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Differential Gene Expression Patterns in HER2/*neu*-Positive and -Negative Breast Cancer Cell Lines and Tissues

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Overexpression of the oncogene HER2/*neu* (c-erbB-2) occurs in up to 30% of breast cancers and is correlated with reduced survival, especially in node-positive disease. The aim of this study was to identify genes associated with the aggressive phenotype of HER2/*neu*-positive breast cancer cells using cDNA microarrays. RNA was extracted from three HER2/*neu*-positive and three HER2/*neu*-negative breast cancer cell lines. Pooled RNA was hybridized in duplicate to the breast specific microarray filters from Research Genetics containing 5184 unique cDNAs. Subsequently, a similar comparison was performed for pooled RNAs from 10 node-positive, ER-positive invasive ductal carcinomas, half of which were HER2/*neu* overexpressers. In HER2/*neu* overexpressing breast cancer cell lines, 90 (1.7%) genes were up-regulated and 46 (0.9%) were down-regulated, compared to cell lines with low HER2/*neu* protein levels. In contrast, in HER2/*neu* overexpressing primary breast cancers, more genes were down-regulated (N = 132, 2.5%) than up-regulated (N = 19, 0.4%). Many of the differentially expressed genes have previously not been known to play a role in human neoplasia, and some of them may represent novel tumor suppressor or oncogenes. No genes were up-regulated, and only a small number of genes were down-regulated both in cell lines and in carcinomas with high HER2/*neu* protein levels. These included transforming acidic coiled-coil containing protein 1, glycogen phosphorylase BB, complement 1q and one EST. The differential expression of select genes was confirmed by Northern blotting (trefoil factor 3) or by immunocytochemistry (glycogen phosphorylase BB, vimentin, KAI1). In an extended validation study, 18 of 41 ER-negative, but none of 46 ER-positive, breast carcinomas were found to express vimentin, and all but one of the vimentin-positive tumors were confined to the HER2/*neu*-negative subgroup (P = 0.0019). Our findings support an important role of the mammary stroma in determining the clinical breast cancer phenotype. *(Am J Pathol 2002, 161:1171–1185)*

HER2/*neu* (c-erbB-2) is a proto-oncogene that is amplified and/or overexpressed in some 30% of human breast cancers. Numerous studies have shown that this event is associated with a more aggressive phenotype. In particular, HER2/*neu* overexpressing tumors are known to be refractory to various types of chemo- and endocrine therapy (often associated with down-regulation of the estrogen receptor; ER) and to be associated with shortened overall survival, especially in node-positive patients.[1–3] However, the mechanism(s) by which HER2/*neu* overexpression confers the more aggressive biological behavior are poorly understood. HER2/*neu* encodes a tyrosine kinase receptor, $p185^{erbB-2}$, which is anchored in the cell membrane. Activation of this receptor leads to transduction of an extracellular signal to the nucleus *via* one of several signaling cascades including the ras/raf/mitogen-activated protein kinase pathway that ultimately leads to intranuclear activation of c-*fos* and c-*jun*.[3] A limited number of additional genes have been found to be up- or down-regulated by HER2/*neu*.[4–6]

The principal aim of our study was to examine the gene expression profile of HER2/*neu*-positive and -negative breast cancer cells on a more global scale. To this end, we used recently developed cDNA microarray technology that allows the simultaneous evaluation of expression, at the mRNA level, of thousands of genes.[7] Several groups have used this technology to study gene expression patterns in human breast cancer.[8–11] We used it to answer the specific question whether HER2/*neu*-positive and -negative breast cancer cells could be distinguished by their gene expression profiles and whether this could be related to differences in biological effects. We used an

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array enriched in genes expressed in normal breast tissue to evaluate the effect of HER2/*neu* status on the expression level of novel genes with physiological functions in the human mammary gland. Some of the differentially expressed genes may be, directly or indirectly, responsible for the more aggressive properties of HER2/*neu* overexpressing tumor cells. In addition to new diagnostic and prognostic markers, this approach may also yield new therapeutic targets. Lastly, to determine whether differential gene expression in HER2/*neu*-positive and -negative breast cancer cells *in vitro* reflects differences in gene expression profiles *in vivo*, we subjected both breast cancer cell lines and primary carcinomas to cDNA microarray analysis. Our results suggest that a significant number of genes are up- or down-regulated in HER2/*neu*-positive breast cancer cells, but only a minority of them have previously been implicated in mammary tumorigenesis or human neoplasia in general. Surprisingly, only a small number of genes were found to be differentially expressed in HER2/*neu*-positive *versus* -negative breast cancer cells both *in vitro* and *in vivo*.

Materials and Methods

Cell Culture

Six commercially available breast carcinoma cell lines (obtained from the ICRF Cell Production Department, Clare Hall, London, UK) were cultured under the recommended optimum conditions (Dulbecco's modified E4 medium (DMEM) (ICRF) supplemented with fetal calf serum (10%) and glutamine (2 mmol/L) except for BT474 which required DMEM:F12 (Life Technologies, Paisley, UK), fetal calf serum (10%), glutamine (2 mmol/L), penicillin (100U/ml), streptomycin (100 mg/ml), insulin (10 μg/ml), and EGF (10 ng/ml)). Three of these cell lines were known to be high expressers of HER2/*neu* (MDA-MB-361, SKBR3 and BT474) with the remaining three demonstrating low $p185^{erbB-2}$ levels (MDA-MB-231, MDA-MB-435 and MDA-MB-468). The cells were harvested by trypsinization before reaching confluence. They were then pelleted and divided for RNA extraction (see below) and for paraffin section immunocytochemistry. Cell blocks were prepared by fixation of the pelleted cells in 10% buffered formalin for 30 minutes, followed by a wash in 70% ethanol for 30 minutes and 100% ethanol overnight at 4°C. The fixed cell buttons were then xylene cleared and embedded in paraffin.

Tissues

Ten breast carcinomas were selected from the ICRF frozen breast cancer archive for cDNA microarray analysis. The tumors had been stored at −80°C for 8 to 10 years following surgical excision. All were classified as invasive ductal carcinomas, of which half had been determined to be HER2/*neu*-positive. The HER2/*neu* status was originally determined by Southern blotting and subsequently confirmed by immunohistochemistry (IHC). All ten carcinomas were of intermediate or high combined grade, ER-positive (ER+) and lymph node positive. We used only ER+ primary tumors (as determined by ELISA and IHC) to focus on the HER2/*neu* pathway, as many studies have shown an inverse relation of ER to HER2/*neu*, which could confound analysis of genes regulated by HER2/*neu in vivo*. A second piece of each tumor was used for frozen section IHC. In addition, a representative formalin-fixed block of each case was selected for paraffin section IHC. A second group of 15 invasive ductal carcinomas was randomly selected from the ICRF frozen breast cancer archive for confirmatory Northern analysis. These tumors had variable grade, HER2/*neu*, ER, and nodal status. Formalin-fixed samples of these tumors were also used for paraffin section IHC. For further validation of the vimentin staining data, IHC for this marker was performed on 70 additional formalin-fixed, paraffin-embedded invasive ductal carcinomas with variable ER and HER2/*neu* status retrieved from the pathology files of the Roswell Park Cancer Institute.

RNA Isolation

Approximately 5×10^6 cultured cells were pelleted, following harvesting at subconfluency, and total RNA was extracted using TRI reagent (σ-Aldrich, Inc., St. Louis, MO), as described in the protocol supplied by the manufacturer. The frozen breast carcinomas were homogenized using an Ultra Turrax T25 homogenizer, and total RNA was extracted by the same procedure as for the cell pellets. For the cell lines, 3.3 μg of total RNA was used for each line for each hybridization. For the frozen carcinomas, 5 μg RNA was used per case. Total RNA from the HER2/*neu*-positive samples was pooled (for a total of 10 μg for the cell lines and 25 μg for the carcinomas) and compared against pooled RNA from the HER2/*neu*-negative samples (see below).

cDNA Microarray Hybridization

GF225 (Research Genetics, Huntsville, AL) is a commercially available "breast-specific" cDNA filter containing 5184 unique cDNAs (0.5 ng per spot), including some 3000 named genes, in addition to 370 control points and housekeeping genes. According to the manufacturer, the array is enriched for genes expressed in human breast tissue. The filter is a 5×7 cm positively charged nylon membrane that may be re-used up to four times with careful handling. All filters used in this study were from the same lot. The hybridization of the filters with the pooled total RNA from HER2/*neu*-positive or -negative cell lines or carcinomas was performed in duplicate. Pre-hybridization, probe preparation (radiolabeled with Redivue [α-^{33}P]dCTP (3000 Ci/mmol; Amersham-Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and hybridization were conducted under the conditions recommended in the manual accompanying the Pathways 2 software (Research Genetics, Huntsville, AL). Unhybridized probe was washed from the GeneFilter at the recommended stringency (0.2X SSC/1% SDS).

