Differential Gene and Protein Expression in Primary Breast Malignancies and Their Lymph Node Metastases as Revealed by Combined cDNA Microarray and Tissue Microarray Analysis

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BACKGROUND. Metastatic disease is a major adverse prognostic factor in breast carcinoma. Lymph node metastases often represent the first step in the metastatic process.

METHODS. To gain insight into the molecular events that underlie breast carcinoma metastasis, the authors compared gene expression profiles, obtained by cDNA microarray analysis, of nine matched primary tumors and metastases after screening for enrichment of tumor cells. Statistical analysis identified genes that are expressed at elevated or decreased levels in metastases relative to the corresponding primary tumors. Multidimensional scaling analysis indicated that in terms of expression levels, primary tumors were tightly clustered, whereas metastases exhibited a greater spread; this finding points to the more heterogeneous nature of metastases. Among the differentially expressed entities were the invasion- and tissue modeling–related genes *IGFBP5*, fibronectin, and *MMP2*; the cell cycle regulatory gene cyclin D1; other genes, such as enolase 2; and an expressed sequence tag similar to angiopoietin 1. To validate and extend these initial findings, the authors constructed a tissue microarray consisting of 100 primary malignancies paired with their lymph node metastases. Antibodies for the IGFBP-5, fibronectin, MMP-2, cyclin D1, and MDM-2 proteins were used to stain tissue array sections.

RESULTS. Consistent with microarray data, statistically significant overexpression of IGFBP-5, down-regulation of cyclin D1, and unchanged MDM-2 levels were observed in metastatic tumor cells. Nonetheless, although fibronectin and *MMP2* mRNA expression levels were decreased in many metastasis specimens, expression levels of the corresponding proteins in the extracellular matrix were elevated in most metastases. Decreased expression of fibronectin and *MMP2* in lymph node metastases was further confirmed by real-time polymerase chain reaction assays performed on five additional specimen pairs.

CONCLUSIONS. The results of the current study suggest that extracellular matrix protein expression and nuclear gene expression are associated via a negative-feedback regulatory mechanism. Therefore, gene expression profiling and tissue array validation should be combined to elucidate molecular events associated with the metastatic process. ***Cancer* 2004;100:1110–22.**

KEYWORDS: metastasis model, microarray, breast cancer, lymph node.

Breast carcinoma is the most common malignancy among women worldwide. Metastasis represents a major adverse step in the progression of breast carcinoma. It has been established that auxiliary

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lymph node metastasis is the most reliable factor for predicting poor prognosis, including early recurrence and short survival duration.[1] Because of the important clinical implications, many assays have been developed with the aim of assessing gene activity in the lymph nodes of patients with breast carcinoma. These assays, which primarily involve markers expressed in primary breast carcinoma tissue, are based on the observation that most genes that are highly expressed in primary breast carcinoma tissue also are expressed in lymph node metastases.[2] Because distant metastases (to bone and other sites) are rare findings at presentation,[3] lymph node metastasis is considered the key event in the metastatic process for patients with breast carcinoma. Thus, it is important to understand the molecular events underlying this process, so that better treatment strategies can be developed based on this understanding.

It has been hypothesized that a preexisting subpopulation of cells in the primary tumor possess high metastatic potential and can migrate to new sites.[4,5] Alternatively, the entire primary tumor may progressively and collectively gain invasive and metastatic capabilities. Regardless, metastasis is believed to be mediated by multifunctional gene products that digest basal membranes, interact with extracellular matrix proteins, protect cells from death, and promote angiogenesis. Previous investigations comparing paired primary tumors and lymph node metastases revealed few unique genetic alterations in lymph node metastases[6,7]; these findings support the 'collective progression' model of metastasis. Nonetheless, only a small group of cells actually migrate from the primary tumor site to the lymph nodes. This suggests that a relatively subtle molecular event or events separate lymph node metastases from their corresponding primary tumors. Thus, a broader and more comprehensive investigation, involving genomic technologies such as cDNA microarrays, is required to characterize these events.

To capture subtle differences and to avoid confounding factors associated with heterogeneities among patients, a study such as the current one ideally would involve paired primary tumors and lymph node metastases, with each pair obtained from a single patient. One major obstacle is the difficulty associated with obtaining a sufficient number of such paired surgical specimens for genomic analysis from a large number of patients. Therefore, microarray analysis, which requires a relatively small number of paired samples, is more practical, but its results must be viewed with caution and must be confirmed by other methods. In the current study, we initially performed microarray analysis of nine paired samples and then validated the results of the analysis for selected genes by analyzing five additional paired samples via the real-time polymerase chain reaction (PCR). We subsequently analyzed 100 paired samples on a tissue microarray by immunohistochemical methods. Using our combined expression microarray screening/tissue array validation method, we identified genes and proteins that were consistently up-regulated or down-regulated in metastases.

Among the genes found to be differentially expressed in primary tumors and metastases was *IGFBP5*. Although another member of the *IGFBP* family, *IGFBP2*, has been implicated in malignant invasion,[8] the current study is the first to find a link between *IGFBP5* and metastatic breast carcinoma. It is noteworthy that microarray and real-time PCR analysis indicated that fibronectin and *MMP2* were expressed at higher levels in primary tumors, whereas tissue array immunohistochemical analysis demonstrated that fibronectin and MMP-2 proteins were expressed at higher levels in the extracellular matrices of metastases; these findings suggest that a negative-feedback control mechanism may be operative.

MATERIALS AND METHODS

Breast Carcinoma Tissue Samples

Primary tumor and lymph node metastasis samples were surgically obtained from patients as part of treatment. A fraction of the tissue samples were snap-frozen in liquid nitrogen immediately after surgical resection. The tissue collection procedure and the use of collected material for research were approved by an institutional review committee at Tianjin Cancer Hospital (Tianjin, China). Tissue samples were stained with hematoxylin and eosin and evaluated by a pathologist (B.S.). Of the 18 paired tissue samples that were obtained, 9 consisted of $>$ 75% tumor cells and thus were used for microarray studies. Fine-needle aspirates from five paired primary tumors and lymph node metastases were collected with the approval of the Institutional Review Board of The University of Texas M. D. Anderson Cancer Center (Houston, TX).

Microarray Assay

RNA isolation, microarray production, hybridization, and image analysis were performed as described previously.[9–11] Two microarrays generated by the Cancer Genomics Core Laboratory at The University of Texas M. D. Anderson Cancer Center were used in the study. The first was the Pathway microarray, which contains 1500 well characterized genes that are involved in the following cellular pathways and functions: apoptosis, the cell cycle, metastasis, DNA repair, immune modulation, cell-cell communication, signal transduction, and cytoskeletal processes. The second was a cDNA

array containing 2300 randomly selected known genes and expressed sequence tags.

