

Differentiation of Lobular *versus* Ductal Breast Carcinomas by Expression Microarray Analysis¹

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ABSTRACT

Invasive lobular and ductal breast tumors have distinct histologies and clinical presentation. Other than altered expression of *E-cadherin*, little is known about the underlying biology that distinguishes the tumor subtypes. We used cDNA microarrays to identify genes differentially expressed between lobular and ductal tumors. Unsupervised clustering of tumors failed to distinguish between the two subtypes. Prediction analysis for microarrays (PAM) was able to predict tumor type with an accuracy of 93.7%. Genes that were significantly differentially expressed between the two groups were identified by MaxT permutation analysis using *t* tests (20 cDNA clones and 10 unique genes), significance analysis for microarrays (33 cDNA clones and 15 genes, at an estimated false discovery rate of 2%), and PAM (31 cDNAs and 15 genes). There were 8 genes identified by all three of these related methods (*E-cadherin*, *survivin*, *cathepsin B*, *TP11*, *SPRY1*, *SCYA14*, *TFAP2B*, and *thrombospondin 4*), and an additional 3 that were identified by significance analysis for microarrays and PAM (*osteopontin*, *HLA-G*, and *CHC1*). To validate the differential expression of these genes, 7 of them were tested by real-time quantitative PCR, which verified that they were differentially expressed in lobular *versus* ductal tumors. In conclusion, specific changes in gene expression distinguish lobular from ductal breast carcinomas. These genes may be important in understanding the basis of phenotypic differences among breast cancers.

INTRODUCTION

Invasive ductal and lobular breast carcinomas are the most common malignancies of the breast, accounting for ~80% and 15% of all invasive breast tumors, respectively (1). The two tumor subtypes are distinguished on the basis of their histology, with ductal tumors tending to form glandular structures, whereas lobular tumors are less cohesive and tend to invade in single file (2). Lobular tumors are often slower growing than ductal tumors, are more often estrogen and progesterone receptor positive (3, 4), have lower vascular endothelial growth factor expression (5), and more frequently have loss of *E-cadherin* (6–8). Genomic analyses have also identified differences, with lobular tumors characterized by loss of chromosome 16q and gain of 1q (7, 9). Such studies suggest that lobular tumor development and progression may follow a distinct pathway from ductal tumors. Although treatment for stage-matched ductal *versus* lobular tumors is similar (10), some studies suggest that metastatic patterns differ between lobular and ductal tumors (11, 12), and lobular tumors may be less responsive to neoadjuvant therapy (13).

The power of expression profiling using microarrays for distinguishing subgroups of breast cancers has been demonstrated by several groups (14–19). Expression profiling and hierarchical clustering of ductal breast cancers have identified classes of tumors with aggres-

sive phenotype and poor prognosis (15, 18). Predictive models based on expression data from node-negative patients can stratify patients into groups that have good prognosis *versus* those with poor prognosis (16, 19). Other studies have found distinct expression patterns based on *BRCA1* and *BRCA2* status (17). To date, however, gene expression differences based on breast cancer histology have not been reported. We report gene expression profiling on a panel of ductal and lobular tumors to identify differentially expressed genes, using several alternative statistical methods to correct for the multiple comparisons problem.

MATERIALS AND METHODS

Samples. Fresh-frozen breast tumors were collected after institutional review board approval from patients treated at UCSF³ and California Pacific Medical Centers. Frozen tumor blocks were trimmed to ensure that a minimum of 70% of the cells present were tumor cells. A total of 106 invasive ductal tumors, 17 invasive lobular tumors, and 6 normal breast samples from reduction mammoplasties were analyzed. Lobular cases were additionally stained for E-cadherin by immunohistochemistry. There were no significant differences between the ductal and lobular groups in distribution of stage or nodal status.

RNA Isolation. Frozen sections were placed directly in Trizol reagent (Invitrogen, Carlsbad, CA), homogenized for 2 min on ice, and RNA was isolated using the manufacturer's protocol. RNA was quantified based on absorbance at 260 nm. Quality of the RNA was assured by measuring 260:280 ratios and reviewing integrity on agarose denaturing gels. Some samples were additionally purified through RNeasy columns (Qiagen, Valencia, CA) according to the manufacturer's protocol. A mixture of equal amounts of RNA from the following cell lines, all available from American Type Culture Collection (Manassas, VA), was used as a common hybridization reference sample: SW872, WM115, NTERA2, MCF7, HEPG2, MOLT4, Hs578t, HL60, OVCAR3, COLO205, and RPMI 8226. The same preparation of reference pool RNA was used for all of the hybridizations.

Expression Microarray Preparation. Microarrays were prepared using standard protocols.⁴ Briefly, clones from the Research Genetics clone set (Research Genetics, Huntsville, AL) were PCR amplified using universal primers (20), and clone band size was verified. Clones were precipitated, dissolved in 3× SSC, and printed on poly-L-lysine (Sigma, St. Louis, MO) coated slides. Microarrays consisted of 32 subarrays, each 18 rows by 18 columns, for a total of 10,368 spots. Slides were stored under vacuum, and postprocessed using standard protocols.⁴ Microarrays from five different print runs (using the same clone preparations) were used in this study. Data were normalized by print before unsupervised clustering to avoid print specific effects (15).

Labeling and Hybridization. For each sample, 5–10 μg of RNA was digested with DNase I (Ambion, Austin, TX) then reverse transcribed with superscript II reverse transcriptase (Invitrogen) in the presence of amino-allyl modified dUTP (Sigma) using 2 μg of random hexamers (Invitrogen) and 1.25 μg of oligodeoxythymidylic acid primers (Invitrogen). BSA (1.5 μg; Ambion) and 125 μmol of ddATP (Invitrogen) were included in the reaction to increase signal and representation of rare transcripts (21). The cDNA product was cleaned through Microcon 30 columns (Millipore, Billerica, MA), coupled to

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³ The abbreviations used are: UCSF, University of California San Francisco; PAM, prediction analysis for microarrays; SAM, significance analysis for microarrays.

⁴ Internet address: <http://derisilab.ucsf.edu/microarray/protocols.html>.

