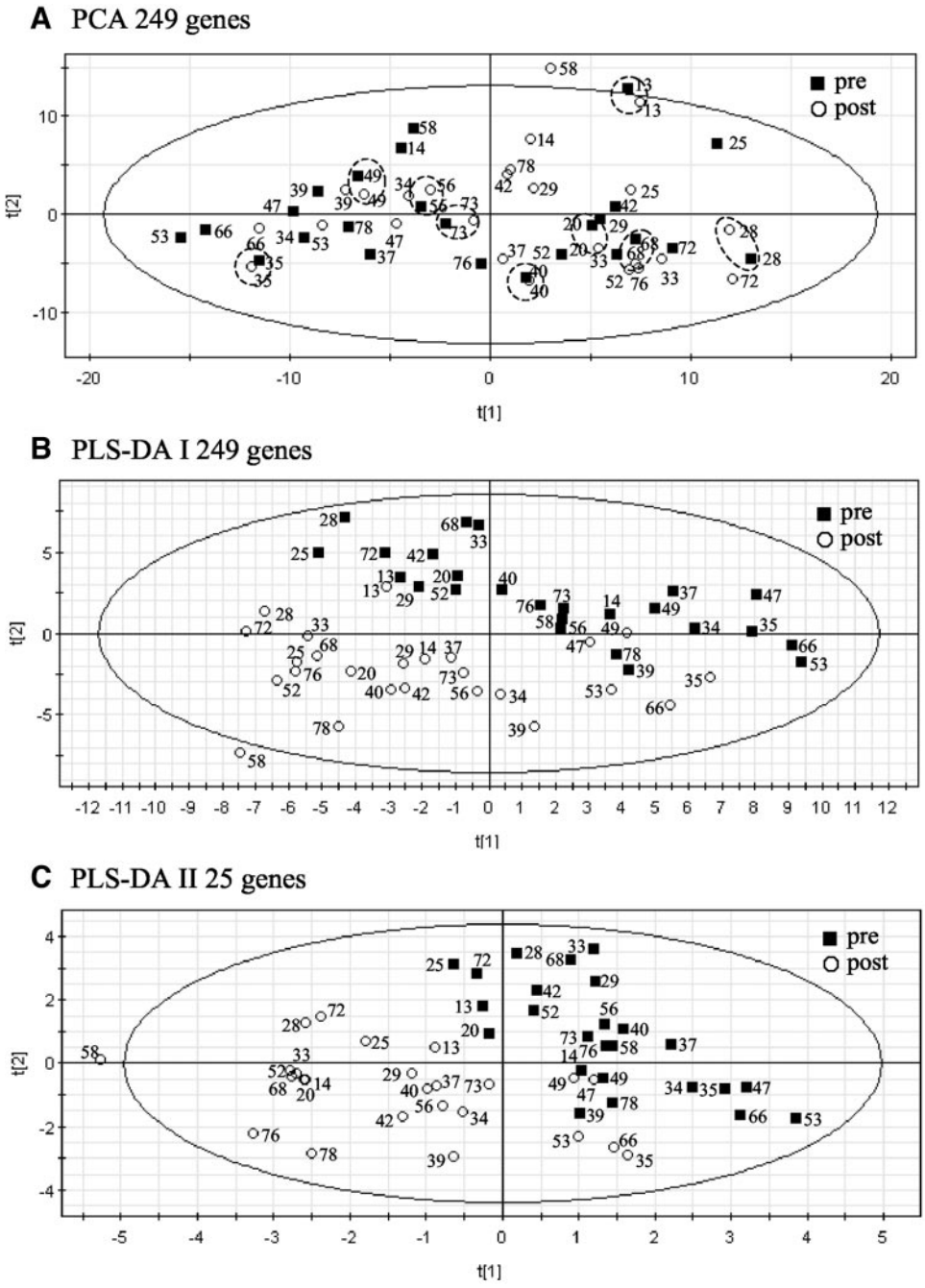

Fig. 3 PLS-DA between pre- and postchemotherapy treatment breast cancer samples. *A*, principal component analysis discrimination with 249 cDNA clones (*PCA 249 genes*) selected from Human Cancer 1.2 Clontech Arrays on the basis of their expression levels. *B*, PLS discrimination with 249 cDNAs (*PLS-DA I 249 genes*). *C*, PLS discrimination with the 25 cDNAs with VIP >1.8 (*PLS-DA II 25 genes*). *A–C*, all 50 of the observations were used. *Horizontal axis*, the first PLS component; *vertical axis*, the second PLS component. *Numbers next to the circles*, codes for patients in Table 1.

divergence between different array platforms. In addition, the role of genes, the expression of which is often changed in primary breast cancers shortly after the beginning of the chemotherapy, should ultimately be validated by the patient's response to treatment and overall outcome.