Real-Time PCR Assay

Real-time PCR analysis was performed using the Applied Biosystems (Foster City, CA) Assay-On-Demand system according to the manufacturer's instructions. Assays were performed with the ABI 7700 TaqMan system (Applied Biosystems).

Tissue Microarray and Immunohistochemical Analysis

After screening H & E–stained slides for optimal tumor content, we constructed a tissue microarray consisting of 100 paired samples (primary tumor/metastatic tumor) using punch cores measuring 0.6 mm in diameter. Immunohistochemical analysis of tissue sections cut from the array block was performed as described previously,[12] using polyclonal antibodies against IGFBP-5 (Santa Cruz Biotechnology, Santa Cruz, CA), fibronectin (Zymed, South San Francisco, CA), MMP-2 (Zymed), cyclin D1 (Zymed), and MDM-2 (Zymed). The staining index was evaluated by pathologists according to previously described criteria.[12]

Microarray Data Analysis and Multidimensional Scaling

Microarray analysis and identification of differentially expressed genes were performed as described previously.[11,13,14] In brief, unreliable replicates were detected as described by Kobayashi et al.,[11] with an exception that allowed slightly more variability between replicated expression measurements for highly expressed genes. This exception involved the acceptance of not only replicates whose absolute differences were less than three times the standard deviation of the differences but also replicates whose absolute normalized differences were less than two times the standard deviation of the normalized differences. Accepted replicates were averaged and normalized to the median.[13] A gene was considered to be significantly overexpressed (underexpressed) if the ratio of the expression level in the metastasis sample to the expression level in the primary tumor sample was ≥ 2 (< 0.5).[14,15]

Primary tumor and metastasis samples were clustered using nonmetric multidimensional scaling (MDS).[16] Noninformative variables—i.e., variables (in this case, genes) that primarily contain noise—can have reductive effects on clustering results. To diminish these effects, we reduced the number of genes before applying the MDS algorithm by considering only genes that exhibited significant differences in at least 3 of 9 patients; doing so left a total of 280 genes. Dissimilarities were calculated as $1 - |\rho|$, where ρ is the Spearman correlation measure between a given pair of gene expression profiles containing 280 genes.

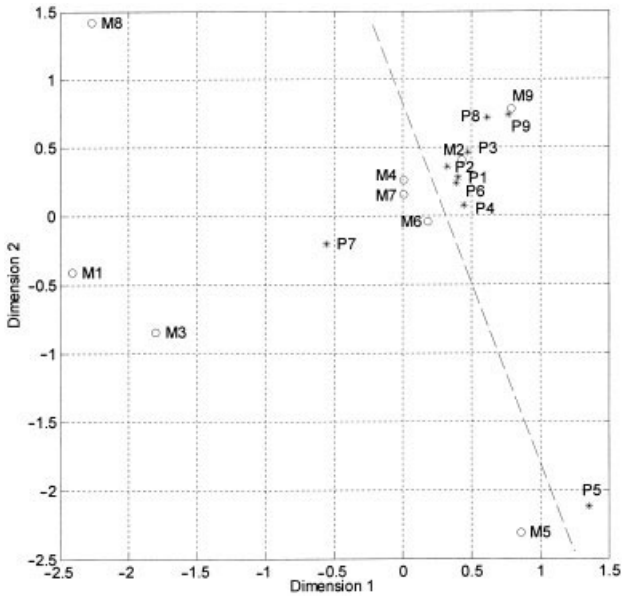

FIGURE 1. Informative genes distinguish metastases from primary breast carcinoma. The 9 matched expression profile pairs (18 expression profiles in total), containing only the 280 most informative genes, were subjected to multidimensional scaling (MDS) analysis. The graph shows clustering results after MDS analysis and illustrates the differences between metastases and primary tumors in the nine paired specimens. The dashed line shows that matched primary tumor and metastasis samples are nearly linearly separable. M: metastasis sample; P: primary tumor sample.

Statistical Analysis of Tissue Microarray Data

Tissue microarray data consisted of pairwise, discrete-valued measurements of the abundance levels of five different proteins in primary tumor and metastasis samples. These measurements were made to determine whether protein levels in metastasis samples were significantly different from the corresponding levels in primary tumor samples. Furthermore, to account for the semiquantitative nature of the data, we used only information regarding the sign of the difference—i.e., whether the measured protein levels were higher or lower in metastasis samples (or equal in both types of samples).

We performed hypothesis testing to determine whether there were significant differences in staining levels between primary tumor and metastasis samples. Because the measurements were not continuous valued, and because a relatively large portion of pairwise differences consequently equaled zero, standard nonparametric tests, such as the Wilcoxon rank test and the Fisher sign test, could not be used reliably.

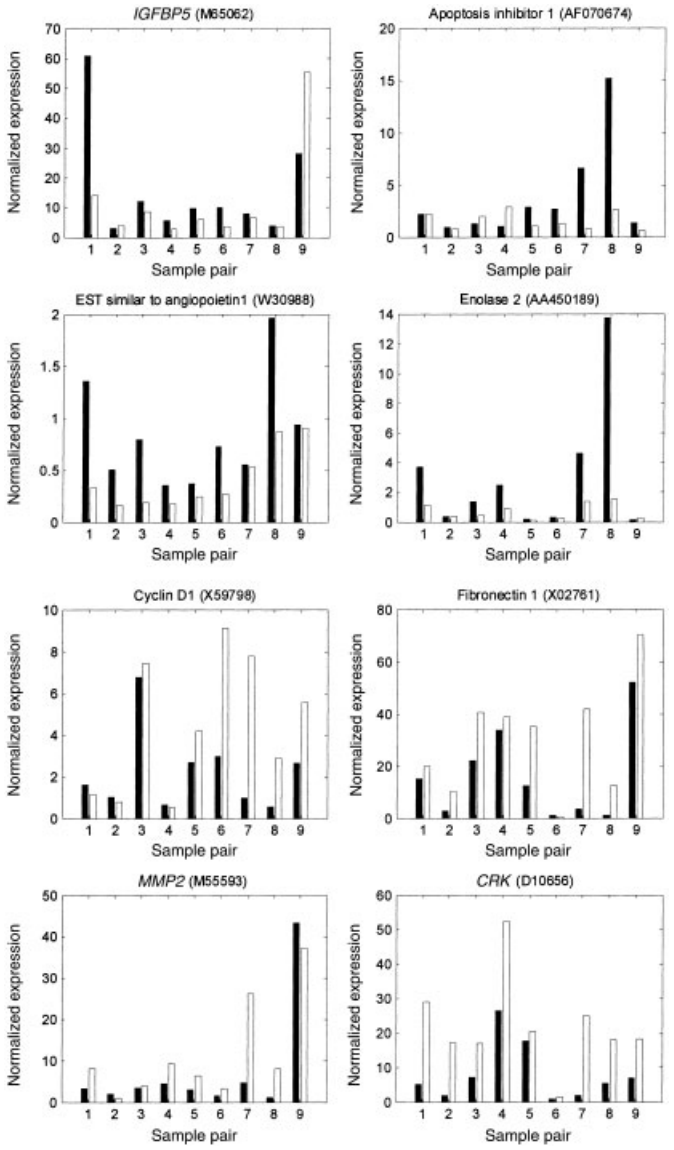

FIGURE 2. Relative gene expression levels in paired samples. Black bars represent metastasis samples, and white bars represent primary tumors. Vertical axes show normalized densities for the indicated genes. EST: expressed sequence tag.