Cy3 dye (Amersham, Piscataway, NJ), and mixed with a Cy5 (Amersham)-labeled reference. The probe mixture was purified through QIAquick PCR columns (Qiagen), and mixed with 2 μg of Cot-1 DNA (Invitrogen) and 10 μg of tRNA (Invitrogen) in a buffer composed of 25 mM HEPES, 3 \times SSC, and 0.03% SDS. The probe was spun through an ULTRAFree MC column (Millipore) heated to 100°C for 2 min, transferred to 37°C for 5 min, and applied under a lifter slip (Erie Scientific, Portsmouth, NH) to the slide surface. Slides were incubated at 65°C overnight in a HybChamber (Gene Machines, San Carlos, CA), and washed in 2 \times SSC, 1% SDS at 55°C for 10 min, 2 \times SSC at room temperature for 10 min, and 0.2 \times SSC at room temperature for 10 min. Slides were briefly rinsed in deionized water and dried with compressed air. Slides were imaged on an Axon 9000B scanner (Axon Instruments, Union City, CA).

Data Analysis. All of the images were analyzed using GenePix pro v3.06 (Axon Instruments). Data were then corrected by subarray (print tip) median centering and LOESS smoothing using the Bioconductor R software package.⁵ Clustering was performed using Eisen's Cluster and TreeView (22). For unsupervised clustering, clones were chosen based on the entire tumor sample set; they were accepted if they were present in at least 80% of cases and showed a \log_2 ratio of less than -2 or >2 in at least one case, resulting in a total of $\sim 4,000$ genes. All of the genes and arrays were median centered and normalized. The entire LOESS smoothed and median centered data set is available.⁶ For the supervised clustering methods described below, the entire set of $\sim 10,000$ genes was used.

The *mt.MaxT* function in the *Multtest* package for Bioconductor R was used to determine family-wise error rate-adjusted *P* values using the *t*-statistic, to test genes for differential expression between the two tumor sets by the method of Westfall and Young (23). Briefly, the class labels (ductal and lobular) are permuted, and the *t*-statistic for each gene is calculated. The maximum *t*-statistic is recorded for 10,000 random permutations, the distribution of maximum *t*-statistics is compared with observed values for the statistic, and the *P* for each gene is estimated as the proportion of the maximum permutation-based *t*-statistics exceeding the observed value.

SAM software was applied to adjust *P* values using a false discovery rate to identify genes that were significantly different between groups (24). SAM computes a penalized *t*-statistic for each gene and then performs a set of permutations to determine the false discovery rate by shuffling the class labels (5000 permutations in our analysis). The δ value was adjusted to give approximately the same number of genes as were identified by PAM.

The PAM R package was used to classify tumors and identify genes that were associated with specific histology (25). PAM ranks genes using a penalized *t*-statistic and uses soft-thresholding to identify a gene set for classification. The number of genes used is controlled by a thresholding parameter, which is determined with a 10-fold cross-validation. The threshold parameter is chosen to minimize the overall error rate (25).

Quality Control and Validation by Quantitative PCR. Multiple hybridizations of the breast cell line BT474 showed high reproducibility. Washing the slides at 55°C in 2 \times SSC/0.1% SDS, a higher stringency than reported by others⁷ additionally increased reproducibility.⁸ Slides washed under these conditions gave a stronger rank correlation when expression array values were compared with expression values for the *ERBB2* gene measured by real-time quantitative PCR across a series of 11 cell lines ($R^2 = 0.85$). As an additional control, replicate clones of selected genes clustered together and showed high correlation. Also, replicate samples were hybridized to slides from different print runs, and these samples showed strong clustering. All of the clones identified by supervised analyses were sequence verified, to avoid misidentification (26, 27). A representative subset of genes identified by array analysis were chosen for validation using commercially available Assays-on-Demand probe-primer sets (Applied Biosystems, Foster City, CA) by real-time quantitative PCR analysis. Briefly, 500 ng of RNA was reversed transcribed with 1.25 units of murine leukemia virus reverse transcriptase (Invitrogen) using 5 μM random primers (Invitrogen) in the presence of 7.5 mM MgCl_2 and 1 unit of ANTI-Rnase (Ambion) at 25°C for 10 min, 48°C 40 min, and 95°C for 5 min. RNA was used from 10 lobular tumor samples, 19 ductal tumor samples,

1 sample pooled from three reduction mammoplasties, 1 sample pooled from three primary breast epithelial cell cultures, 1 sample pooled from four primary carcinoma-associated breast fibroblast samples, and 2 samples from primary normal breast fibroblast samples. For real-time quantitative PCR, 4 μl of the cDNA product was used to measure gene expression using an ABI Prism 7900 Sequence Detection System (Applied Biosystems) and the Assay-on-Demand probe-primer sets. One-tailed *t* tests were used to determine significant differences in expression measured by quantitative PCR between ductal and lobular samples.

RESULTS

Hierarchical Clustering of Lobular Tumors. Seventeen lobular and 6 reduction mammoplasty (normal) samples were analyzed by hierarchical clustering on a subset of ~ 3500 genes to explore tumor grouping and gene clustering in the lobular samples. These genes were chosen on the basis of being present in at least 80% of the lobular and normal cases.

Several clusters of related genes were evident by hierarchical clustering, the largest of which was related to proliferation (Fig. 1). This cluster included genes such as *cyclin E1*, *cdc6*, *PCNA*, *B-myb*, histones and histone related centromeric proteins (*CENPE*), the *chek1* mitotic checkpoint gene, and *GART* (involved in purine biosynthesis). Three groups of tumors were evident. Group I, which included the normal samples, was characterized by low levels of expression of genes in the proliferation-associated cluster, group II tumors showed the highest levels of expression, and group III showed mixed expression levels.

A cluster including matrix metalloproteinase *MMP2* and its inhibitor, *TIMP2*, was also evident. Expression of genes in this cluster were highest in tumors from group I, with low levels of expression in group II tumors, and low levels in most tumors in group III.

A cluster of genes related to estrogen receptor (*ESR1*) expression was seen, and included multiple copies of *ESR1* as well as *LIV-1*, which has been shown previously to cluster with *ESR1* (14, 28). Tumors in cluster I showed low levels of expression of *ESR1* and related genes, whereas tumors in clusters II and III showed mixed expression levels. All of the lobular tumors in this study were classified as estrogen receptor-positive by immunohistochemistry except for S36 (* in Fig. 1). Interestingly, this tumor showed high levels of *ESR1* gene expression relative to the other lobular tumors.