Because paired Student's *t* test and PLS-DA are completely different in their algorithms, gene lists in corresponding Table 2 *versus* Table 4 are not identical. Thus, although the prostate differentiation factor (*MIC-1*) is absent in the PLS-DA (Table 4), it was obviously stimulated in almost all posttherapy samples. However, several genes reappear in the Tables, which demonstrates their potential significance for the correct classification of samples. The PLS-DA could confirmed that genes for $p21^{WAF1/CIP1}$, interferon γ-inducible protein 30, BGT family member 2, DNA ligase I, growth arrest-specific 6, thymidylate synthetase, prostate differentiation factor, DNA repair protein XRCC9, and some others were often regulated under the therapy.

Several up-regulated genes are associated with cell cycle arrest and regulation of proliferation. Cyclin-dependent kinase inhibitor 1 (*$p21^{WAF1/CIP1}$*) was shown to be over-expressed in

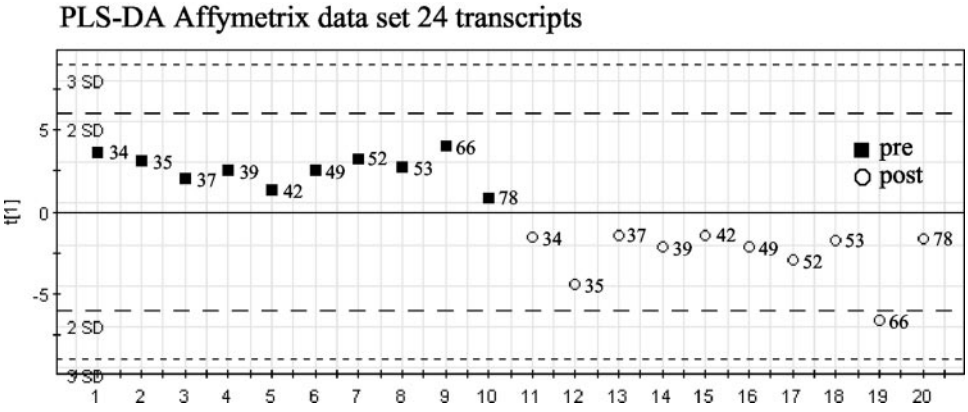

Fig. 4 PLS-DA between pre- and postchemotherapy treatment with 24 cDNAs selected from the Affymetrix arrays data set on the basis of their VIP value >2. All 20 of the observations corresponding to 10 pre-and posttreatment pairs of 10 patients were used. *Horizontal axis*, probe number; *vertical axis*, the PLS component. *Numbers next to the circles*, codes for patients in Table 1.

some breast tumors, whereas normal breast tissues were p21-negative (77). A wide range of stress stimuli including chemotherapeutic agents have been shown to induce expression of the *cyclin-dependent kinase inhibitor p21* (42, 78). In addition to its growth-inhibitory role in cell cycle progression $p21^{WAF1/CIP1}$ has a function in apoptosis prevention. $p21^{WAF1/CIP1}$ regulates the outcome of the p53-mediated response to DNA damage by preventing damaged cells from becoming apoptotic as might occur under chemotherapy. Interestingly, Korn *et al.* (43), analyzing gene expression profiles of breast cancer described by Perou *et al.* (10), found 17 genes including $p21^{WAF1/CIP1}$ that were up-regulated after chemotherapy.

Relatively little is known about the role of MIC-1 in cancer. It reduces cell-to-cell adhesion and may support a tumor cell dissemination (79). Additionally, nonsteroidal anti-inflammatory drugs that inhibit tumor progression also induce *MIC-1* expression (80).