Therefore, we tailored the bootstrap-based hypothesis testing method,[17] which is a general and widely used nonparametric approach, to the problem at hand. The null hypothesis was that the mean of the difference in pairwise staining levels equaled zero. *P* values were obtained using a two-tailed hypothesis test with 100,000 bootstrap iterations.

RESULTS

Gene Expression Fingerprints of Primary Breast Carcinoma and Lymph Node Metastases

One of the goals of the current study was to determine whether primary breast carcinomas and their metastases have distinct gene expression signatures. After performing microarray assays, we analyzed global gene expression patterns using the MDS method. When all genes in the microarray were considered, MDS failed to distinguish metastases from primary malignancies (data not shown). We then selected genes that exhibited significant differential expression in at least three pairs of metastases and corresponding primary tumors. When these 280 informative genes were considered, MDS analysis using the Spearman correlation measure showed a relatively tight clustering of primary tumors and a broader distribution of gene expression levels in metastases. Figure 1 shows clustering results from MDS analysis (with only the 280 most informative genes considered) of the 9 matched expression profile pairs (18 expression profiles in total). Because MDS attempts to preserve the distances between samples, the two-dimensional clustering result shown in Figure 1 directly depicts the

TABLE 1
Genes Overexpressed[a] in Metastases Relative to Corresponding Primary Tumors

Gene accession no.	Gene name/description	Biologic process	Molecular function
Genes overexpressed in metastases in 5 of 9 sample pairs			
W30988	EST, weakly similar to angiopoietin 1	—	—
T96688	Homeobox-containing protein	—	—
R25074	Transmembrane 4 superfamily protein	—	—
R32802	Secretory carrier membrane protein 2 (SCAMP2)	Post-Golgi transport; intracellular protein transport	Protein transporter activity
AA450189	Enolase 2 (gamma, neuronal)	Glycolysis	Phosphopyruvate hydratase activity; magnesium ion binding; lyase activity
X78925	Zinc finger (C2H2)	Transcription regulation, DNA dependent; development	DNA binding
U35376	Zinc finger protein 85	—	Transcription co-repressor activity; DNA binding; transcription factor activity
AF070674	Apoptosis inhibitor 1	Anti-apoptosis; cell surface receptor–linked signal transduction; apoptosis	Apoptosis inhibitor activity
Genes overexpressed in metastases in 4 of 9 sample pairs			
L13616	Focal adhesion kinase pp125 (FAK)	—	—
S53268	Mel-transforming oncogene (RAB8 homolog)	—	—
NM_003726	Src kinase–associated phosphoprotein (SKAP55)	Immune response; signal transduction	Protein–tyrosine kinase activity; SH3/SH2 adaptor protein activity; protein binding
AB011421	Serine/threonine kinase 17b (apoptosis inducing)	Induction of apoptosis, protein amino acid phosphorylation; apoptosis	ATP binding; protein serine/threonine kinase activity; transferase activity
AF070629	RAB2, member of *ras* oncogene family	Small GTPase–mediated signal transduction; ER-to-Golgi transport; intracellular protein transport	GTP binding; RAB small monomeric GTPase activity; protein transporter activity
X62466	CDW52 antigen (CAMPATH-1 antigen)	—	—
M27878	Zinc finger protein 84 (HPF2)	Transcription regulation, DNA dependent	Zinc ion binding; DNA binding
M68520	Cyclin-dependent kinase 2	Regulation of DNA replication; G2/M transition in mitotic cell cycle; positive regulation of cell proliferation; start control point of mitotic cell cycle; oncogenesis	Cyclin-dependent protein kinase activity; protein kinase activity
H94857	GCN5-like protein 1	Biologic process unknown	Molecular function unknown
AF093826	*Plasmodium berghei* GTP-binding protein (rab6)	—	—
AA476272	Putative DNA-binding protein A20	Apoptosis	Apoptosis inhibitor activity; zinc ion binding; DNA binding
H10964	Peroxisomal biogenesis factor 12	—	Protein binding; zinc ion binding
R66310	Peptidylglycine alpha–amidating monooxygenase	Protein modification	Peptidylglycine monooxygenase activity; electron transporter activity
M36768	Gamma enolase	Glycolysis	Phosphopyruvate hydratase activity; magnesium ion binding; lyase activity
H00756	Selectin-L (lymphocyte adhesion molecule I)	Defense response; cell adhesion; cell motility; heterophilic cell adhesion	Sugar binding; selection; cell adhesion molecule activity
AF195942	STK15 serine/threonine kinase (STK15)	—	—
AA026631	Ran GTPase-activating protein 1	Signal transduction	Ran GTPase activator activity
AA262211	Human mRNA for KIAA0008 gene	Biologic process unknown; cell-cell signaling	Molecular function unknown
AA459308	Elastin gene	Respiratory gaseous exchange; circulation; histogenesis and organogenesis; cell shape and size control; cell proliferation	Extracellular matrix structural constituent
X55181	ETS2 gene	—	—
AA427490	Long (electrocardiographic) QT syndrome 2	Cation transport; potassium ion transport; regulation of heart rate; hearing; muscle contraction; two-component signal transduction system (phosphorelay)	Delayed rectifier potassium channel activity; DNA binding; two-component sensor molecule activity
NM_002382	MAX protein (MAX)	Oncogenesis; transcription from Pol II promoter	Transcription cofactor activity; transcription coactivator activity; transcription factor activity
AA279429	Endothelin-converting enzyme 1	—	—

(*continued*)

TABLE 1
(*continued*)