Several other gene clusters were present (data not shown). A cluster of *ERBB2* and related genes included *TOBI*, *NSF*, *COX11*, *CLTC*, and an expressed sequence tag (AA485411), all of which map to the same chromosomal region as *ERBB2* (17q12–21). Lobular tumors B413 and S93 showed relatively high levels of expression of *ERBB2*. Other gene clusters included those related to *ZNF217* expression, and *TIMP3* and *CD44* expression. These clusters showed mixed expression levels in the three tumor groups.

Tumors with different stages, grades, nodal status, and outcomes were found in every group, indicating that tumor clustering was not driven by clinical parameters. Two of the lobular cases were progesterone receptor negative by immunohistochemistry, and both were found in the group I cluster that showed low levels of proliferation.

Supervised Analysis of Lobular Cancer *versus* Normal Breast. Multiple comparisons may result in many genes appearing significant by chance alone. Several approaches for significance adjustments have been used in the analysis of microarray experiments. We used two similar tests, *MaxT* and *SAM*, to identify genes that distinguished between the lobular and normal samples. Both use permutation to identify significant differences between different groups. *MaxT* identified 49 clones that were significantly different in lobular *versus* normal breast. The 10 most significantly differentially expressed

⁵ Internet address: www.bioconductor.org.

⁶ Internet address: ml.ucsf.edu/people/waldman/Korkola/lobular.htm.

⁷ Internet address: cmgm.stanford.edu/pbrown/protocols/5_hyb_human.html.

⁸ J. Korkola and F. Waldman, unpublished observations.

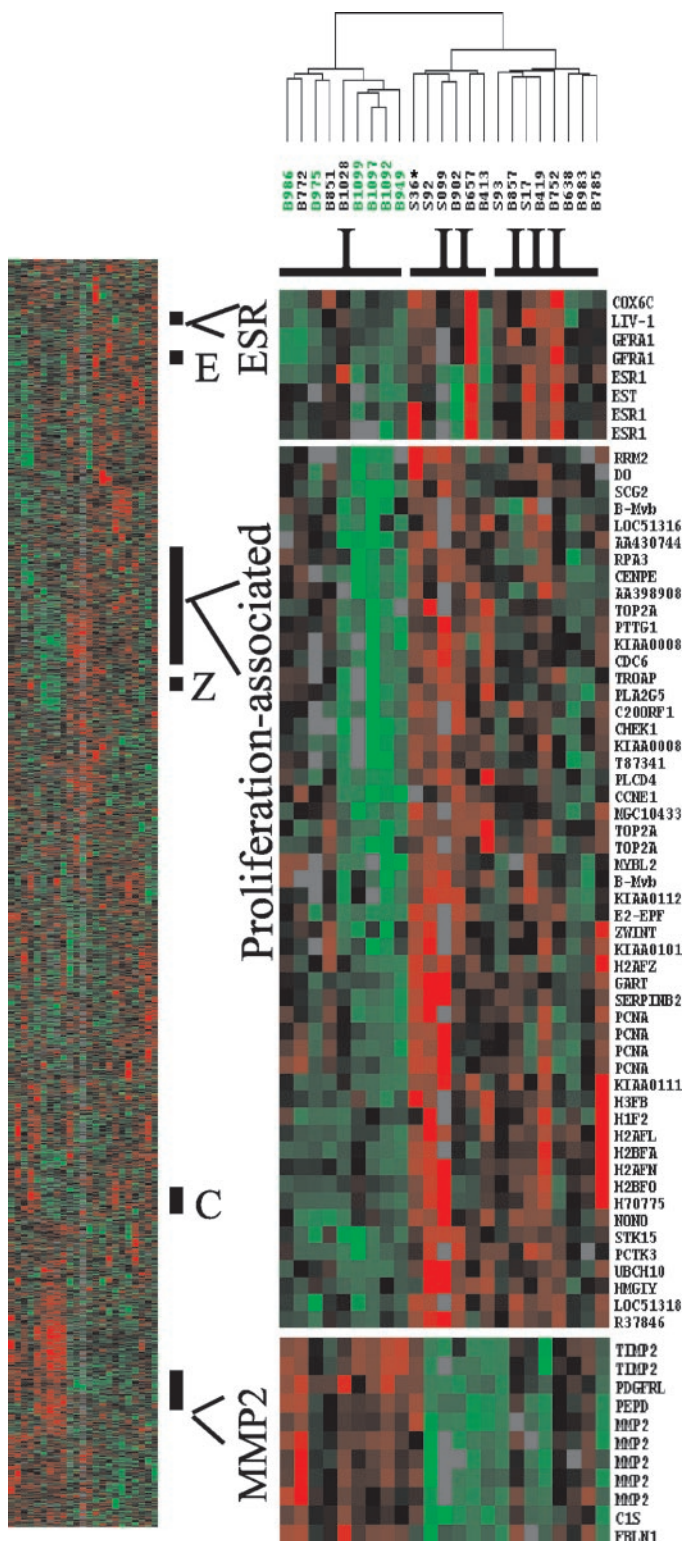


Fig. 1. Unsupervised hierarchical clustering of lobular tumors (black) and normal breast samples (green). Right, clustering of tumors into three groups (I, II, and III). The cluster map for all 3500 genes is shown on the left. Gene clusters included those related to proliferation, MMP2, and estrogen receptor (ESR) shown on left, magnified images on right. Other clusters included those related to ERBB2 (E), CD44 (C), and ZNF217 (Z; magnified images not shown).

genes by MaxT analysis are shown in Table 1. SAM analysis also identified 47 of these 49 clones as being differentially expressed, and an additional 88 clones. For these 135 genes, SAM estimated the false

discovery rate to be 0.5% (0.62 genes). Of 135 clones, 131 were more highly expressed in the normal than lobular samples.

Hierarchical Clustering of Lobular *versus* Ductal Tumors. Unsupervised hierarchical clustering did not distinguish the 17 lobular tumors from the 109 ductal tumors. Whereas the normal samples clustered together in one group, lobular cases were found in multiple groups. As was found with the lobular only clustering, a tumor group characterized by low expression levels of proliferation-associated genes was evident. Normal breast samples were confined to this cluster, which also included lobular and ductal cases.