Data Analysis

Following hybridization and stringent washing, the GeneFilter was exposed to a phosphor screen for periods of 1 hour, 3 hours, 5 hours, 10 hours, and 16 hours, and the subsequent images were scanned into a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Comparisons between the images of HER2/*neu*-positive and -negative filters were made in triplicate using the Pathways 2 software and normalizing against all data points. Filters displaying similar maximum intensity and background radioactivity were selected for comparison against one another. Images generated from the HER2/*neu*-negative RNA species were coded green, and the HER2/*neu*-positive images were coded red. The images were then superimposed, and gene expression ratios calculated by the program. The expression ratios of all housekeeping genes and control points were used to calculate the mean and SD of each comparison. In the Pathways system, genes more highly expressed in the HER2/*neu*-positive samples were assigned positive ratios (≥ 1), while genes more highly expressed in the HER2/*neu*-negative samples had negative values (≤ -1). All expression ratios lying 3 SD above (up-regulated) or below (down-regulated) the mean were selected and a combined table of possible significant data points from all three comparisons was produced. From this table all data points appearing only once were discarded. Subsequently all genes whose signal intensity was less than twice the background on both filters were eliminated from the list. The final lists were thus comprised of genes that were expressed above background levels and that were significantly differentially expressed in HER2/*neu*-negative and -positive samples in at least two of three comparisons.

Northern Blot Analysis

Total RNA from the cell lines and carcinomas (20 μg/lane) was run on a 1% agarose gel in MEA (0.2 mol/L MOPS, 0.05 mol/L sodium acetate, 0.01 mol/L sodium EDTA, pH 7)/formaldehyde, and transferred by capillary action onto Hybond-N+ nitrocellulose paper (Amersham-Pharmacia Biotech). Total RNA extracted from the 15 random breast carcinomas was also Northern blotted. A PCR product was obtained for trefoil factor 3 (TTF3) (plasmid kindly supplied by Dr. Karin Oien, Beatson Institute, Glasgow, UK) using primers 5′-CAGTCCTGAGCTGCGTCCCG-3′ and 5′-CAGGCACGAAGAACTGTCCTCG-3′ under previously described conditions[12] and purified by phenol-chloroform extraction. DECA Template GAPDH-mouse (10 ng/μl; Ambion Inc., Austin, TX) was included as a positive control. Approximately 20 ng of each probe was radiolabeled with Redivue [α-^{32}P]dCTP (3000 Ci/mmol; Amersham-Pharmacia Biotech) using the random prime labeling system Rediprime II (Amersham-Pharmacia Biotech), as laid out in the supplied protocol. Prehybridization and hybridization were performed at 65°C, in the presence of PerfectHyb Plus (σ-Aldrich, Inc.), and blots were washed in increasingly stringent conditions (1X SSC/0.1% SDS twice, 0.5X SSC/0.1% SDS twice) before exposure to autoradiographical film at –70°C. The labeled filters were exposed for 16 and 22 hours (cell lines) or 65 hours (carcinomas), respectively.

Immunohistochemistry (IHC)

Immunohistochemical evaluation of glycogen phosphorylase isotype BB (GPBB) and vimentin expression was performed on 5-μm paraffin sections which were dewaxed, rehydrated, and quenched in 0.3% H_2O_2 in methanol for 20 minutes. For GPBB staining, the sections were reacted with monoclonal antibody (mAb) 8.F.313 (U.S. Biological, Swampscott, MA) at 0.1 μg/ml for one hour at room temperature (RT), following a 20 minutes antigen retrieval step in sub-boiling 0.1 mol/L EDTA (pH 8.0). The detection reaction followed the ChemMate protocol from DAKO (Ely, UK). A GPBB overexpressing colon carcinoma served as a positive external control, and benign breast epithelium and macrophages served as positive internal controls. For vimentin staining, the sections were reacted with mAb V9 (DAKO) at a 1:500 dilution for 2 hours at RT, following a 20 minute antigen retrieval step in sub-boiling 0.01 mol/L citrate buffer (pH 6.0). The detection reaction followed the Vectastain Elite ABC Kit protocol (Vector Laboratories, Burlingame, CA). Normal connective tissue was used as an external positive control, and benign stromal cells served as ubiquitous internal positive controls. Immunohistochemistry for KAI1 was performed on 6 μm frozen sections which were quenched in 10% H_2O_2 for 20 minutes and blocked with 10% FCS for 20 minutes. The sections were then reacted with mAb 50F11 (PharMingen, San Diego, CA) at 0.1 μg/ml for 1 hour at RT. The detection reaction used the DAKO ChemMate kit. Benign breast epithelium served as a positive external control, and inflammatory cells represented positive internal controls. For all three markers, diaminobenzidine was used as chromogen and hematoxylin as counterstain. The GPBB and vimentin immunostains were semiquantitatively assessed by multiplying the percentage of positive cells by the average staining intensity (1+, 2+, 3+), with a theoretical range in scores from 0 to 300. In the extended cohort of carcinomas ($N = 87$), cases were scored as vimentin positive if more than 5% of neoplastic cells stained. The KAI1 immunostains of the frozen breast cancer sections were categorized as negative if less than 10% of neoplastic cells showed membrane reactivity.

Results

cDNA Microarray Analysis of Differential Gene Expression in Breast Cancer Cell Lines

We compared the gene expression profile of three HER2/*neu*-positive breast cancer cell lines with that of three HER2/*neu*-negative breast cancer cell lines to specifically identify genes whose expression level may be linked to the HER2/*neu* status. Duplicate hybridizations to the

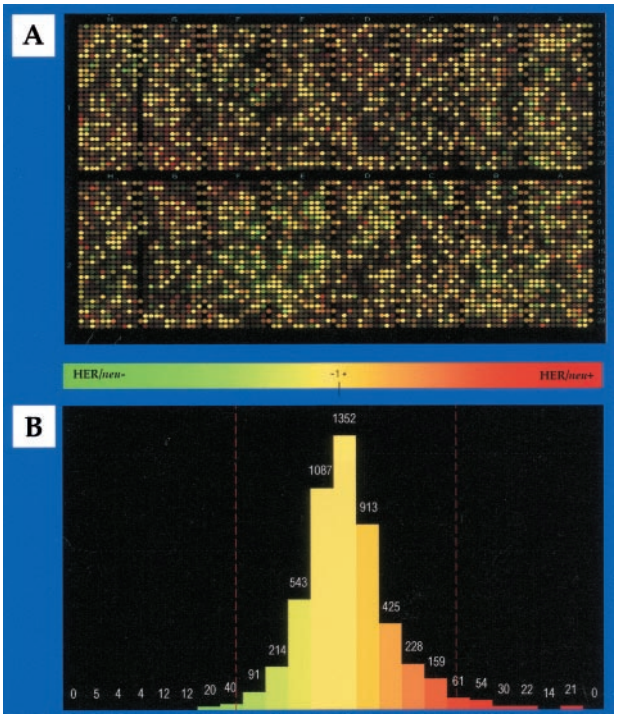

Figure 1. Representative comparison of gene expression profiles in pooled HER2/*neu*-negative and -positive breast cancer cell lines. **A:** Pathways generated green/red overlay. The green spots represent genes more highly expressed in pooled HER2/*neu*-negative breast cancer cell lines, the red spots represent genes more highly expressed in pooled HER2/*neu*-positive breast cancer cell lines, and the yellow spots indicate genes expressed at similar levels. **B:** Histogram derived from **A**. Cutoffs were determined by statistical analysis and are indicated by red lines. In this analysis, 202 genes (3.9%) were up-, and 94 genes (1.8%) were down-regulated in HER2/*neu*-overexpressing breast cancer cells *in vitro*.