Gene accession no.	Gene name/description	Biologic process	Molecular function
AA149096	Hemopoietic cell kinase	Mesoderm development; protein amino acid phosphorylation; intracellular signaling cascade	Protein–tyrosine kinase activity; ATP binding; protein serine/threonine kinase activity; transferase activity
AA400474	Zona pellucida–binding protein (sp38)	—	—
AA258396	PQ-rich protein	—	—
AA430382	Nucleoside phosphorylase	DNA modification; nucleobase, nucleoside, nucleotide, and nucleic acid metabolism	Phosphorylase activity; purine-nucleoside phosphorylase activity; transferase activity, transferring glycosyl groups
H62162	Hepsin	Cell growth and/or maintenance; proteolysis and peptidolysis	Trypsin activity; scavenger receptor activity; chymotrypsin activity; hydrolase activity
NM_005000	NADH dehydrogenase (ubiquinone) 1 alpha 5 (NDUFA5)	—	NADH dehydrogenase (ubiquinone) activity
NM_005050	ATP-binding cassette, subfamily D, member 4 (ABCD4)	Transport	Nucleotide binding; ATP binding; ATP-binding cassette transporter activity; transporter activity
H58873	Glucose transporter gene	—	—
AA406422	Human mRNA for KIAA0276 gene, partial cds	—	—
Genes overexpressed in metastases in 3 of 9 sample pairs			
NM_004431	EPHA2 mRNA	—	—
U17163	Ets variant gene 1	Cell growth and/or maintenance; transcription regulation, DNA dependent; transcription from Pol II promoter	Transcription factor activity
AF100338	NF-kB subunit	Induction of apoptosis; apoptosis; negative regulation of cell cycle	Protein binding; apoptosis regulator activity
M65062	Insulinlike growth factor–binding protein 5	—	—
U37546	Human IAP homolog C (MIHC)	—	—
X54156	Tumor protein p53	—	—
M80397	Polymerase (DNA directed), delta 1	Response to ultraviolet radiation; DNA replication; DNA repair	Nucleotide binding; delta DNA polymerase activity; 3′-5′ exonuclease activity; DNA binding; transferase activity; hydrolase activity
J02931	Coagulation factor III (thromboplastin, tissue factor)	Blood coagulation; immune response	Blood coagulation factor activity; hematopoietin/interferon-class (D200-domain) cytokine receptor activity
J02958	Met protooncogene	Protein amino acid phosphorylation; development; cell proliferation; signal transduction	Hepatocyte growth factor receptor activity; protein–tyrosine kinase activity; ATP binding; receptor activity; transferase activity
U57650	Inositol polyphosphate-5-phosphatase, 145 kD	Phosphate metabolism; signal transduction	Inositol polyphosphate-5-phosphatase activity
X60673	Adenylate kinase 3	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolism	Adenylate kinase activity; GTP binding; ATP binding; kinase activity; transferase activity
U28014	Caspase 4, apoptosis-related cysteine protease	Induction of apoptosis; proteolysis and peptidolysis; apoptosis	Thiol protease; caspase 4 activity
M91196	Interferon consensus sequence–binding protein 1	Transcription regulation, DNA dependent; immune response; negative regulation of transcription from Pol II promoter	RNA polymerase II transcription factor activity, enhancer binding
X66975	Polypyrimidine tract–binding protein	mRNA splicing; nuclear mRNA splicing (via spliceosome)	Polypyrimidine tract binding; heterogeneous nuclear ribonucleoprotein
XM_041991	Ataxin 2–related protein	—	—
NM_003355	Mitochondrial uncoupling protein 2	Mitochondrial transport; proton transport; transport	Uncoupling protein activity; binding; transporter activity

(*continued*)

TABLE 1
(*continued*)

Gene accession no.	Gene name/description	Biologic process	Molecular function
NM_003356	Mitochondrial uncoupling protein 3	Mitochondrial transport; proton transport; transport; respiratory gaseous exchange; lipid metabolism; energy pathways	Uncoupling protein activity; binding; transporter activity
NM_004417	Dual-specificity phosphatase 1	Cell cycle; response to oxidative stress; protein amino acid dephosphorylation	Non-membrane-spanning protein tyrosine phosphatase activity; hydrolase activity; MAP kinase phosphatase activity
AF019770	Macrophage inhibitory cytokine 1 (MIC1)	—	—
NM_001406	Ephrin B3 (EFNB3),	Development; neurogenesis; cell-cell signaling	Transmembrane ephrin receptor activity
K03218	V-src viral oncogene homolog	—	—
M13194	ERCC1	Nucleotide excision repair; embryogenesis and morphogenesis; DNA repair	Endodeoxyribonuclease activity
AF015950	Telomerase reverse transcriptase	Telomere binding	Telomerase activity; telomeric template RNA reverse transcriptase activity; ribonucleoprotein
X62535	Diacylglycerol kinase, alpha, 80 kD	—	—
X15949	Interferon regulatory factor 2	Transcription regulation, DNA dependent; immune response; cell proliferation; negative regulation of transcription from Pol II promoter	RNA polymerase II transcription factor activity; transcription factor activity
U53832	Interferon regulatory factor 7	Response to virus; transcription regulation, DNA dependent; negative regulation of transcription from Pol II promoter	Specific RNA polymerase II transcription factor activity; transcription factor activity
D86550	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1	Protein amino acid phosphorylation; neurogenesis	Protein-tyrosine kinase activity; ATP binding; protein serine/threonine kinase activity; transferase activity
X17576	NCK adaptor protein 1	Signal complex formation; intracellular signaling cascade; positive regulation of T cell proliferation; T cell activation; positive regulation of actin polymerization	Cytoskeletal adaptor activity; receptor binding; receptor signaling complex scaffold activity
L15309	Zinc finger protein 141 (clone pHZ-44)	Transcription regulation, DNA dependent; embryogenesis and morphogenesis	Specific RNA polymerase II transcription factor activity; DNA binding
X85786	Regulatory factor X, 5 (influences human leukocyte antigen Class II expression)	Transcription regulation, DNA dependent; transcription from Pol II promoter	Transcription coactivator activity; transcription factor activity
X14787	Thrombospondin 1	Blood coagulation; cell adhesion; development; neurogenesis; cell motility	Heparin binding; structural molecule activity; endopeptidase inhibitor activity; calcium ion binding; signal transducer activity; cell adhesion molecule activity

EST: expressed sequence tag; ATP: adenosine triphosphate; GTP: guanosine triphosphate; ER: endoplasmic reticulum; NADH: nicotinamide adenine dinucleotide (reduced form); kD: kilodalton.
[a] By a factor of ≥ 2 relative to expression levels in primary tumor samples.

underlying similarities and dissimilarities among samples. That is, samples that are closer to each other in Figure 1 have smaller differences between each other in terms of actual expression values. The dashed line in Figure 1 demonstrates that matched primary tumor and metastasis samples are nearly linearly separable from each other. Nonetheless, in certain cases (Pairs 2, 5, and 9), matched primary tumor and metastasis samples exhibited more similar expression profiles.

Identification of Candidate Differentially Expressed Genes

Our analysis indicated that most genes were expressed at similar levels in primary tumors and their metastases. Of the 3800 genes analyzed on cDNA arrays, only 280 exhibited twofold (or greater) differences in expression in at least 3 sample pairs. Nonetheless, the patterns of differential expression were relatively heterogeneous. Some genes consistently exhibited substantial differential expression in matched pairs. For other genes, however, the differences between sample

pairs were somewhat minor, but the trend of change was consistent (Fig. 2; Tables 1, 2).