Lobular tumors frequently have absent or altered expression of *E-cadherin* (6). Of the 17 lobular tumors, 15 showed low levels of *E-cadherin* by expression microarray analysis. The exceptions were cases B638 and B413, which showed weak to moderate levels of expression by microarray. Although B638 did not show any staining for *E-cadherin* by immunohistochemistry, B413 showed diminished but positive membranous staining. All of the other lobular cases showed no *E-cadherin* staining by immunohistochemistry. Histological review of B413 revealed mixed invasive lobular cancer and ductal carcinoma *in situ*.

Expression of *E-cadherin* was associated with expression of *ZNF217* ($r = 0.488$). *E-cadherin* and *ZNF217* are not located on a common chromosomal region and have not been reported previously to have coregulated expression.

All of the lobular cases except one were estrogen receptor-positive. There was a group of tumors that consisted almost entirely of estrogen receptor-negative ductal samples in the unsupervised clustering (data not shown). To determine whether clustering could separate the receptor-positive ductal from lobular cases, clustering was performed excluding the estrogen receptor-negative samples. This clustering did not show improved separation of the lobular from ductal samples (data not shown). There were no tumor groups that formed that were related to other clinical parameters such as stage, nodal status, or outcome with unsupervised clustering. As described in "Materials and Methods," there were no significant differences in these clinical parameters between the tumor types.

Distinguishing Lobular and Ductal Tumors by Differential Gene Expression

MaxT Analysis. MaxT permutation analysis identified 20 clones representing 10 genes that had adjusted P values of <0.05 (compared with 1733 clones with $P < 0.05$ before permutation correction), as shown in Table 2. *E-cadherin* (*CDH1*, 5 clones) was identified as having significantly lower expression levels in lobular than ductal tumors. This gene is frequently lost or altered in lobular breast cancer. Significantly lower levels of *cathepsin B* (*CTSB*, 7 copies) were also

Table 1 Genes differentially expressed between lobular cancer and normal breast tissue

Gene	Genbank accession no.	Normal:lobular ratio ^a	MaxT P^b
<i>Matrix metalloendopeptidase^c</i>	R98851	7.08	0.030
<i>Bullous pemphigoid antigen 1</i>	H44785	7.53	0.003
<i>Keratin 5</i>	AA160507	11.86	0.005
<i>Metallothionein 1L</i>	A1289110	2.52	0.005
<i>Platelet activating factor acetylhydrolase 1B</i>	AA464238	0.43	0.005
<i>KIAA0353</i>	AA877815	7.24	0.006
<i>Regulator of G protein signaling 2</i>	A1675670	3.79	0.007
<i>Metallothionein 1L</i>	N80129	3.15	0.007
<i>MAP3K7 interacting protein 1</i>	BC050554	4.37	0.008
<i>Potassium channel KCNMB1</i>	AA029299	5.04	0.009

^a The calculated ratio between normal and lobular samples.

^b The adjusted P from MaxT permutation analysis.

^c For *matrix metalloendopeptidase*, 6 clones were found to be significant by both SAM and MaxT analysis; the least significant MaxT adjusted P is shown for this clone.

Table 2 Genes that differentiate lobular from ductal breast cancers

Gene	Genbank accession no.	PAM ^a	SAM ^b	MaxT ^c	lob:ductal ^d	MaxT p value ^e
<i>E-cadherin</i>	H97778 or AI671174	6	6	5	0.325	0.003
<i>Osteopontin</i>	AA775616	5	5	0	0.326	0.084
<i>Survivin</i>	AA460685	1	1	1	0.548	0.048
<i>Cathepsin B</i>	AA598950	7	8	7	0.554	0.005
<i>HLA-G</i>	AI565209	1	1	0	0.558	0.250
<i>HLA-F</i>	AA988615	0	1	0	0.577	0.550
<i>H2BFO</i>	AA010223	0	1	0	0.580	0.550
<i>Chromosome condensation 1</i>	AA291398	1	1	0	0.586	0.120
<i>Triose phosphate isomerase 1</i>	AA663983	1	1	1	0.623	0.045
<i>Calreticulin</i>	H99170	0	2	0	0.643	0.260
<i>β-Actin</i>	M10278	0	1	0	0.664	0.052
<i>Expressed sequence tag</i>	R26172	0	0	1	1.485	0.040
<i>Transmembrane 4 SFM2</i>	N93505	0	0	1	1.547	0.042
<i>Sprouty 1</i>	AA055440	1	1	1	1.697	0.038
<i>Four and a half Lim domains 1</i>	AA455925	1	0	0	1.906	1.000
<i>Alcohol dehydrogenase 3</i>	AI420743	1	0	0	2.050	0.150
<i>Insulin like growth factor 1</i>	AA456321	1	0	0	2.050	0.370
<i>Small inducible cytokine A14</i>	R96626	1	1	1	2.130	0.024
<i>Elastin</i>	AA459308	1	0	0	2.323	0.420
<i>Trans.Factor AP2B</i>	AA018906	1	1	1	2.536	0.012
<i>Thrombospondin 4</i>	AA423957	2	2	1	2.583	0.009

^a The number of replicates for each gene identified by PAM analysis that distinguish lobular *versus* ductal tumors. Note for *E-cadherin* (*CDH1*) two different clones were identified in the analysis.

^b The number of replicates for each gene identified by SAM analysis.

^c The number of replicates for each gene identified by MaxT permutation analysis.

^d The average lobular:ductal tumor expression level as measured by microarray.

^e The maxT adjusted *P* for each gene. For samples with multiple clones, the least significant *P* is shown.

associated with lobular histology. Other genes identified included *transcription factor AP2B* (*TFAP2B*), *triosephosphate isomerase 1* (*TP11*), and *thrombospondin 4* (*THBS4*).

SAM Analysis. SAM uses a false discovery rate permutation approach for *P* adjustment to identify genes that are significantly differentially expressed between two or more classes. For 33 clones identified by SAM, it was estimated that the false discovery rate was 2.0%.

PAM Analysis. PAM was also used to classify the tumors. PAM builds a classifier based on a ranking of genes using a penalized t-statistic, then determines the misclassification error rate through cross-validation. The optimal classifier included 31 cDNA clones (15 unique genes). Adding more genes to the classifier increased the error rate, whereas fewer genes did not have enough power to discriminate between classes. This gene set showed excellent performance, classifying 15 of 17 lobular and 103 of 109 ductal samples correctly for an overall success rate of 93.7%.