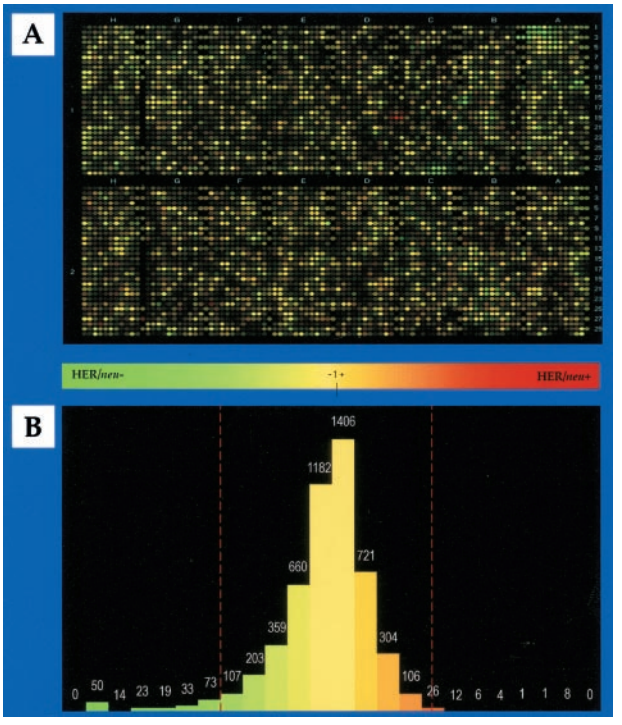

Figure 2. Representative comparison of gene expression profiles in pooled HER2/*neu*-negative and -positive breast cancers. **A:** Pathways generated green/red overlay. The green spots represent genes more highly expressed in pooled HER2/*neu*-negative breast cancers, the red spots represent genes more highly expressed in pooled HER2/*neu*-positive breast cancers, and the yellow spots indicate genes expressed at similar levels. **B:** Histogram derived from **A**. Cutoffs were determined by statistical analysis and are indicated by red lines. In this analysis, 40 genes (0.8%) were up-, and 219 genes (4.2%) were down-regulated in HER2/*neu*-overexpressing breast carcinomas.

cDNA microarrays and exposures of the hybridized filters to the phosphor imaging screen for variable periods of time produced multiple images for each RNA species. Three different pairs of images were used to detect differential gene expression, and in each case the cut-offs were determined by statistical analysis (see Materials and Methods). On average, 211 genes (4.1%) were up-regulated and 99 genes (1.9%) were down-regulated in HER2/*neu*-positive breast cancer cell lines. Figure 1 illustrates a representative comparison. Over half of the genes were found to be differentially expressed in only one of three comparisons and thus were disregarded, while 136 genes appeared in two or three analyses. Among these, 90 genes were up-regulated. These included 37 unknown genes (ESTs, KIAAs) and 53 named genes (Table 1). The up-regulated genes included several genes that are known to play a role in mammary tumorigenesis such as mammaglobin, heat shock protein 70 (HSP70), trefoil factor 3 (TFF3) and tumor protein D52. Five other genes (α1-catenin, CD52, serine protease inhibitor Kunitz type I, interferon receptor 1 and histone acetyltransferase) have known roles in other types of neoplasia but not breast cancer. The largest group of named genes has no defined role in human neoplasia. It includes well-characterized genes such as hepatocyte nuclear factor 3 (HNF3), ribophorin II, ferritin, and myosin. The 46 consistently down-regulated genes are listed in Table 2. Of these, 22 were named genes including several genes (eg, metallothionein 1E, transforming acidic coiled coil containing protein 1 (TACC1), vimentin) previously implicated in breast cancer. Five genes (tissue factor pathway inhibitor (LAC1), α2-glycoprotein 1, aldo-keto reductase family 1 member C1, GPBB, high mobility group protein isoforms I and Y) were described in other types of human tumors. Again, the largest group of named genes has previously not been implicated in neoplasia.

cDNA Microarray Analysis of Differential Gene Expression in Primary Breast Carcinomas

This analysis was performed as described for the cell lines. Pooled RNA from five HER2/*neu*-positive breast cancers was compared against pooled RNA from five HER2/*neu*-negative cancers. All carcinomas were of intermediate to high grade ductal type, ER-positive and node-positive. In triplicate analysis, on average 38 genes were up- and 257 genes were down-regulated in HER2/*neu* overexpressing breast cancers. Figure 2 illustrates a representative comparison. Almost half of the genes appeared in only one analysis and were not further considered. 151 genes were identified as differentially expressed in two or three analyses. The 19 up-regulated genes are listed in Table 3. Only six of these genes have

Table 1. Up-Regulated Genes in HER2/*neu*-Positive Breast Cancer Cell Lines (N = 90)

Accession number	Gene	BC	OC	Chromosome location	Average expression ratio
AA991451	Mammaglobin 1	X		11q13	3.45
AA864271	mRNA from cDNA clone DKFZp586B1810			9	3.41
AI000670	EST			U/K	3.28
H59916	CD24	X		6q21	3.23
AA454810	Membrane component, chromosome 1, surface marker 1			1p32-31	3.22
AA918089	EST			U/K	3.19
AA504201	mRNA from cDNA clone DKFZp586J2118			1	3.09
H68664	mRNA from cDNA clone DKFZp564D246			7	3.02
AA93874	KIAA0344			12	3.00
AA994785	TNF superfamily, member 10			3q26	3.00
AA608567	ATPase, H+ transporting, lysosomal (vacuolar proton pump), member J			U/K	2.85
W80724	EST			17	2.79
AA991871	Hepatocyte nuclear factor 3a (HNF3a)			14q12-13	2.78
AA664179	Keratin 18	X		12q13	2.78
AI000971	EST			U/K	2.69
H81115	BAC clone			16	2.66
AA083485	Ribosomal protein L19	X		17p12-q11	2.64
N62666	EST			4	2.63
AA682392	EST			17	2.59
AA487253	Myosin, light polypeptide, regulatory, non-sarcomeric			18	2.59
AA485441	EST			17	2.57
AA495790	Ras homolog gene family, member B (RhoB)			2pter-p12	2.54
AA489232	ATPase, H+ transporting, lysosomal (vacuolar proton pump) membrane sector associated M8–9 protein			X	2.54
AA490497	HCG-1			13	2.50
AA630449	Epididymal secretory protein			14	2.49
AI299426	EST			11	2.49
AA488658	Heat shock protein 70 (HSP70)	X		6p21.3	2.46
R28294	Glycine cleavage system protein H			2	2.45
W86202	EST			U/K	2.45
AA683102	RAD21			5	2.44
AA918050	EST			U/K	2.43
AI005519	Ribosomal protein S24			10q22-23	2.41
AA453783	KIAA0187			U/K	2.39
AA182845	TNF-α inducible cellular protein			10	2.38
AA693571	Minichromosome maintenance deficient 3-associated protein			21q22.3	2.37
H99123	EST			U/K	2.37
W73966	EST			5	2.35
AA991856	Ribophorin II			20q12-13.1	2.34
AA446103	Lectin mannose-binding, 1			18q21.3-22	2.32
AA430653	EST			5	2.31
N91817	EST			9	2.31
AI025126	EST			7	2.30
AA683050	Ribosomal protein S8			1p34.1-32	2.30
AA932521	FK506-binding protein 4			12	2.29
AA459100	Tumor protein D52	X		8q21	2.29
AA676957	α1-Catenin		X	5q31	2.28
AA495936	Microsomal glutathione S-transferase 1			12	2.26
AA973283	CDW52 (CAMPATH-1 antigen)		X	1p36	2.26
AA905624	KIAA0182			16	2.25
H05099	EST			U/K	2.24
T78584	Splice factor			U/K	2.24
AA418813	EST			U/K	2.24
AA047338	Proteasome subunit α6			14q13	2.24
AA455300	Cold shock domain protein A			12p13.1	2.23
AA040742	Poly(A)-binding protein 2			14q11.2-13	2.23
AA991162	EST			U/K	2.22
N74131	Trefoil factor 3 (human secretory protein P1B)	X		21q22.3	2.21
AA873604	Cysteine-rich protein 1, intestinal			7q11.23	2.20
AA975209	Serine protease inhibitor, Kunitz type I		X	15	2.19
AA488627	H2A histone family, member Y			5q31.1-32	2.19
AA488349	Interferon receptor 1		X	21q22	2.18
R70263	EST			U/K	2.18
AA478949	Discs (*Drosophila*), large homolog 5			10q23	2.15
AA976544	KIAA0788			17	2.13