Evaluation of Candidate Gene Products Using Tissue Immunohistochemistry Microarrays

We selected the following candidates for tissue microarray analysis: IGFBP-5, fibronectin, MMP-2, cyclin D1, and MDM-2 (Fig. 3). Expression microarray analysis indicated that IGFBP-5 levels were increased in five of nine metastasis samples; MMP-2 and fibronectin levels were decreased in five of nine and four of nine metastasis samples, respectively; and cyclin D1 levels were decreased in four of nine metastasis samples. Expression of MDM-2 was similar in primary tumor and metastasis samples. In our immunohistochemical assays, as in our gene expression assays, heterogeneity in differential expression patterns was observed. Data on the number of individual samples in which each candidate gene product exhibited increased, decreased, or unchanged expression can be seen in Table 3. Although 100 sample pairs were assembled on the tissue array, different numbers of sample pairs were unevaluable for each candidate. Thus, the total number of sample pairs was different for each candidate shown in Table 3.

IGFBP-5 protein expression was elevated in lymph node metastasis samples compared with primary tumor samples in 26 of 57 pairs. In 22 pairs, there was no difference in IGFBP-5 expression levels, and in 9 cases, IGFBP-5 expression levels were decreased in metastases. Immunostaining was primarily localized to the cytoplasm of malignant cells. Statistical analysis indicated that metastases exhibited significantly elevated expression of IGFBP-5.

Regarding fibronectin, we observed increased expression in metastases in 55 of 84 sample pairs, similar expression in 17 of 84 pairs, and decreased expression in 12 of 84 pairs. Thus, fibronectin, which was detected in the matrices of tissue samples, was significantly overexpressed in the majority of metastases. This finding differs from the gene expression pattern observed on cDNA microarrays (i.e., decreased fibronectin expression in metastases). *MMP2* transcript levels on cDNA microarrays also were decreased in metastasis samples. Immunohistochemical staining, however, indicated that MMP-2, like fibronectin, was up-regulated in metastases. The opposing RNA and protein expression patterns for fibronectin and *MMP2*/MMP-2 suggest that the protein products exert negative-feedback control over gene expression.

To confirm that *MMP2* and fibronectin mRNA levels actually were decreased in lymph node metastases compared with matched primary tumors, we collected five additional paired samples by fine-needle aspiration and isolated total RNA from them. We then performed real-time PCR analysis of *MMP2* and fibronectin expression levels in these paired samples. In all five sample pairs, *MMP2* and fibronectin mRNA levels were reduced in metastases (Table 4).

Sixty-one of 86 sample pairs exhibited no difference in cyclin D1 expression. In 9 of 86 cases, expression was increased in metastasis samples, whereas in 16 of 86 cases, expression was decreased in metastases. Although there was a trend toward decreased expression of cyclin D1 in metastases, it was not statistically significant.

Similar numbers of sample pairs exhibited increased, decreased, and unchanged expression of MDM-2 in metastases. This finding suggests that there is no difference in MDM-2 expression between primary tumor and metastasis samples, a conclusion that is consistent with cDNA microarray results.

DISCUSSION

Metastasis is a major step in the progression of malignant disease. In breast carcinoma, lymph node metastasis is an indicator of poor prognosis and represents the beginning of the progression from local to systemic disease, which poses serious challenges with regard to treatment. The mechanism by which metastasis occurs still is not well understood. It has been hypothesized that dramatic genetic changes occur in a small population of cells in the primary tumor and that these changes lead to the emergence of metastases.[4,5] This hypothesis would predict a greater degree of difference in gene expression profiles between primary tumors and metastases. A second hypothesis suggests that more collective and gradual changes result in the development of metastases. This hypothesis would predict more subtle differences in gene expression profiles between primary tumors and metastases.

A transcriptome comparison of nine pairs of primary tumor and lymph node metastasis samples did not strongly support either of the two hypotheses regarding metastasis. In roughly half of all cases, the paired primary tumor and metastasis samples were closely matched; this finding supports the second hypothesis. In the remaining cases, the difference between primary tumor and metastasis samples was more evident; this finding supports the first hypothesis. One of the primary observations of the current study was that gene expression patterns were highly heterogeneous among the set of sample pairs. This finding may suggest that metastases are derived from two different mechanisms, corresponding to the two stated hypotheses. Studies of additional paired tissue samples may reveal whether two distinct groups of

TABLE 2
Genes Underexpressed[a] in Metastases Relative to Corresponding Primary Tumors

Gene accession no.	Gene name/description	Biologic process	Molecular function
Genes underexpressed in metastases in 6 of 9 sample pairs			
AA520978	Ubiquitin-conjugating enzyme E2H	—	—
D10656	V-crk oncogene homolog	—	—
AF060865	Zinc finger protein 205	Transcription regulation, DNA dependent	Zinc ion binding; DNA binding
Genes underexpressed in metastases in 5 of 9 sample pairs			
NM_003118	Secreted protein, acidic, cysteine rich (osteonectin)	Ossification	Collagen binding; calcium ion binding
NM_000422	Keratin 17	Epidermal differentiation	Structural constituent of cytoskeleton
M55593	Matrix metalloproteinase 2	—	—
AA437191	Coagulation factor VIIIc (hemophilia A)	Blood coagulation	Blood coagulation factor activity
R01638	HYA22	Transcription regulation, DNA dependent; biologic process unknown	DNA binding; molecular function unknown
R53294	Serine hydroxymethyltransferase	Glycine metabolism; L-serine catabolism; one-carbon compound metabolism	Glycine hydroxymethyltransferase activity; transferase activity
AA504713	Tubulin-folding cofactor E	—	—
Genes underexpressed in metastases in 4 of 9 sample pairs			
X59798	Cyclin D1	G1/S transition in mitotic cell cycle; cell growth and/or maintenance; cell cycle regulation; cytokinesis	—
NM_006475	Osteoblast-specific factor 2 (fasciclin 1–like) (OSF2)	Skeletal development; cell adhesion	Cell adhesion molecule activity
X02761	Fibronectin 1	Cell adhesion; cell motility; signal transduction	Cell adhesion molecule activity
NM_006435	Interferon-induced transmembrane protein 2	Immune response	—
NM_021034	Interferon-induced transmembrane protein 3	Immune response	—
NM_003507	Frizzled (*Drosophila*) homolog 7	Frizzled signaling pathway; oncogenesis	Frizzled receptor activity
X01677	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Glycolysis	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity; oxidoreductase activity
K00558	Tubulin	Microtubule-based movement	GTP binding; structural molecule activity
M90657	Transmembrane 4 superfamily member 1	—	—
M36981	Nonmetastatic cells 2 (NM23B)	Negative regulation of cell proliferation; GTP biosynthesis; transcription regulation, DNA dependent; UTP biosynthesis; CTP biosynthesis; negative regulation of cell cycle; nucleoside triphosphate biosynthesis	Nucleoside diphosphate kinase activity; ATP binding; transcription factor activity; kinase activity; transferase activity
U75503	Adenosine deaminase, RNA specific	—	—
NM_003255	Tissue inhibitor of metalloproteinase 2 (TIMP2)	—	Metalloendopeptidase inhibitor activity
M98343	Ems1 (p80/85 src substrate)	—	—
NM_005507	Cofilin 1 (nonmuscle) (CFL1) mRNA	Rho protein signal transduction; actin cytoskeleton organization and biogenesis	Actin-modulating activity
AA448599	Coagulation factor XIII A	Blood coagulation; peptide cross-linking	Blood coagulation factor activity; acyltransferase activity; protein–glutamine gamma–glutamyltransferase activity; calcium ion binding; transferase activity
AA280677	Major histocompatibility complex, Class II, DM beta	Development	DNA binding
AA196465	Sarcolipin (SLN)	Small-molecule transport	—
W67174	Integrin, beta 1 (fibronectin receptor)	Homophilic cell adhesion; cellular defense response; cell matrix adhesion	Cell adhesion receptor activity
AA013094	K^+ channel beta subunit (Kvb1.3)	Potassium ion transport; ion transport	Potassium channel regulator activity; voltage-gated potassium channel activity
AA401693	M130 antigen	—	Scavenger receptor activity
T63686	Oculocerebrorenal syndrome (Lowe syndrome)	Lipid metabolism	Phosphoinositide-5-phosphatase activity; hydrolase activity
T55353	Tumor necrosis factor type 2 receptor–associated protein (TRAP3)	—	—