BTG2 (*PC3*) is a member of a family of genes endowed with antiproliferative properties. Current evidence suggests a physiologic role of BTG2 (PC3) in the control of cell cycle arrest after DNA damage and other types of cellular stress or before the cell differentiation (81–83).

Differences in gene expression levels were confirmed by semiquantitative and/or real-time RT-PCR for several genes. It demonstrates that measurement of gene expression profiling with arrays is able to detect changes in gene expression patterns after treatment if one is looking for the prediction of treatment effectiveness.

In conclusion, we showed that it is feasible to study the changes in gene expression under the chemotherapy treatment

Table 5 Twenty-four genes with expression levels involved in discrimination between before-and-after EC or ET chemotherapy, ordered by *VIP* as defined by SIMCA-P software with Affymetrix data set

GenBank accession no.	Gene name	VIP	*P* value (*t* test, paired)
AF016266.1	*TRAIL receptor 2 mRNA, complete cds.*	1.15	0.00233
AA883493	*Consensus includes gb:AA883493/KIAA0761 protein*	1.13	0.00890
BC001149.1	*Similar to KIAA0266 gene product, complete cds.*	1.12	0.00101
NM_014964.1	*KIAA1065 protein* (*KIAA1065*), *mRNA*	1.10	0.00711
NM_005955.1	*Metal-regulatory transcription factor 1* (*MTF1*)	1.08	0.00703
NM_002778.1	*Prosaposin* (*PSAP*)	1.07	0.00285
NM_018449.1	*AD-012 protein* (*LOC55833*)	1.02	0.0109
U67195.1	*Tissue inhibitor of metalloproteinase-3, complete cds.*	1.01	0.00456
AK000826.1	*Consensus includes gb:cDNA FLJ20819 fis, clone ADSE00511*	1.01	0.00534
NM_000389.1	*Cyclin-dependent kinase inhibitor 1A* (*p21, Cip1*) (*CDKN1A*)	1.00	0.0243
BC004247.1	*ras-related C3 botulinum toxin substrate 1* (*Rac1*)	0.99	0.0118
AI769416	*Consensus includes gb:AI769416/FEA_EST*	0.98	0.0120
AF261137.1	*HT031 mRNA, complete cds.*	0.98	0.0362
NM_021074.1	*NADH dehydrogenase* (*ubiquinone*) *flavoprotein 2* (*24kD*) (*NDUFV2*)	0.96	0.0139
NM_012245.1	*ski-interacting protein* (*SNW1*)	0.95	0.00629
NM_006938.1	*Small nuclear ribonucleoprotein D1 polypeptide* (*16kD*) (*SNRPD1*)	0.95	0.00159
NM_000107.1	*Damage-specific DNA binding protein 2* (*48kD*) (*DDB2*)	0.95	0.0138
BC001286.1	*Similar to dCMP deaminase, clone MGC:5160, complete cds.*	0.94	0.0154
AF135266.1	*p8 protein homolog* (*COM1*), *complete cds.*	0.94	0.0124
NM_006736.1	*Heat shock protein, neuronal DNAJ-like 1* (*HSJ1*)	0.94	0.0282
BE560202	*Consensus includes gb:BE560202/FEA_EST*	0.94	0.0266
NM_013329.1	*GC-rich sequence DNA-binding factor candidate* (*GCFC*)	0.94	0.0314
NM_005163.1	*v-akt murine thymoma viral oncogene homolog 1* (*AKT1*)	0.93	0.00880
BE513151	*Consensus includes gb:BE513151/FEA_EST*	0.87	0.0313

Abbreviation: cds., coding sequence.