Validation of Tumor-Associated Genes by Quantitative PCR Analysis. To validate the findings from the expression arrays, 7 genes were tested by real-time quantitative PCR analysis. The genes selected were *E-cadherin*, *survivin*, *thrombospondin 4*, *β-actin*, *transcription factor AP2B*, *cathepsin B*, and *osteopontin*. Control genes were *PPP1CA* and *GAPDH*. Fig. 2A shows the average δ Ct (normalized cycle number, relative to *PPP1CA*) for lobular *versus* ductal tumors. For every gene that was differentially expressed by microarray, quantitative PCR found significant differences in gene expression. This was true for genes that were more highly expressed in ductal tumors than in lobular (*β-actin*, *E-cadherin*, *cathepsin B*, and *osteopontin*) and those that were more highly expressed in lobular samples (*TFAP2B* and *thrombospondin 4*). The linear fold change between ductal and lobular samples was calculated based on measurements from either quantitative PCR or microarray reading (Fig. 2B). There was good agreement between the two, although quantitative PCR tended to have larger magnitude differences than microarrays.

To determine whether these differentially expressed genes were preferentially expressed in stromal or epithelial cells, RNA from cultured fibroblasts and epithelial cells were tested by quantitative PCR (Fig. 3). *β-Actin*, *cathepsin B*, and *thrombospondin 4* showed higher levels of expression in the fibroblasts than epithelial cells,

whereas *E-cadherin*, *osteopontin*, and *TFAP2B* showed higher levels of expression in epithelial cells. *Survivin* showed comparable expression levels in both cell types. To test whether culturing had an effect on gene expression in these samples, an uncultured pooled reduction mammoplasty sample was also tested for expression of these genes by real-time PCR. *Osteopontin*, *TFAP2B*, and *thrombospondin 4* were all expressed at lower levels in the cultured samples than in the reduction mammoplasty, whereas *survivin* was expressed at higher levels in the cultured samples. *E-cadherin*, *cathepsin B*, and *β-actin* did not show any changes in expression between the cultured and uncultured samples.

Hierarchical Clustering on the Basis of Genes Identified by Supervised Approaches. Unsupervised clustering using the 39 clones identified by MaxT, SAM, or PAM showed good discrimination of lobular from ductal tumors (Fig. 4). All of the lobular tumors except 2 were found in a large cluster consisting of 15 lobular tumors and 21 ductal tumors. Within this cluster, a smaller subcluster of 9 tumors was evident and consisted entirely of lobular samples. Clustering based on clones identified only by MaxT, SAM, or PAM all led to similar tumor grouping, with enrichment for lobular tumors in one tumor group (data not shown).

DISCUSSION

Expression analysis using microarrays has proven valuable in the study of tumor biology, identifying genes associated with tumor phenotype, response to treatment, and prognosis (14–17). The technique allows high-throughput analysis of expression for tens of thousands of genes in a single sample, is relatively easy to perform, and tends to give reproducible and accurate results. Expression analysis can detect alterations in both tumor and surrounding stroma (29). Whereas the large number of genes assayed is a strength of the approach, it does require statistical methods to correct for random associations. RNA expression analysis fails to identify post-translational modifications that impact protein expression and function. Furthermore, microarray analysis can be complicated by problems with clone misidentification. Studies that take these potential pitfalls into account have the ability to identify complex gene expression