(*continued*)

TABLE 2
(*continued*)

Gene accession no.	Gene name/description	Biologic process	Molecular function
AA436409	TAR RNA-binding protein (TRBP)	—	—
AA412500	Transcription elongation factor S-II, hS-II-T1	—	—
AA455272	ITBAI protein	Biologic process unknown	Molecular function unknown
AA035796	SWI/SNF complex 60 kD subunit (BAF60c)	Chromatin modeling; regulation of transcription from Pol II promoter	Transcription coactivator activity
NM_006745	Sterol-C4-methyl oxidase–like protein (SC4MOL)	Steroid metabolism; fatty acid metabolism; metabolism; sterol biosynthesis	C4-methyl sterol oxidase activity
AA478066	Kinase Myt1 (Myt1)	—	—
U27112	HT-1080 protein	Cell cycle arrest; positive regulation of cell proliferation	—
W15277	Ribosomal protein L31	Protein biosynthesis	Structural constituent of ribosome; RNA binding
AA608514	Transcriptional activation factor TAFII32 mRNA	Nucleosome assembly; chromosome organization and biogenesis (sensu Eukarya)	DNA binding
R13925	CBP20	Transport; mRNA-nucleus export; mRNA processing; snRNA-nucleus export	RNA-cap binding
NM_002731	cAMP-dependent kinase beta (PRKACB)	Protein amino acid phosphorylation; signal transduction	cAMP-dependent protein kinase activity; ATP binding; protein serine/theronine kinase activity; transferase activity
AA282445	DENN	Cell surface receptor–linked signal transduction	Death receptor–interacting protein activity
AA101792	Phosphatidylinositol glycan	Glycosylphosphatidylinositol anchor biosynthesis	Ethanolaminephosphotransferase activity; molecular function unknown
Genes underexpressed in metastases in 3 of 9 sample pairs			
AA234982	Sarcoglycan, delta (35 kD dystrophin-associated glycoprotein)	Muscle development	—
AA428859	Glutathione transferase zeta 1 (GSTZ1)	Tyrosine catabolism; phenylalanine catabolism; aromatic amino acid family metabolism	Maleylacetoacetate isomerase activity; glutathione transferase activity; glutathione peroxidase activity; transferase activity; isomerase activity
T87012	Immunoglobulin-associated alpha	Defense response; cell surface receptor–linked signal transduction	Transmembrane receptor activity
U35113	Metastasis-associated protein 1 (MTA1)	Transcription regulation, DNA dependent; protein biosynthesis; signal transduction	Structural constituent of ribosome; transcription factor activity
NM_002211	Integrin beta 1 subunit	Homophilic cell adhesion; cellular defense response; cell matrix adhesion	Cell adhesion receptor activity
X04571	Epidermal growth factor	Activation of mitogen-activated protein kinase; epidermal growth factor receptor signaling pathway; positive regulation of cell proliferation; chromosome organization and biogenesis (sensu Eukarya); DNA replication	Epidermal growth factor receptor–activating ligand activity; growth factor activity; calcium ion binding
D38551	RAD21 (*Schizosaccharomyces pombe*) homolog	Meiotic recombination; chromosome segregation; cell cycle; mitosis; double-strand break repair; apoptosis	Protein binding
M91196	Interferon consensus sequence–binding protein	Transcription regulation, DNA dependent; immune response; negative regulation of transcription from Pol II promoter	RNA polymerase II transcription factor activity, enhancer binding
NM_000362	Tissue inhibitor of metalloproteinase 3	Proteolysis and peptidolysis	Metalloendopeptidase inhibitor activity
NM_003000	Succinate dehydrogenase subunit B	Aerobic respiration; electron transport; tricarboxylic acid cycle	Electron transporter activity; succinate dehydrogenase (ubiquinone) activity; oxidoreductase activity; quinol; fumarate oxidoreductase activity
K00558	Tubulin	Microtubule-based movement	GTP binding; structural molecule activity
Z13009	Cadherin 1, E-cadherin (epithelial)	—	Calcium-dependent cell adhesion molecule activity; tumor suppressor activity; cell adhesion molecule activity
M96995	Growth factor receptor–bound protein 2	—	—

(*continued*)

TABLE 2
(*continued*)

Gene accession no.	Gene name/description	Biologic process	Molecular function
AF072752	Integrin, beta-like 1 (with EGF-like repeat domains)	Cell adhesion	Cell adhesion receptor activity
U33822	MAD1 (mitotic arrest deficient, yeast, homolog)–like 1	—	
U48705	Cell adhesion kinase	—	—
U34360	Lymphoid nuclear protein related to AF4	Development; oncogenesis	Transcription-activating factor
L10844	Cell division cycle 42 (GTP-binding protein, 25 kD)	—	—
M98343	Ems1 sequence (mammary tumor–and squamous cell carcinoma–associated (p80/85 src substrate)	—	—
X70683	SRY (sex-determining region Y) box 4	Transcription regulation, DNA dependent	Transcription factor activity
X52541	Early growth response 1	Transcription regulation, DNA dependent	Transcription factor activity
M25280	Lymph node homing receptor	Defense response; cell adhesion; cell motility; heterophilic cell adhesion	Sugar binding; selectin; cell adhesion molecule activity

GTP: guanosine triphosphate; UTP: uridine triphosphate; CTP: cytidine triphosphate; ATP: adenosine triphosphate; kD: kilodalton; snRNA: small nuclear RNA; cAMP: cyclic adenosine monophosphate.
[a] By a factor of ≥ 2 relative to expression levels in primary tumor samples.