(*Table 1 continues*)

Table 1. *Continued*

Accession number	Gene	BC	OC	Chromosome location	Average expression ratio
AI369331	Protein tyrosine phosphatase, non-receptor type substrate 1			20p13	2.12
AA873427	Ferritin, light polypeptide			19q13.3-13.4	2.12
AA485959	Keratin 7	X		12q12-14	2.09
R77432	Mannose-P-dolichol utilization defect 1			17p13.1-12	2.09
AI001180	Oxidase (cytochrome c) assembly-like 1			14q11.2	2.09
AA676460	EST			U/K	2.08
AI090094	Human factor VIII gene L1 element insertion DNA			U/K	2.07
AA282263	KIAA0556			16	2.06
AA620580	Proteasome subunit β3			2q35	2.05
AA485994	DEAD/H box polypeptide			22q13.1	2.05
AA962301	KIAA0215			11	2.05
AA994796	LIM and SH3 protein 1	X		17q11-21.3	2.04
AA478066	Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase (PKMYT1)			U/K	2.01
H14805	EST			U/K	1.99
AA598797	Histone acetyltransferase		X	2q31.2-33.1	1.99
AA505111	Copine III			8	1.97
AA291069	EST			U/K	1.97
H16256	Dihydropyrimidinase-like 3			5q32	1.96
AA668189	Small nuclear ribonucleoprotein polypeptide F			U/K	1.95
AA195002	PAC clone			U/K	1.95
N74574	EST			U/K	1.94
AA669341	Unactive progesterone receptor			1	1.94
H95976	KIAA0251			16	1.94
H67086	Similar to *S. cerevisiae* SSM4			5p15.2	1.91
AA608514	H3 histone family, 3B			17q25	1.90
AA404619	CD73	X		6q14-21	1.85

BC, breast cancer-associated gene; OC, other cancer-associated gene; U/K, unknown.

known functions, and two of these have been linked to human neoplasia (Max, stanniocalcin 2). There was no overlap with the list of genes up-regulated in HER2/*neu*-positive breast cancer cell lines. 132 genes were consistently down-regulated, and of these, 58 genes are unidentified (Table 4). A number of the down-regulated genes (eg, TGF-β1, KAI1) are well described in breast cancer. Other genes such as latent TGF-β binding protein 1 (LTBP1) and melanoma antigen p15 were previously found to be involved in other human malignancies. Again, most of the named genes have no known function in human neoplasia. Only four genes (GPBB, TACC1, complement 1q, EST R83355) were found to be down-regulated both in HER2/*neu*-positive breast cancer cell lines and in HER2/*neu*-overexpressing breast cancers. Interestingly, six genes were down-regulated in the carcinomas but up-regulated *in vitro* (RAD 21, serine protease inhibitor Kunitz type 1, factor VIII gene L1 element insertion DNA, ribophorin II, EST AI000971, one chromosome 16 BAC clone).

Confirmatory Studies: Northern Analysis

The up-regulation of TFF3 expression in HER2/*neu*-positive breast cancer cell lines was confirmed by Northern analysis. In the pooled RNA samples used for microarray analysis, relatively abundant TFF3 mRNA was detected in the HER2/*neu*-positive but not in the HER2/*neu*-negative cell lines (Figure 3A). TFF3 expression was then studied in the individual cell lines used in the microarray analysis. Two HER2/*neu*-negative cell lines failed to express TFF3. In contrast, positive bands were detected for HER2/*neu*-positive cell lines BT474 and MDA-MB-361 (Figure 3B). Because insufficient RNA was available, TFF3 expression could not be studied in the ten breast carcinomas used in the gene expression profiling experiments. However, Northern analysis was performed on 15 breast cancers that were not controlled for histological type or grade, receptor or nodal status. In this random sample, TFF3 expression could be detected in both HER2/*neu*-positive (4/6) and -negative (4/9) specimens (Figure 3C).

Confirmatory Studies: Immunohistochemistry

IHC was used to further demonstrate the specificity of the cDNA microarray results and to confirm the differential expression of three additional genes that, like TFF3, were known to have important physiological functions and whose expression was previously found to be deregulated in human neoplasia. GPBB was one of only a few genes found to be down-regulated in HER2/*neu*-positive breast cancer cells both *in vitro* and *in vivo*. The differential expression could also be demonstrated at the protein level. The average immunoreactivity of the three HER2/*neu*-negative cell lines was more than twice than that of the three HER2/*neu* overexpressers (Figure 4, A and B). Seven of the 10 cancers in the gene expression cohort could be stained for GPBB. The HER2/*neu*-negative carcinomas had average IHC scores that were about three times as high as those of the HER2/*neu*-positive tumors

Table 2. Down-Regulated Genes in HER2/*neu*-Positive Breast Cancer Cell Lines ($N = 46$)

Accession number	Gene	BC	OC	Chromosome location	Average expression ratio
AA872383	Metallothionein 1E	X		16q13	−3.85
N93476	Endothelial differentiation sphingolipid G-protein-coupled receptor 1			1pter-qter	−3.32
AA486321	Vimentin	X		10p13	−3.31
AI022299	High-mobility group protein 17			1p36.1	−3.24
AA634006	Aortic-type smooth muscle α-actin gene	X		10q22-24	−3.11
H63077	Annexin I	X		9q11-22	−2.98
AA127217	EST			1	−2.78
H79839	EST			U/K	−2.61
T50282	Tissue factor pathway inhibitor (LAC1)		X	2q13-32.1	−2.61
AA991578	Vitelliform macular dystrophy (bestrophin)			11q13	−2.55
AI44462	EST			U/K	−2.54
AI017394	EST			12	−2.52
AA862465	α2-glycoprotein 1, zinc		X	7q22.1	−2.48
AA427954	EST			12	−2.48
AA111865	EST			8	−2.39
R93124	Aldo-keto reductase family 1, member C1		X	10p15-14	−2.38
AA913304	Heat shock transcription factor 2			6	−2.36
AA878089	EST			U/K	−2.35
AA436384	EST			1	−2.31
AI089149	KIAA0448			1	−2.28
AA922705	Glycogen phosphorylase isotype BB		X	20p11.2-11.1	−2.27
AA010360	EST			4	−2.25
AA496007	EST			5	−2.20
R97503	EST			3	−2.19
H79022	EST			U/K	−2.19
AA989257	Interleukin 4	X		12q13	−2.18
T71284	Complement 1q			1p36.1	−2.18
AI347695	Mitochondrial translational release factor 1			13q14.1-14.3	−2.17
AA456063	EST			12	−2.16
AA448261	High-mobility group protein isoforms I & Y		X	6p21	−2.06
AA865147	EST			4	−2.06
AI357378	Macrophage lectin 2			U/K	−2.04
R48312	EST			1	−2.03
R83355	EST			6	−2.00
AA459364	EST			8	−2.00
AA259151	EST			U/K	−1.99
AA634028	SB class II histocompatibility antigen α-chain			6p21.3	−1.98
AI000935	EST			11	−1.98
AA862717	Cas-Br-M (murine) ectropic retroviral transforming sequence B			3q	−1.97
H15570	EST			U/K	−1.96
AA677200	EST			11	−1.95
AA97960	EST			20	−1.91
AA961361	Transforming acidic coiled coil containing protein 1 (TACC1)	X		8p11	−1.90
H50623	MHC class II HLA-DR7-β-chain			6p21.3	−1.88
AA931758	G0S2 protein			1q32.2-41	−1.87
N34316	EST			17	−1.84
AI264651	mRNA sequence from H.sapiens clone 24649			19	−1.83

BC, breast cancer-associated gene; OC, other cancer-associated gene; U/K, unknown.