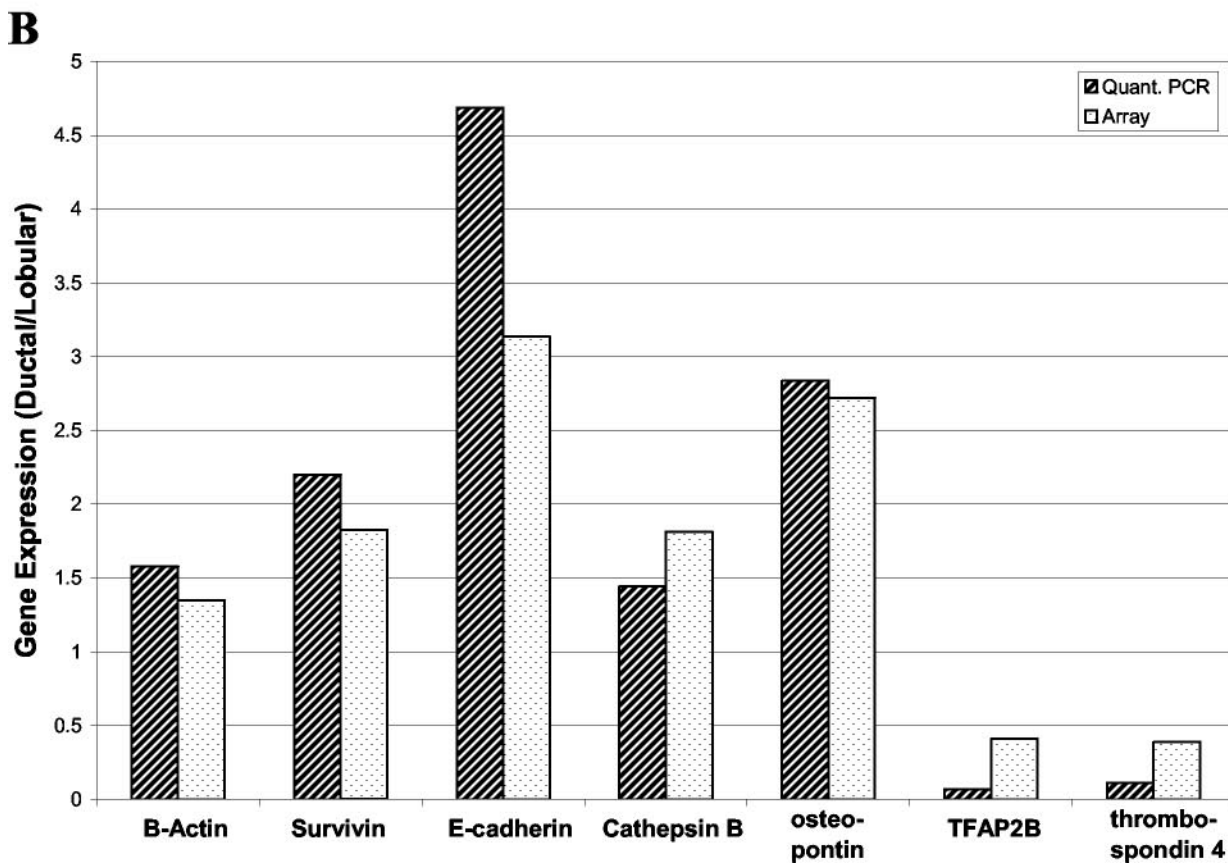
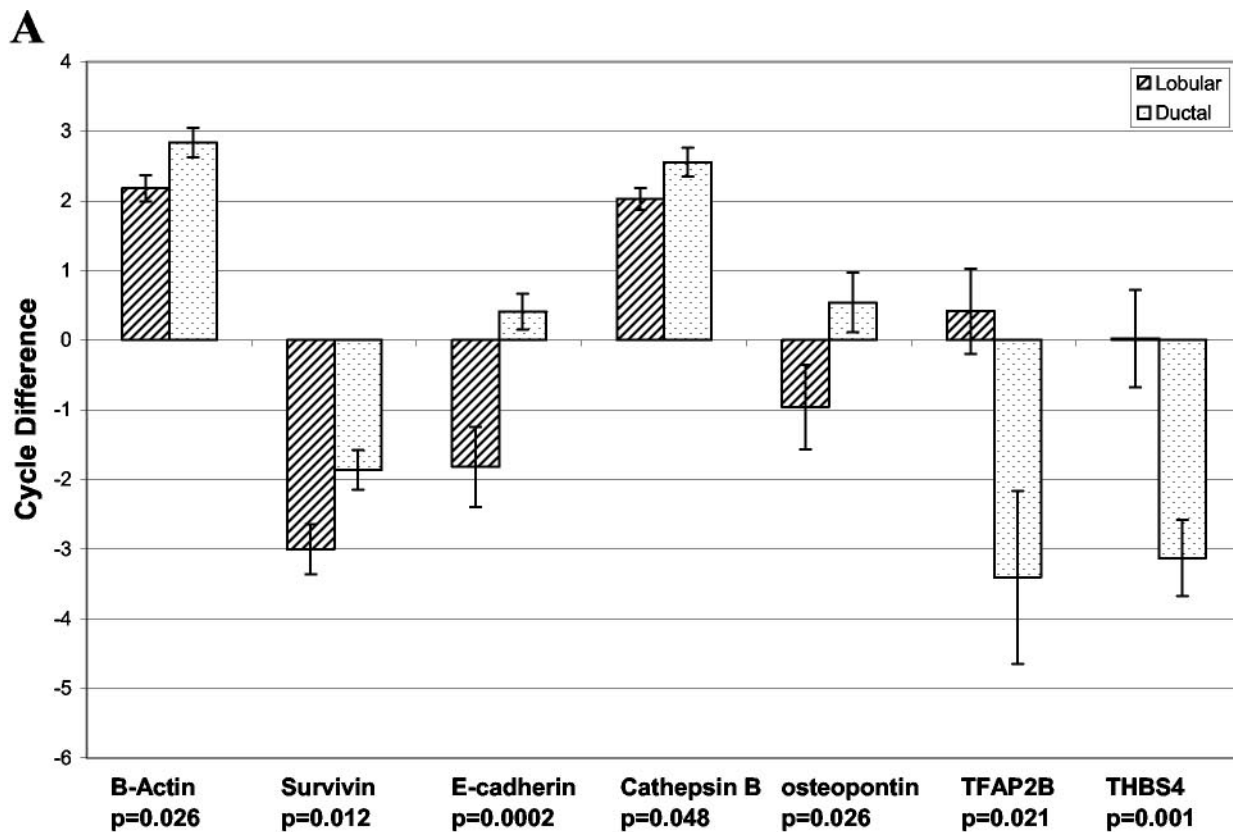


Fig. 2. A. Cycle difference (test gene – control gene *PPP1CA*) for lobular (▨) *versus* ductal samples (▤) for 7 test genes as measured by quantitative PCR. Error bars, ± 1 SE; *P*s reflect one-tailed *t* tests between lobular ($n = 10$) and ductal ($n = 19$) samples. B, relative gene expression in ductal *versus* lobular tumors for the 7 test genes. (▨), real-time quantitative PCR; (▤), microarray.