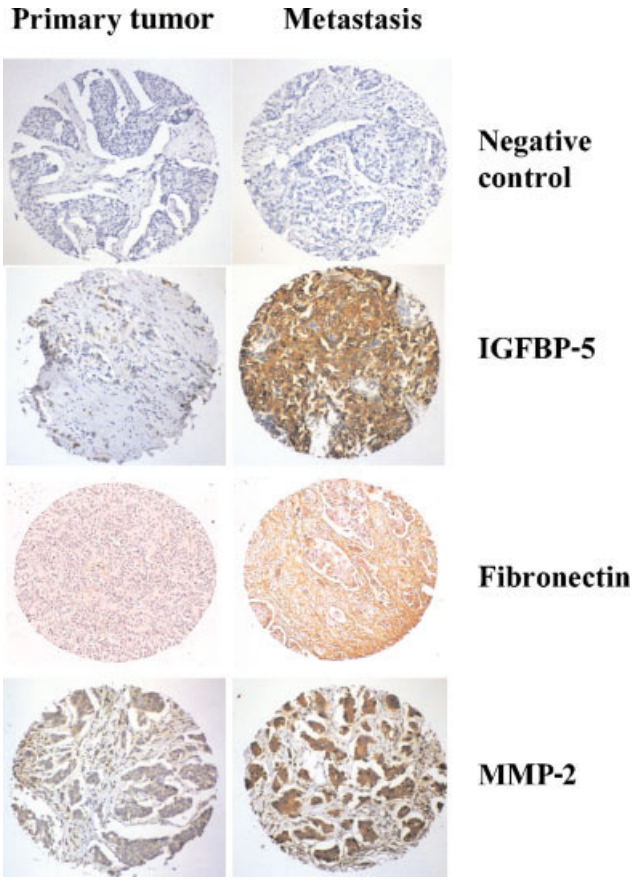

FIGURE 3. Immunohistochemical analysis of selected markers on a tissue array.

metastases (characterized by distinct differences in expression signatures with respect to the primary tumor) exist and whether these groups are associated with separate prognoses.

The second noteworthy finding of the current study is that expression at the transcript level does not always correspond to expression at the protein level; this finding highlights the complexity inherent in transcriptional and translational regulation. In the current study, fibronectin provided the best example of this complexity. Our microarray and real-time PCR data indicated that fibronectin transcription was consistently decreased in metastases. This finding was unexpected, because fibronectin is a key extracellular matrix protein in cell migration. Tissue array analysis subsequently revealed that fibronectin protein expression actually was significantly elevated in metastases. These opposing RNA and protein expression patterns suggest that negative-feedback control of fibronectin gene expression may be operative; under such control, a decrease in gene expression may actually signify an increase in protein expression. We also cannot exclude the possibility that fibronectin was secreted by other cells and deposited in the extracellular matrix in the lymph nodes. Both possibilities are supported by reports in the literature.[18,19]

In the current study, MMP-2, another extracellular matrix protein, also exhibited opposing expression patterns at the transcript and protein levels. In contrast, we observed a strong correlation between transcript and protein expression for the other genes and gene products assayed. It is noteworthy that cyclin D1

TABLE 3
Summary of Tissue Array Data for the Five Candidate Proteins

Protein name	No. of evaluable sample pairs	No. of sample pairs (%)			*P* value
		M = P	M < P	M > P	
IGFBP-5	57	22/57 (39)	9/57 (16)	26/57 (46)	0.0018
Fibronectin	84	17/84 (20)	12/84 (14)	55/84 (65)	$< 1 \times 10^{-5}$
MMP-2	81	29/81 (36)	15/81 (19)	37/81 (46)	0.0011
Cyclin D1	86	61/86 (71)	16/86 (19)	9/86 (10)	0.1589
MDM-2	79	32/79 (41)	20/79 (25)	27/79 (34)	0.3091

M = P: expression levels are equal in metastasis and primary tumor samples; M < P: expression level is lower in metastasis sample than in primary tumor sample; M > P: expression level is higher in metastasis sample than in primary tumor sample.

TABLE 4
Expression of Fibronectin and MMP-2 in Lymph Node Metastases Relative to Primary Breast Tumors[a]

Primary tumor sample no.	Metastasis sample no.	Fibronectin expression[b] (range)	MMP-2 expression[b] (range)
02-028	02-027	0.00531 (0.005–0.006)	0.0101 (0.008–0.012)
02-041	02-042	0.469 (0.354–0.624)	0.493 (0.420–0.578)
02-052	02-053	0.243 (0.189–0.313)	0.118 (0.107–0.130)
02-080	02-081	0.228 (0.193–0.270)	0.173 (0.143–0.209)
03-007	03-009	0.186 (0.138–0.253)	0.0158 (0.012–0.020)

[a] Cyclophilin was used as an endogenous control. All measurements were made in triplicate.
[b] In metastasis samples relative to primary tumor samples.

exhibited decreased expression in many metastasis samples, as this finding suggests that increased cell proliferation is not an intrinsic feature of metastasis. Thus, both tissue microarray analysis and cDNA microarray analysis are important for obtaining a complete picture of molecular events.

Another notable finding is that IGFBP-5 often was overexpressed in metastases. IGFBP-5, a member of the IGFBP family, has several unique features. It has been reported that IGFBP-5 can bind extracellular matrix proteins and can also enter the nucleus to act as a potential transcriptional factor.[20,21] In addition, IGFBP-5 expression has been shown to activate PI-3 kinase[22] and enhance the survival of breast carcinoma cells.[23] Furthermore, the *IGFBP5* gene recently was identified as one of the 70 signature genes correlated with poor survival in patients with breast carcinoma.[24]

Reports by others[25] and our own observations (data not shown) indicate that IGFBP-5 is not expressed in normal breast epithelial cells under normal conditions. Nonetheless, IGFBP-5 is expressed in early development and is important in mammary gland remodeling,[26,27] which shares features with cell invasion. In this sense, IGFBP-5 is similar to another IGFBP family member, IGFBP-2, which normally is expressed in fetal glial cells during early neuronal development and shut down in adult glial cells.[28] IGFBP-2 is reactivated in invasive glioblastoma cells.[29]

It is noteworthy that our microarray and tissue array studies of paired breast carcinoma samples revealed similar patterns of IGFBP-2 expression in primary tumor and metastasis samples (data not shown). Nonetheless, IGFBP-2 levels are elevated in breast carcinoma cells relative to normal breast epithelial cells. It is possible that IGFBP-2 overexpression is an early event in breast carcinoma progression and that IGFBP-5 expression plays a more important role in sustained invasive activity. The exact role of IGFBP-5 in breast carcinoma invasion and metastasis requires further functional studies. IGFBP-5 has been shown to be a nuclear protein that also has a membrane receptor; these findings suggest intriguing directions for further study. It is conceivable that IGFBP-5 may bind to the membrane receptor and that the complex may then migrate to the nucleus to activate a new set of genes required for lymph node and distant metastases. If this hypothesis is true, then IGFBP-5 may be a good target for the prevention of breast carcinoma metastases.