(Figure 4, C and D). Immunocytochemistry for vimentin produced similar findings. The three HER2/*neu*-negative breast cancer cell lines stained 2.5 times more intensely than the three HER2/*neu* overexpressers (Figure 5, A and B). To test whether this inverse correlation also existed in primary breast carcinomas, we stained 17 of the 25 ICRF tumors and 70 additional invasive ductal carcinomas from the pathology files of the Roswell Park Cancer Institute for vimentin. Of 87 tumors, 18 (21%) were positive, and these cases were exclusively confined to the ER-negative group ($P < 0.0001$, Table 5). Among the ER-negative carcinomas, almost all (17/18, 94%) of the vimentin-positive cases were confined to the HER2/*neu*-negative subgroup, and only one of 13 HER2/*neu*-positive cancers (8%) expressed this filament ($P = 0.0019$, Table 5, Figure 5 C, D). Lastly, among the down-regulated genes in HER2/*neu*-positive breast cancers, KAI1 had one of the highest expression ratios. IHC was performed on frozen sections from all ten tumors in the gene expression cohort. Extensive down-regulation of KAI1 expression was demonstrated both in the HER2/*neu*-positive and in the -negative carcinomas (Figure 6).

Discussion

HER2/*neu* has become an important breast cancer biomarker because it predicts for a more aggressive clinical phenotype, and it also correlates with a tumor's response to systemic therapy.[13–15] Although a number of genes and pathways have been identified that are down-

Table 3. Up-Regulated Genes in HER2/*neu*-Positive Breast Carcinomas ($N = 19$)

Accession number	Gene	BC	OC	Chromosome location	Average expression ratio
AA598631	EST			X	4.45
AA490477	Smooth muscle myosin heavy chain isoform Smemb			17	4.40
R96579	EST			U/K	3.78
AA026152	Vesicle trafficking protein			3	3.53
AA677406	EST			U/K	3.47
W72838	EST			U/K	3.22
AA278402	KIAA0465			1	2.67
AA937783	KIAA0461			1	2.66
AA598572	Spleen tyrosine kinase			9q22	2.35
AA903500	EST			20	2.19
AA448660	EST			11	2.14
AA113016	EST			11	2.11
AA629688	EST			U/K	2.09
T98503	EST			20	2.08
N68443	Max		X	14q23	2.05
AA233901	EST			18	2.04
AI376502	Rab13			12q13	1.94
AA676408	Stanniocalcin 2	X		5	1.93
W31717	EST			10	1.92

BC, breast cancer-associated gene; OC, other cancer-associated gene; U/K, unknown.

stream targets of HER2/*neu*, the biological basis of the increased virulence of breast cancers overexpressing this oncogene remains elusive. The principal objective of our study was to identify differentially expressed genes that may shed light on this question and that may serve as novel diagnostic, prognostic or therapeutic markers in the future.

With the recent development of cDNA microarray technology, it has become possible to study gene expression in cells and tissues of interest on a global scale. This technology has been used to arrive at a molecular classification of several tumor types including breast cancer.[9] We used it to answer a more specific question, ie, whether HER2/*neu*-negative and -positive breast cancer cells could be distinguished by their gene expression profiles. In an attempt to cancel out many of the random differences in gene expression that exist between individual breast cancer cell lines and tumors, respectively, and to specifically identify genes whose expression level may be linked to the HER2/*neu* status, we pooled RNA from $p185^{erbB-2}$ overexpressing specimens and compared it to pooled RNA from HER2/*neu*-negative samples. An analysis of individual specimens may have revealed a large number of genes whose expression ratios are not related to HER2/*neu* activity. While pooling of RNAs produces an average of the expression ratios, this approach is more likely to identify genes that are linked to HER2/*neu* status across the pooled specimens. It is validated by our ability to identify a number of genes with a known role in mammary neoplasia and to verify the differential expression of certain genes by other methods and in an independent, expanded series of tumors. Whereas single cDNA microarray analyses may be subject to significant experimental variability,[16] the validity and reliability of our results were increased by our performing the hybridizations in duplicate and the comparisons in triplicate. We chose to use the GF225 microarray from Research Genetics, in part because it contains a large number of breast specific cDNAs. This would allow us to assess the effects of transformation on known and unknown genes involved in differentiation and proliferation. Our data complement an earlier report by Oh et al, [6] who studied the effect of HER2/*neu* overexpression on gene expression in breast and ovarian cancer cells by differential hybridization. In that analysis, only 19 of 16,000 cDNA clones were found to be differentially expressed. Recently, Kauraniemi et al[17] used a microarray containing 636 cDNA clones from chromosome 17 including 217 ESTs from 17q12 to identify a small number of genes that were co-amplified and co-overexpressed with HER2/*neu* in breast cancer. Most of these genes, including HER2/*neu* itself, were not represented on the GF225 array. The latter does contain cDNAs for GRB7 and MLN64, two genes that Kauraniemi et al[17] reported to be highly expressed in HER2/*neu*-positive BT474, SKBR3 and MDA-MB-361 cells. However, the level of expression of these genes in our three HER2/*neu*-negative breast cancer cell lines was not examined, and we did not find them to be significantly differentially expressed in our analysis.

A similar number of genes were found to be differentially expressed in HER2/*neu* overexpressing breast cancer cells in culture ($N = 136$ (2.6%)) and in HER2/*neu*-positive breast carcinomas ($N = 151$ (2.9%)) compared to the HER2/*neu*-negative specimens. However, one of the most significant findings in our study is the paucity of genes appearing in both cell lines and tumors. *In vitro*, twice as many genes were up- than down-regulated in HER2/*neu* overexpressing breast cancer cells (Figure 1). In contrast, only 13% of the differentially expressed genes in HER2/*neu*-positive carcinomas were up-regulated compared to HER2/*neu*-negative cancers (Figure 2). Only four genes were found to be down-regulated, and none were up-regulated in HER2/*neu*-positive specimens both *in vitro* and *in vivo*. In addition, a small number

Table 4. Down-Regulated Genes in HER2/*neu*-Positive Breast Carcinomas ($N = 132$)

Accession number	Gene	BC	OC	Chromosome location	Average expression ratio
AA644693	Ariadne-2 (*Drosophila*) homolog			3p21.2-21.3	−34.83
AA136125	Spermine synthase	X		Xp22.1	−25.05
AA22309	KAI1 (CD82)	X		11p11.2	−14.53
AA975832	EST			U/K	−12.35
AA070226	Selenoprotein P, plasma, 1		X	5q31	−11.65
AA010400	Ets variant gene 4		X	17q21	−11.14
AA633569	Ribosomal protein L26			17p	−9.57
H26183	CCAAT/enhancer binding protein (C/EBP), β			20q13.1	−8.49
AA400476	Mitotic centromere-associated kinesin (kinesin-like 6)	X		1	−7.78
AA279440	Tafazzin			Xp28	−5.67
AA279941	Zinc finger protein 42		X	19q13.2-13.4	−5.55
N35050	TNF, member 12			17p13.3	−5.49
N34048	KIAA0691			19q13.4	−5.40
AA044390	Uridine diphosphoglucose pyrophosphorylase			2p14-13	−5.07
T90778	EST			19	−4.93
AA136710	Lactoyl glutathione lyase (glycoxylase 1)	X		6p21.3-21.2	−4.74
R08932	Golgi transport complex protein			7q31	−4.61
AA968664	Melanoma antigen p15		X	4	−4.37
AA664101	Aldehyde dehydrogenase 1	X		9p21	−4.28
H39187	EGF-like-domain, multiple 2			1p21	−4.27
W73790	Immunoglobulin λ-like polypeptide 2			22q11.23	−4.13
AA129777	Solute carrier family 16, member 3			22q12.3-13.2	−4.10
R59615	EST			18	−4.10
AA917766	EST			10	−4.03
AA056013	Microfibril-associated glycoprotein-2			12p13.1-12.3	−3.99
AA454657	Interleukin 11 receptor α		X	9p13	−3.97
AA878048	Keratin 15			17q21.1	−3.86
H73586	KIAA0262			12	−3.65
AA886199	DEME-6	X		U/K	−3.63
AA447770	KIAA0409			11	−3.57
AA670422	ADP-ribosylation factor 3			12q13	−3.52
AA465522	EST			11	−3.49
AA960842	EST			8	−3.46
H38650	Solute carrier family 2, member 5 (GLUT5)	X		1p36.2	−3.40
R50337	Solute carrier family 19, member 1	X		21q22.3	−3.40
H24650	Laminin-γ1		X	1q31	−3.38
T86708	Solute carrier family 4, member 1, anion exchanger			17q21-22	−3.38
AA464856	Inhibitor of DNA binding 4			6p22-21.3	−3.25
AA133584	JM1			Xp11.23	−3.24
AA418737	EST			U/K	−3.21
AA417806	EST			U/K	−3.21
AA885642	H2B histone family, member B			6p21.3	−3.12
AA683102	RAD21			5	−3.11
R45254	EST			6	−3.10
AA464140	ATP-dependent RNA helicase			17q21.1	−3.08
H04202	KIAA0635			4	−3.07
AA598621	Signal recognition particle receptor ("docking protein")			11q23-24	−3.03
AA974805	EST			7	−3.00
AA487575	Calcium & integrin binding protein			15q25.3-26	−2.99
R36467	TGF-β1	X		19p13.1	−2.99
H74265	Protein tyrosine phosphatase, receptor type, c			1q31-32	−2.99
H51574	Arachidonate 5-lipoxygenase		X	10	−2.95
W56266	Cot oncogene	X		10p11.2	−2.93
H17551	RNA (guanine-7-)-methyltransferase			18p11.22-11.23	−2.93
AA989457	EST			U/K	−2.87
AA283023	EST			4	−2.86
AA426264	Ki-67	X		10q25-ter	−2.81
AA455507	KIAA0618			7	−2.81
AA150403	EST			3	−2.81
AA974801	Capping protein (actin filament) muscle Z-line, α1			1	−2.78
AA417994	EST			16	−2.78
AA620527	EST			1	−2.78
AI338952	EST			U/K	−2.76
AA101617	Fos-like antigen 2			2p23-22	−2.75
AI000138	EST			U/K	−2.75
AA418674	Fibrillin 1			15q21.1	−2.70
AA453816	Folate receptor 2 (fetal)	X		11q13.3-13.5	−2.68
AA487236	EST			U/K	−2.67