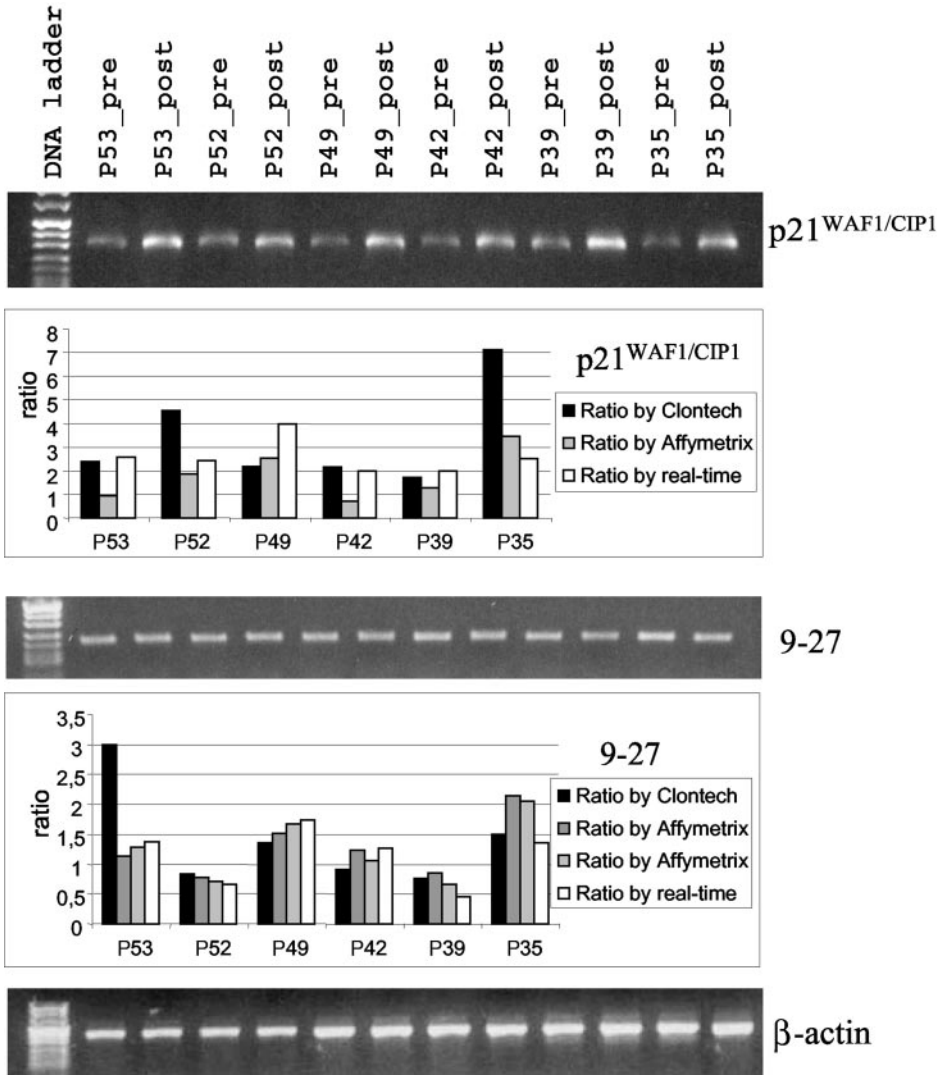

Fig. 5 Validation of gene expression in pre- and post-treatment breast cancer biopsies of six patients (*labeled at the top*: *P53_*, *P52_*, *P49_*, *P42_*, *P39_*, *P35_pre* and *post*) . Expression differences detected by cDNA array were verified by sqRT-PCR for cyclin-dependent kinase inhibitor 1 ($p21^{WAF1/CIP1}$) and interferon-induced transmembrane protein 9-27 (*9-27*). β-*actin*, control, used to standardize sqRT-PCR. *Bar graphs*, comparison of cDNA (Clontech), Affymetrix GeneChip arrays, and real-time RT-PCR results. *On Y axis*, ratios (in absolute units) of expression levels detected by cDNA and oligonucleotide array hybridization from the six different breast tumors pre- and posttreatment, compared after-*versus*-before, for $p21^{WAF1/CIP1}$ and *9-27* genes. Data for two different features on the Affymetrix HG-U133A for *9-27* were available. *Insets with black, gray, and white squares*, symbols key for ratios of differential gene expression obtained by Clontech, Affymetrix, and real-time RT-PCR methods, respectively.

with cDNA or oligonucleotide arrays. The complex cellular and tissue responses to therapy have been demonstrated. To ultimately define whether these changes have a predictive potential for treatment effectiveness, additional information about the pathologic outcome should be added into the data analysis, which will be the topic for our next report.[6]

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