REFERENCES

1. Fisher ER, Palekar A, Rockette H, Redmond C, Fisher B. Pathologic findings from the National Surgical Adjuvant Breast Project (Protocol No. 4). V. Significance of axillary nodal micro- and macrometastases. *Cancer.* 1978;42:2032–2038.
2. Noguchi S, Aihara T, Motomura K, Inaji H, Imaoka S, Koyama H. Detection of breast cancer micrometastases in axillary lymph nodes by means of reverse transcriptase-polymerase chain reaction. Comparison between MUC1 mRNA and keratin 19 mRNA amplification. *Am J Pathol.* 1996;148:649–656.
3. Wingo PA, Tong T, Bolden S. Cancer statistics. *CA Cancer J Clin.* 1995;45:8–30.

4. Weiss L, Holmes JC, Ward PM. Do metastases arise from preexisting sub-populations of cancer cells? *Br J Cancer*. 1995;47:81–89.
5. Fidler IJ, Hart IR. Biological diversity in metastatic neoplasms: origins and implications. *Science*. 1982;217:893–895.
6. Bonsing BA, Devilee P, Cleton-Janson AM, Kuipers-Dijkshoorn N, Fleuren GJ, Cornelisse CJ. Evidence for limited molecular genetic heterogeneity as defined by allelotyping and clonal analysis in nine metastatic breast carcinomas. *Cancer Res*. 1993;53:3804–3811.
7. Chen LC, Kuriso W, Ljung BM, Goldman ES, Moore D, Smith HS. Heterogeneity for allelic loss in human breast cancer. *J Natl Cancer Inst*. 1992;84:505–510.
8. Wang H, Wang H, Shen W, et al. Insulin-like growth factor binding protein 2 enhances glioblastoma invasion via activation of invasion enhancing genes. *Cancer Res*. 2003;63:4315–4321.
9. Shmulevich I, Hunt K, El-Naggar A, et al. Tumor specific gene expression profiles in human leiomyosarcoma: an evaluation of intratumor heterogeneity. *Cancer*. 2002;94:2069–2075.
10. Hu L, Wang J, Baggerly K, et al. Obtaining reliable information from minute amounts of RNA using cDNA microarrays. *BMC Genomics*. 2002;3:16.
11. Kobayashi T, Yamaguchi M, Kim S, et al. Microarray reveals differences in both tumor and vascular specific gene expression in de novo CD5+ and CD5- diffuse large B-cell lymphomas. *Cancer Res*. 2003;63:60–66.
12. Wang H, Wang H, Zhang W, Fuller GN. Tissue microarrays: applications in neuropathology research, diagnosis, and education. *Brain Pathol*. 2002;12:95–107.
13. Shmulevich I, Zhang W. Binary analysis and optimization-based normalization of gene expression data. *Bioinformatics*. 2002;18:555–565.
14. Iyer VR, Eisen MB, Ross DT, et al. The transcriptional program in the response of human fibroblasts to serum. *Science*. 1999;283:83–87.
15. DeRisi J, van den Hazel B, Marc P, et al. Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants. *FEBS Lett*. 2000;470:156–160.
16. Borg I, Groenen P. Modern multidimensional scaling: theory and application. New York: Springer, 1997.
17. Efron B, Tibshirani RJ. An introduction to the bootstrap. London: Chapman & Hall, 1993.
18. Soose M, Wenzel S, Padur A, Oberst D, Stolte H. Fibronectin expression in human mesangial cell cultures and its alterations by adriamycin. *Cell Biol Toxicol*. 1995;11:51–63.
19. Ozaki T, Moriguchi H, Nakamura Y, Kamei T, Yasuoka S, Ogura T. Regulatory effect of prostaglandin E2 on fibronectin release from human alveolar macrophages. *Am Rev Respir Dis*. 1990;141:965–969.
20. Arai T, Parker A, Busby W Jr., Clemmons DR. Heparin, heparan sulfate, and dermatan sulfate regulate formation of the insulin-like growth factor-I and insulin-like growth factor-binding protein complexes. *J Biol Chem*. 1994;269:20388–20393.
21. Schedlich LJ, Young TF, Firth SM, Baxter RC. Insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 share a common nuclear transport pathway in T47D human breast carcinoma cells. *J Biol Chem*. 1998;273:18347–18352.
22. Miyake H, Nelson C, Rennie PS, Gleave ME. Overexpression of insulin-like growth factor binding protein-5 helps accelerate progression to androgen-independence in the human prostate LNCaP tumor model through activation of phosphatidylinositol 3′-kinase pathway. *Endocrinology*. 2000;141:2257–2265.
23. McCaig C, Perks CM, Holly JM. Signalling pathways involved in the direct effects of IGFBP-5 on breast epithelial cell attachment and survival. *J Cell Biochem*. 2002;84:784–794.
24. van't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature*. 2002;415:530–536.
25. Wood TL, Yee D. IFGs and IGFBPs in the normal mammary gland and in breast cancer. *J Mammary Gland Biol Neoplasia*. 2000;5:1–5.
26. Wood TL, Richert MM, Stull MA, Allar MA. The insulin-like growth factors (IGFs) and IGF binding proteins in postnatal development of murine mammary glands. *J Mammary Gland Biol Neoplasia*. 2000;15:31–42.
27. Tonner E, Barber MC, Travers MT, Logan A, Flint DJ. Hormonal control of insulin-like growth factor-binding protein-5 production in the involuting mammary gland of the rat. *Endocrinology*. 1997;138:5101–5107.
28. Lee WH, Michels KM, Bondy CA. Localization of insulin-like growth factor binding protein-2 messenger RNA during postnatal brain development: correlation with insulin-like growth factors I and II. *Neuroscience*. 1993;53:251–265.
29. Fuller GN, Rhee CH, Hess K, et al. Reactivation of insulin-like growth factor binding protein II expression during glioblastoma transformation revealed by parallel gene expression profiling. *Cancer Res*. 1999;59:4228–4232.