(*Table 4 continues*)

Table 4. *Continued*

Accession number	Gene	BC	OC	Chromosome location	Average expression ratio
AA132874	EST			15	−2.67
AI337445	Collagen I α1			17q21.31-22	−2.64
AA922705	Glycogen phosphorylase isoform BB		X	20p11.2-11.1	−2.63
AA046713	Ribosomal protein S3		X	11q13.3-13.5	−2.63
AA488674	Myeloid cell leukemia sequence 1 (Bcl-2 related)		X	1q21	−2.61
R73542	EST			U/K	−2.60
T98783	Latent transforming growth factor β-binding protein 1 (LTBP1)		X	2p12-q22	−2.59
H53025	Calumenin			7q32	−2.57
AA975209	Serine protease inhibitor, Kunitz type 1		X	15	−2.57
AA504772	S-adenosylmethionine decarboxylase 1	X		6q21-22	−2.56
AA436384	EST			1	−2.55
AI000971	KIAA0344			12	−2.55
AA872041	EST			U/K	−2.54
AA608548	Template activating factor-1α			U/K	−2.52
N50738	EST			12	−2.52
W72591	EST			U/K	−2.52
AI262140	Activated p21 cdc42Hs kinase (ACK)			3	−2.51
H81115	BAC clone			16	−2.50
AA915976	EST			U/K	−2.50
AA978328	EST			21	−2.46
H40880	EST			16	−2.46
R14855	Structure specific recognition protein I		X	11q12	−2.45
AA905588	Axin 1		X	16p13.3	−2.45
AA857103	EST			U/K	−2.45
AI074017	EST			U/K	−2.45
H20676	EST			1	−2.42
R83355	EST			6	−2.42
AA521423	EST			U/K	−2.40
H11003	Endothelin 1	X		6p24-23	−2.39
AA479967	EST			3	−2.39
H80325	EST			14	−2.39
AA457223	EST			20	−2.36
R46218	EST			U/K	−2.36
W81139	EST			U/K	−2.35
AA877815	KIAA0353			15	−2.35
W74337	KIAA0684			1	−2.34
AA991889	EST			U/K	−2.33
AA018457	Glutamate decarboxylase 1 (GAD1)			2q31	−2.31
AA486533	Early growth response I	X		5q31.1	−2.31
AA485893	Ribonuclease, RNase A family, 1 (pancreatic)			14	−2.29
AA995174	EST			12	−2.29
AA043552	EST			7	−2.28
W73810	Epithelial membrane protein 3			19q13.3	−2.27
AA291163	Glutaredoxin (thioltransferase)		X	5q14	−2.27
AA064668	Rab8			19p13.2-cen	−2.22
N48345	EST			U/K	−2.22
AI140997	EST			U/K	−2.21
AA282983	EST			U/K	−2.21
AA988959	Max-interacting protein 1		X	10q25	−2.21
AI090094	Factor VIII gene L1 element insertion DNA			U/K	−2.20
AA961361	Transforming acidic coiled-coil containing protein 1 (TACC1)	X		8p11	−2.20
R54643	EST			3	−2.20
AA644679	Dynein, cytoplasmic, light polypeptide		X	14q24	−2.19
AA158162	EST			11	−2.16
T71284	Complement 1q			1p36	−2.16
AA465180	EST			2	−2.16
AI369623	Regulator of G-protein signalling 3			9q31-33	−2.11
AA479060	EST			6	−2.11
R23752	Ribosomal protein S12			19q13.1	−2.08
AA449667	Milk fat globule-EGF factor 8 protein			15q25	−2.08
AA644191	ADP-ribosylation factor-like 3			10q23.3	−2.10
AA121836	EST			15	−2.10
AA991856	Ribophorin II			20q12-13.1	−2.01
AA406603	KIAA0761			1	−1.94

BC, breast cancer-associated gene; OC, other cancer-associated gene; U/K, unknown.

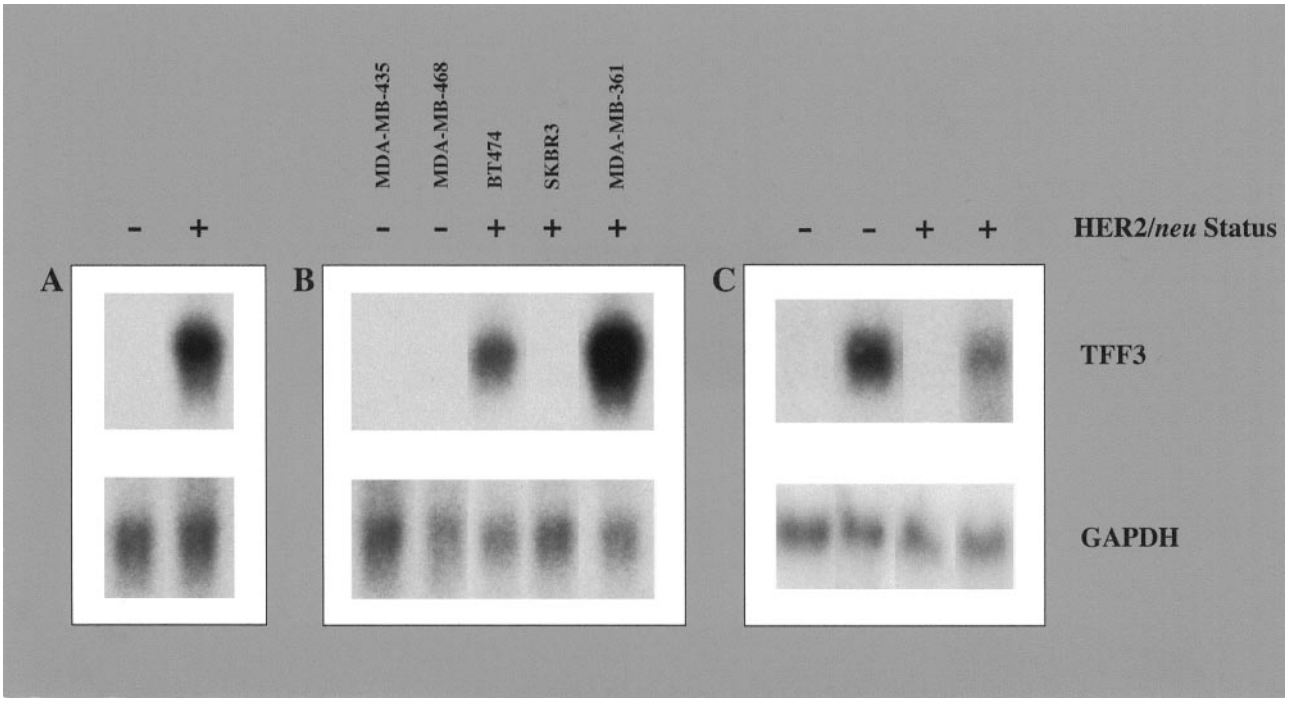

Figure 3. Northern blot analysis of trefoil factor 3 (TFF3) expression in breast cancer cell lines and carcinomas. Northern blots of HER2/*neu*-negative (-) and -positive (+) breast cancer cell lines and carcinomas (20 μg total RNA per lane) were hybridized with TFF3 and GAPDH probes. **A:** Pooled RNA from three HER2/*neu*-negative and three -positive breast cancer cell lines. TFF3 is only expressed in the latter. **B:** TFF3 expression in five of the six breast cancer cell lines. TFF3 is expressed in two of the three HER2/*neu*-positive, but in neither of the two HER2/*neu*-negative lines. **C:** Representative TFF3 expression patterns in randomly selected HER2/*neu*-negative and HER2/*neu*-positive breast carcinomas.

of genes were induced in HER2/*neu*-positive cell lines but repressed in the carcinomas.