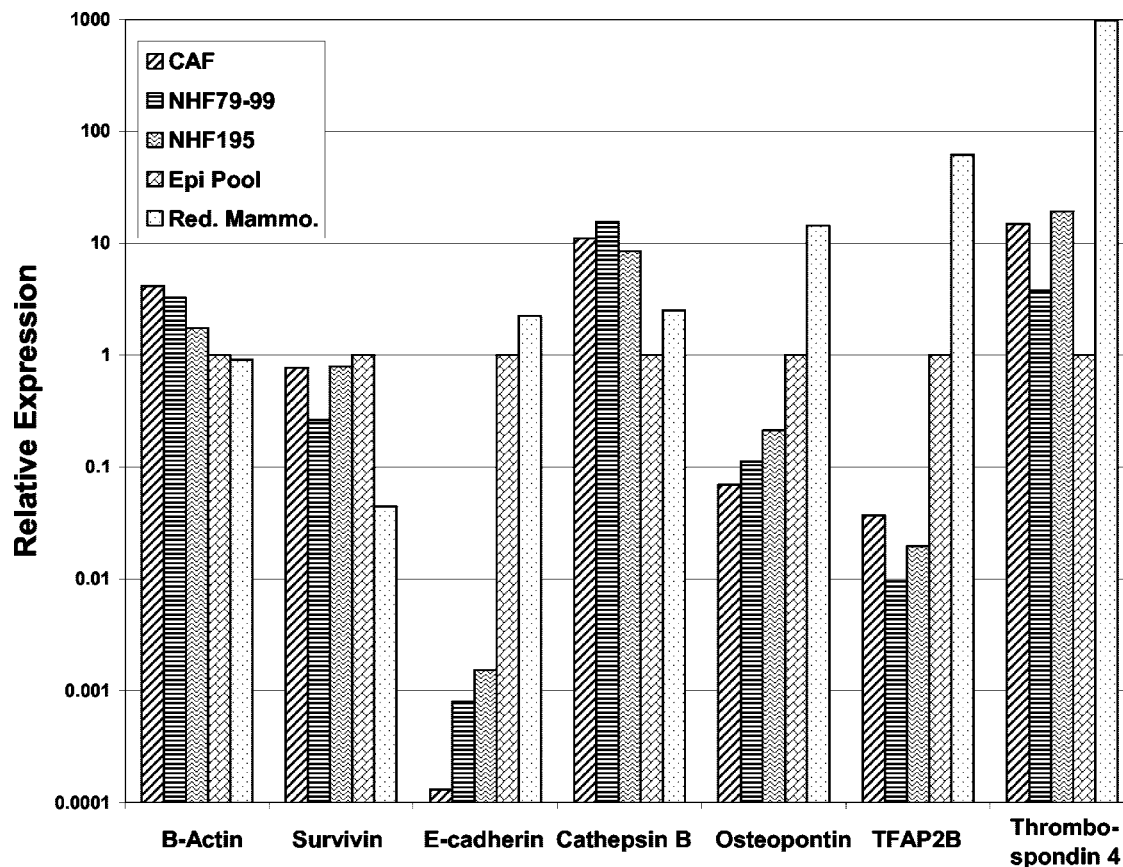


Fig. 3. Relative gene expression by quantitative PCR for the 7 test genes. Expression levels were normalized to the *PPP1CA* control gene. All expression levels are shown relative to the cultured epithelial cell pool sample (set to a value of 1.0). *CAF*, pooled breast carcinoma associated fibroblast cultured sample; *NHF 79-99* and *NHF 195*, normal breast associated fibroblast cultured samples; *Epi. Pool*, pooled normal breast epithelial cultured sample; *Red. Mammo.*, pooled reduction mammoplasty sample.

relationships and allow classification of samples based on their molecular profiles.

Lobular tumors are a distinct subset of breast tumors, based on histology, genetics, and biology. They are often hormone receptor-positive and tend to be more slowly proliferating than ductal tumors (3, 4). Lobular tumors have distinct DNA copy number changes compared with ductal cancers, suggesting a separate pathway of tumor formation (7, 9). Whereas gain of 1q and loss of 16q are among the most common chromosomal changes in ductal cancer, array comparative genomic hybridization analysis shows that these alterations occur in ~100% of lobular tumors.⁸ The loss of 16q is of particular interest, because this is the site of the *E-cadherin* gene, which is lost or altered in almost all of the lobular tumors (6-8). Another feature that distinguishes lobular from ductal breast tumors is the rarity of amplification of *cyclin D1* and *ERBB2* in lobular cancer, both of which occur frequently in ductal tumors (30-33).

Supervised analysis was able to identify many genes that were differentially expressed between lobular and normal samples. Many of the genes showing higher expression in normal *versus* tumor samples were related to nonepithelial cell types, likely reflecting the enrichment for cells of epithelial origin in the tumor compared with normal. For example, genes that are more highly expressed in fat (*FABP4* and *caveolin 1*), muscle (*myosin*), and immune cells (*kit*) were more highly expressed in the normal than in lobular tumors. There were also a number of genes involved in cell adhesion and extracellular matrix degradation that were more highly expressed in normal, reflecting a possible change in stromal-epithelial interactions and signaling in lobular tumors.

We chose three supervised methods to distinguish lobular from

ductal tumors; no other supervised approaches were used. SAM and MaxT use similar *t* tests and permutations to identify differentially expressed genes, but the calculation in SAM analysis adds a small correction factor (24). This results in a less stringent method, which likely accounts for the larger number of significant genes identified. Indeed, several of the genes identified by SAM were of borderline significance according to MaxT analysis. In contrast, PAM analysis does not test statistical significance of differentially expressed genes, but rather selects genes that best classify two groups of samples. This lack of significance likely explains why several of the genes identified by PAM analysis had relatively high *Ps* according to MaxT.

PAM correctly classified 15 of 17 lobular and 103 of 109 ductal cases. Case B413 was one of the misclassified lobular samples and showed atypical clinicopathological features, with a mixed lobular-ductal carcinoma *in situ* histology, positive *E-cadherin* expression by microarray analysis, positive but diminished *E-cadherin* staining by immunohistochemistry, a normal copy number for chromosome 16q, and overexpression of *ERBB2*. The atypical phenotype of this sample suggests a possible histological misclassification.

The gene that had the strongest association with lobular histology was *E-cadherin*, which has been described previously as being differentially expressed between the two tumor types (6). *E-cadherin* interacts with other *cadherins* and *catenins* for maintenance of tissue architecture and signaling through the adenomatous polyposis coli pathway (34, 35), and mutations, deletions, and methylation silencing can lead to loss of this function (6, 36). Loss of *E-cadherin* function is thought to directly contribute to the histological appearance of lobular tumors, which are characterized by a lack of cohesive architecture with single cells invading through the stroma (2). All but two