The differences in the specific genes regulated in HER2/*neu*-positive cell lines and tumors, with so few in common, may be related to three major factors. The ten carcinomas subjected to microarray analysis were carefully controlled for histological type and grade, nodal status, and ER content, leaving HER2/*neu* status as the major discriminating variable. In contrast, the six cell lines selected for this study were derived from quite divergent tumors representing different stages of disease, and they showed variable aggressiveness in experimental systems.[18] Secondly, there is mounting evidence that the stroma plays a critical role in breast tumor formation,[19,20] and it is likely that the stromal compartment in HER2/*neu*-negative breast cancers has a gene expression profile that differs from that in tumors overexpressing the oncogene. Previous transcriptional profiling reports that included both cell lines and primary carcinomas also indicated significant differences in the gene expression patterns.[21,22] However, Perou et al[8] found good concordance of gene expression profiles in their cell lines and non-microdissected cancers. This may be due to the choice of genes on the arrays, which differ markedly in principle of selection. To evaluate the contribution to the breast carcinoma gene expression pattern of the stroma versus the malignant epithelium, separate cDNA microarray analyses of microdissected tumor tissues may need to be carried out. It will also be of interest to analyze breast cancer cell lines co-cultivated with stromal cells or in three-dimensional growth models. Indeed, the comparison of gene expression profiles between two- and three-dimensional growth would be important for understanding the relationship of gene expression *in vitro* and *in vivo*. Finally, it has been recognized that long-term growth *in vitro* creates significant changes in the genotype and phenotype of the cultured cells, and it is possible that the gene expression profile of an established cell line is significantly different from that of its parental cells *in vivo*. The role of transforming genes in switching off normal expression patterns has been poorly studied, but our results suggest that this may be a major effect of HER2/*neu in vivo* and may therefore have a role in producing aberrant patterns of tissue architecture and more aggressive clinical characteristics. This phenomenon may have been shown in our study because we used an array for normal breast, in contrast to oncogene enriched or selected arrays.

One of the few genes that were down-regulated in HER2/*neu*-positive breast cancer cells both *in vitro* and *in vivo* was GPBB. We were able to confirm the differential expression of this enzyme by immunohistochemistry (Figure 4). Previous reports indicated up-regulation of GPBB in a high proportion of certain types of human tumors including gastric and colorectal carcinomas.[23,24] The physiological role of this enzyme in malignant tissues is poorly understood, but it has been suggested that elevated levels of GPBB may provide emergency glucose supply under anoxic conditions.[25] Our study newly implicates GPBB in breast tumorigenesis, but its mechanism of action and biological relevance remain to be demonstrated. In contrast to gastrointestinal mucosa, normal

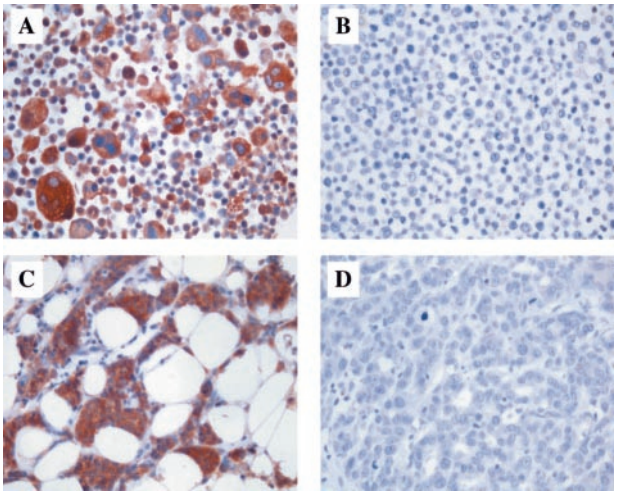

Figure 4. Glycogen phosphorylase BB (GPBB) expression in HER2/*neu*-negative and -positive breast cancer cell lines and carcinomas. Immunohistochemical stains of cell lines MDA-MB-435 (**A**) and MDA-MB-361 (**B**) as well as a HER2/*neu*-negative (**C**) and -positive (**D**) primary breast carcinoma. There is strong cytoplasmic reactivity in the HER2/*neu*-negative breast cancer cells (**A**, **C**) but almost no staining in the HER2/*neu*-positive specimens (**B**, **D**). Original magnifications, ×400.

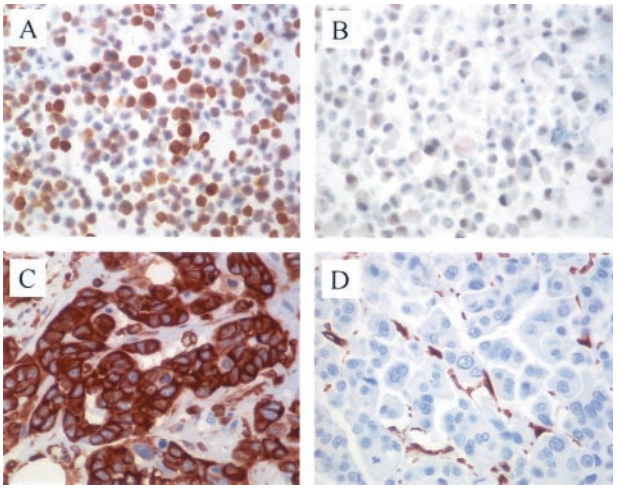

Figure 5. Vimentin expression in HER2/*neu*-negative and -positive breast cancer cell lines and carcinomas. Immunohistochemical stains of cell lines MDA-MB-468 (**A**) and SKBR3 (**B**) as well as a HER2/*neu*-negative (**C**) and -positive (**D**) primary breast carcinoma. There is strong cytoplasmic reactivity in the HER2/*neu*-negative breast cancer cells (**A**, **C**) but absence of reactivity in the HER2/*neu*-positive tumor cells (**B**, **D**). Original magnifications, ×400.

breast epithelium contains high levels of this enzyme (not shown). It is conceivable that down-regulation of GPBB is partly responsible for the higher prevalence of necrosis seen in HER2/*neu*-positive *in situ* and invasive breast cancers, possibly due to reduced glucose levels in the neoplastic cells. This marker is prototypic of a significant number of genes that we found to be down- or up-regulated in HER2/*neu*-positive breast cancer specimens and that had previously been reported to be involved in the development of other tumor types. This group of 26 genes also included α1-catenin, CDW52, interferon receptor 1, tissue factor pathway inhibitor, and melanoma antigen p15. Thus, cDNA microarray analysis of breast cancer cell lines and tissues can identify genes whose importance may not be confined to other tumor types.

Supporting the sensitivity of the microarray approach, our analyses revealed 32 genes that had previously been implicated in breast cancer development. These included well characterized genes such as HSP70, metallothionein 1E, glycoxylase and TGF-β1, as well as more recently described genes such as annexin 1, mitotic centromere-associated kinesin and DEME-6, whose pathobiological significance in mammary tumorigenesis needs to be further defined. One of these genes, TFF3 (intestinal trefoil factor, human secretory protein 1.B), was overexpressed in HER2/*neu*-positive breast cancer cells *in vitro*, and we confirmed this finding by Northern analysis (Figure 3A). TFF3 was not expressed in HER2/*neu*-negative cell lines, but was expressed at high levels in two of three HER2/*neu*-positive lines (Figure 3B). TFF3 was not found to be up-regulated in HER2/*neu*-positive breast carcinomas by microarray analysis. Indeed, Northern analysis of randomly selected tumors revealed a range of mRNA levels both in the HER2/*neu*-positive and in the –negative samples (Figure 3C). This finding suggests that *in vivo*, TFF3 expression may depend not only on the HER2/*neu* status of the neoplastic cells but also on stromal factors. The protein is structurally related to pS2 (TFF1), and it has been best studied in the gastrointestinal tract.[12] It was also shown to be expressed by breast cancer cell lines and tumors, and the expression

Table 5. Vimentin Expression in Breast Carcinomas as a Function of ER and HER2/*neu* Status

		Vimentin		
		Negative	Positive	*P**
All cancers (*N* = 87)				
	Negative ER	23 (26%)	18 (21%)	<0.0001
	Positive	46 (53%)	0	
ER-negative cancers (*N* = 41)				
	Negative HER2/*neu*	11 (27%)	17 (41%)	0.0019
	Positive	12 (29%)	1 (2%)	

*Fisher's exact test.

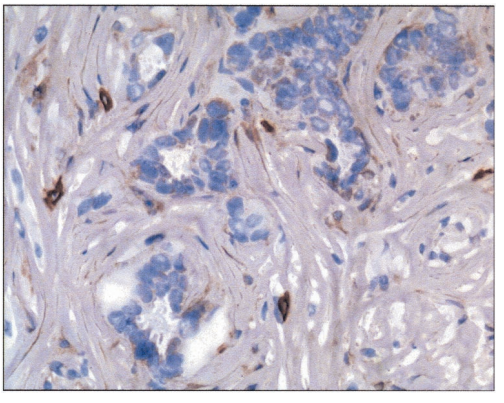

Figure 6. KAI1 down-regulation in a breast carcinoma. Representative immunohistochemical stain of a frozen section of an invasive ductal carcinoma. There is complete absence of membrane staining in the neoplastic cells. Admixed endothelial and inflammatory cells act as positive internal controls. Original magnification, ×400.

level was reported to be higher in ER+ cells.[26,27] Our data support the finding by May et al that TFF3 was not detectable in SKBR3 and MDA-MB-231 cells.[26] Elevated levels of TFF3 in HER2/*neu*-positive breast cancer specimens may induce a more aggressive phenotype by several mechanisms. Trefoil factors in general have been found to be motogenic, and it has been suggested that TFF3 levels may affect the metastatic potential of tumor cells.[12] The motogenic effect of TFF3 may be mediated by interaction of this molecule with E-cadherin, β-catenin, and associated proteins.[28] Moreover, in colorectal carcinomas, TFF3 expression may confer resistance to chemotherapy.[29] Whether this effect is also present in breast carcinomas, is unknown.

As discussed above, down-regulation of GPBB and up-regulation of TFF3 can be linked, at least in theory, to the more virulent clinical behavior of HER2/*neu*-positive breast cancers. Our microarray analysis revealed several other genes whose differential expression may be related to this phenotype. Tables 1 to 4 include a surprisingly small number of *bona fide* oncogenes and tumor suppressor genes, and this may partly reflect under-representation of these genes on the GF225 cDNA microarray. One of the few suppressors that was on the list of down-regulated genes in HER2/*neu*-positive breast cancers was KAI1 (CD82). This gene suppresses the invasive and metastatic properties of several tumor types such as prostate cancer and melanoma.[30,31] We previously demonstrated KAI1 down-regulation in lymphomas and squamous and colon carcinomas.[32,33] Moreover, this gene was down-regulated in a subset of breast carcinomas,[34] and transfection of the gene into breast cancer cells suppressed invasion and metastasis.[35] We confirmed the down-regulation of KAI1 in five HER2/*neu*-positive breast cancers by frozen section immunohistochemistry (Figure 6). However, there was equally dramatic reduction in KAI1 expression in the five HER2/*neu*-negative carcinomas despite higher mRNA levels, suggesting some degree of dissociation of KAI1 mRNA and protein levels. Significantly, the microarray identified KAI1 as one important gene whose expression is deregulated in breast cancer.

$p185^{erbB-2}$ overexpression in breast cancer was associated with differences in the gene expression pattern of several structural and functional pathways. These included elements of the extracellular matrix and tumor basement membrane (laminin-γ1, microfibril-associated glycoprotein 2, fibrillin 1, collagen I α1), components of the TGF-β pathway (TGF-β1, LTBP1, HNF3), stress related genes (glutathione S-transferase, HSP70, annexin I, high mobility group protein isoforms I and Y, complement 1q, stanniocalcin 2), and components of the cytoskeleton (keratins 7, 15 and 18, myosin light and heavy chains, smooth muscle actin, vimentin). In the latter three pathways, some components were up-, while others were down-regulated. In contrast, all four stromal matrix/basement membrane proteins were down-regulated, suggesting that in HER2/*neu*-positive tumors, the stroma may be less resistant to invasion by the neoplastic cells.

We confirmed the elevated expression of vimentin in HER2/*neu*-negative breast cancer cell lines by immunocytochemistry (Figure 5, A and B). Our results are in agreement with a report by Dandachi et al[36] indicating absence of vimentin expression in SKBR3(A) and MDA-MB-361 cells and positive reactivity in the MDA-MB-231 cell line. To test whether the inverse association between vimentin expression and HER2/*neu* status also existed *in vivo*, we studied 87 invasive ductal carcinomas with previously determined ER and HER2/*neu* status. Aberrant vimentin expression could be demonstrated in 18 of 41 (44%) ER-negative, but in none of 46 ER-positive carcinomas, consistent with many previous reports of the inverse correlation between vimentin expression and ER status.[36–38] Significantly, all six cell lines used in our analyses were reported to be ER-negative.[18] The overall vimentin positivity rate (18/87, 21%) is similar to previously reported frequencies.[36,37] Our observation that, in ER-negative cancers, vimentin expression is almost completely limited to tumors with low $p185^{c\text{-}erbB\text{-}2}$ levels, is a novel finding. We are aware of only one other study that included vimentin and HER2/*neu* in the same group of breast cancers. Dandachi et al[36] found no correlation between these two markers in their whole cohort of carcinomas, however, the tumors were not stratified by ER status. Previous reports linked vimentin positivity to more aggressive tumor characteristics including high grade and elevated proliferative activity, as well as to high levels of epidermal growth factor receptor (EGFR), another member of the erbB gene family.[37–39] Whether vimentin positivity adversely affects survival, is controversial.[37,38] In an experimental system, inappropriate expression of vimentin in MCF-7 cells led to increased motility, invasiveness, clonogenic potential, tumorigenicity and proliferation.[40] Thus, aberrant expression of vimentin in the neoplastic cells, with or without concomitant EGFR overexpression, may be an alternate mechanism of conferring aggressive biological characteristics on ER-negative breast cancers that do not overexpress HER2/*neu*. The vimentin gene is located at a chromosomal site (10p) that is amplified in a subset of breast cancers.[41] Why aberrant expression of vimentin preferentially occurs in ER- and HER2/*neu*-negative breast cancers, and whether this phenomenon is indeed due to gene amplification, remains to be elucidated.

In summary, our study has revealed a significant number of genes representing multiple biochemical pathways whose level of expression in breast cancer cell lines and tissues is affected by the HER2/*neu* status. cDNA microarray analysis is a useful screening technique for identifying novel genes not previously implicated in mammary tumorigenesis. These include genes described in other types of tumors and an even larger number of genes (N = 100) which have no previous known role in human neoplasia. Our experiments have unveiled 129 uncharacterized genes (ESTs/KIAAs), some of which showed very high expression ratios and may be important determinants of the biological and clinical behavior of HER2/*neu*-positive breast cancers. Some of these unknown genes that are down- or up-regulated in $p185^{erbB-2}$ overexpressing cells reside at chromosomal loci commonly deleted or amplified in human breast cancer and thus may prove to be important tumor suppressor

or oncogenes, respectively. Our data also demonstrate that the gene expression profiles of breast carcinoma cells *in vitro* and *in vivo* were not congruent, implying a major role for the mammary stroma.

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