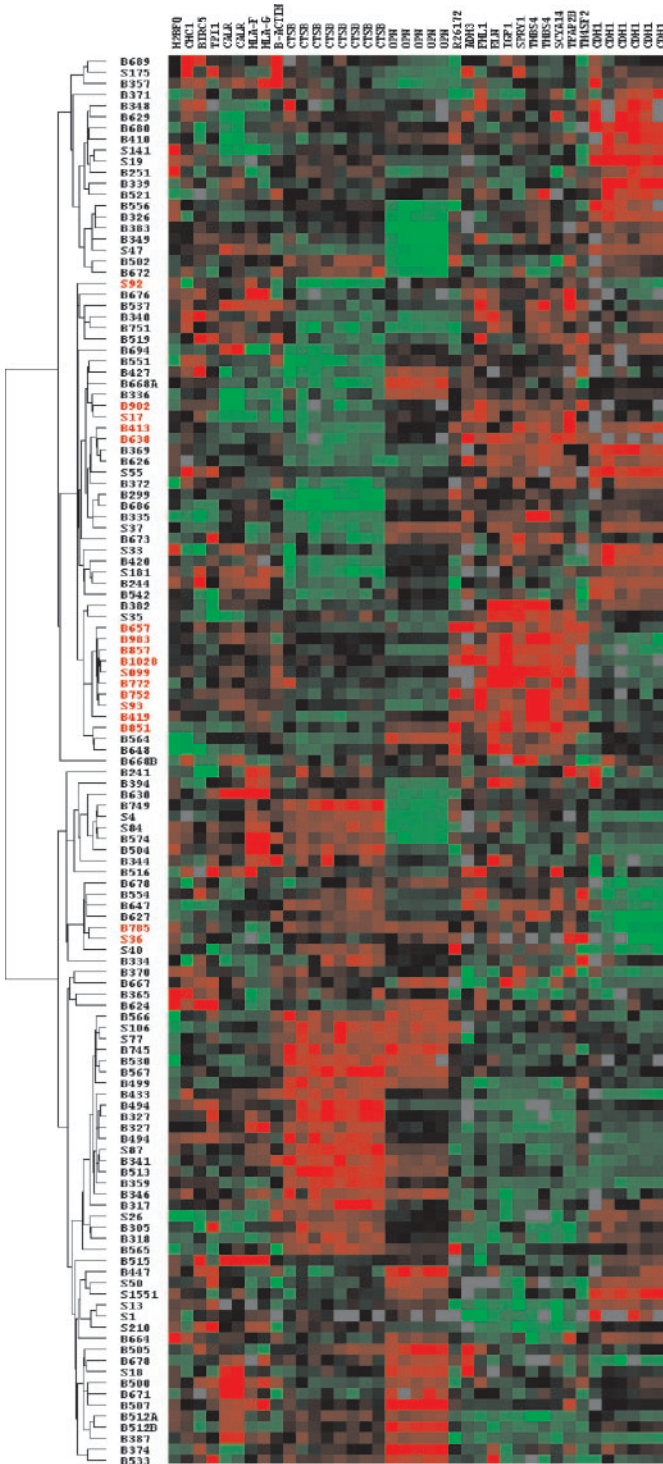


Fig. 4. Hierarchical clustering of lobular and ductal tumors based on the 39 clones identified by any of MaxT, SAM, or PAM. Fifteen of 17 lobular samples (red) are found in a single cluster, with 9 of 17 in one tight central cluster that is distinct from ductal samples (black). Note blocks of similar expression in replicate clones for osteopontin (*OPN*), cathepsin B (*CTS B*), and E-cadherin (*CDH1*).

of the lobular tumors showed very low levels of expression of *E-cadherin* by microarray analysis.

TFAP2B is a transcription factor expressed in both normal and malignant breast tissue (37). One of the target genes of *TFAP2* is *E-cadherin* (38), which raises the possibility that *TFAP2B* is up-regulated in lobular tumors in response to low levels of *E-cadherin* protein. In addition to *E-cadherin*, *TFAP2* family members are

thought to control expression of the *IGF1* receptor (39) and the antiapoptotic gene *BCL2* (40). High levels of *IGF1* were found to be predictive of lobular histology by PAM, whereas low levels of the antiapoptotic gene *BIRC5* (*survivin*; Ref. 41) were found in lobular relative to ductal tumors. This may reflect different mechanisms by which lobular and ductal tumors promote growth and escape apoptosis. It has been observed that *BCL-2* overexpression is often associated with *BIRC5* expression (41), which may explain why levels of *BCL-2* were not predictive of lobular *versus* ductal histology.

Osteopontin and *cathepsins* have been reported to be inversely correlated with members of the *thrombospondin* family (42, 43). Consistent with these observations, low levels of *cathepsin B* and *osteopontin*, and high levels of *thrombospondin 4* were found in lobular tumors when compared with ductal tumors. These genes belong to families that have been implicated in control of adhesion, invasion, and metastasis (42–49). The differential expression of these genes associated with regulation of cell adhesion and invasion suggest a model in which lobular tumors may achieve invasive growth by loss of *E-cadherin*, whereas ductal tumors more commonly overexpress *cathepsins* and *osteopontin*, and down-regulate *thrombospondins* to achieve invasiveness.

Previous reports of expression analysis of breast cancers using microarrays have focused on identification of gene sets that are associated with a more aggressive phenotype (14, 18), familial breast cancer (50, 51), or poor prognosis (16). The current study has identified gene clusters that are very similar to ones reported elsewhere. The “normal-like” cluster that described by Perou *et al.* (28) was similar to the low-proliferation cluster that we observed in our data. There were also some elements of the basal-like, luminal-like, and *ERBB2* clusters described by Perou *et al.* (28) present in our data, but the tumors did not form groups as distinct as in their study, likely because our clustering was performed on a much larger set of genes than their “intrinsic” gene set. Several studies have identified genes such as *GATA-3*, *v-myb*, and *LIV-1* as being associated with *estrogen receptor* expression (14, 16, 28), which we observed when our ductal and lobular tumors were clustered. Our study, however, is the first to describe gene expression differences between breast tumors of different pathologically defined histological subtypes.

We have identified a set of genes that are differentially expressed in lobular *versus* ductal tumors. To determine in which cell compartment these genes were expressed, cultured fibroblasts and epithelial cells were tested for gene expression by real-time quantitative PCR. All of the genes except *survivin* showed differential gene expression between the fibroblasts and epithelial cells, suggesting that differences in expression observed between ductal and lobular tumors depends not only on expression in the tumor cells but also in the surrounding stromal cells. It is important to note that whereas there were detectable changes in gene expression between these two cell types, the reduction mamoplasty sample showed expression levels that were different from both the epithelial and fibroblast samples for several of the genes. This suggests that culturing the cells had an effect on expression of these genes, or that expression of the genes may be driven by a cell type other than epithelial cells or fibroblasts (such as adipocytes).

Lobular and ductal tumors are currently treated similarly and have similar outcomes. The inability of unsupervised clustering to distinguish between the two tumor types suggests that they have many expressed genes in common, and, thus, subtle differences in gene expression may be responsible for the phenotypic differences between them. In contrast, supervised analysis of tumors based on expression array analyses identified a gene set that distinguishes

these two subtypes of breast tumors. In particular, ductal and lobular tumors showed striking differences in the expression of genes associated with cell adhesion and invasion, suggesting that they may achieve invasive growth through separate mechanisms. Several of the genes identified may prove to be useful for diagnosis and may be attractive targets for therapies directed against the specific tumor types.

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