Chromosome 8 BAC Array Comparative Genomic Hybridization and Expression Analysis Identify Amplification and Overexpression of *TRMT12* in Breast Cancer

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Genomic changes in chromosome 8 are commonly observed in breast cancer cell lines and tumors. To fine map such genomic changes by comparative genomic hybridization (CGH), a high resolution (100 kb) chromosome 8 array that can detect single copy changes was developed using Phi29 DNA polymerase amplified BAC (bacterial artificial chromosome) DNA. The BAC array CGH resolved the two known amplified regions (8q21 and 8q24) of a breast cancer cell line (SKBR3) into nine separate regions including six amplicons and three deleted regions, all of which were verified by Fluorescence in situ hybridization. The extent of the gain/loss for each region was validated by qPCR. CGH was performed with a total of 8 breast cancer cell lines, and common regions of genomic amplification/deletion were identified by segmentation analysis. A 1.2-Mb region (125.3–126.5 Mb) and a 1.0-Mb region (128.1–129.1 Mb) in 8q24 were amplified in 7/8 cell lines. A global expression analysis was performed to evaluate expression changes associated with genomic amplification/deletion: a novel gene, *TRMT12* (at 125.5 Mb), amplified in 7/8 cell lines, showed highest expression in these cell lines. Further analysis by RT-qPCR using RNA from 30 breast tumors showed that *TRMT12* was overexpressed >2 fold in 87% (26/30) of the tumors. *TRMT12* is a homologue of a yeast gene encoding a tRNA methyltransferase involved in the posttranscriptional modification of $tRNA^{Phe}$, and exploring the biological consequence of its altered expression, may reveal novel pathways in tumorigenesis. This article contains Supplementary Material available at http://www.interscience.wiley.com/jpages/1045-2257/suppmat. Published 2007 Wiley-Liss, Inc.[†]

INTRODUCTION

Genetic alterations are the basis for tumor initiation and progression, and the identification of such changes may reveal the underlying mechanism of cancer and will help identify molecular targets for therapeutic prevention. In the past, conventional cytogenetic techniques have been used to detect changes in copy number and assess structural rearrangements of individual chromosomes in tumor cells at a resolution of 10–20 Mb (Pinkel et al., 1986; Kallioniemi et al., 1992; Liyanage et al., 1996). Higher resolution can be achieved with bacterial artificial chromosome (BAC) arrayCGH where a library of clones is used instead of metaphase chromosomes. Each BAC clone contains a large insert of human genomic DNA, and earlier BAC arrays contained about 2,500 clones located throughout the genome with a resolution of about 1.4 Mb (Snijders et al., 2001; Albertson, 2003). Higher resolution can be achieved by increasing the number of clones to cover the chromosomal region. High-density BAC arrays for specific area of a chromosome have been constructed to identify genes in a region of interest (Albertson et al., 2000; Orsetti et al., 2004) and a high resolution BAC array has been constructed for the entire genome (Ishkanian et al., 2004).

Recently, BAC arrays and cDNA arrays of varying resolution have been used to study the genomic changes in breast cancer cell lines and/or tumor samples (Naylor et al., 2005; Fridlyand et al., 2006; Shadeo and Lam, 2006; Yao et al., 2006), along with expression changes (Yao et al.,

All of the data from the BAC and expression arrays are deposited at NCBI (http://www.ncbi.nlm.nih.gov/geo/) and the GEO accession number is GSE6567.

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2006). In each case, copy number changes were assessed across the genome and one of the most common regions of amplification was located on chromosome arm 8q. Two studies using arrays that consisted of either 2,462 BACs or 4,134 BACs distributed across the genome analyzed 62 breast tumor samples or a combination of 47 tumor and 18 cell lines, respectively, identified minimal amplified regions of 3.4 Mb and 8.8 Mb at 8q24.1 (Naylor et al., 2005; Fridlyand et al., 2006). This region was also identified in two other studies using higher resolution arrays consisting of either 14,160 cDNAs or the 32K SMRT BAC array (Shadeo and Lam, 2006; Yao et al., 2006). Another minimal amplified region identified in the higher resolution studies was located at 8q21-q22 (Shadeo and Lam, 2006; Yao et al., 2006). The third minimal amplified region identified in two out of four studies was located at 8q24.3 (Naylor et al., 2005; Shadeo and Lam, 2006). Yao et al. (2006) combined the results from two different platforms in an effort to correlate amplified regions with overexpressed genes in 30 tumor samples: 14,160 cDNA array for comparative genomic hybridization (CGH) and a serial analysis of gene expression (SAGE) library for expression. The lower resolution studies listed the *MYC* oncogene as a possible candidate in the 8q24 region; however, the study including expression data indicated that *MYC* is amplified but not overexpressed and suggest possible candidates genes could be *KIAA0196* or *ZHX1* (Yao et al., 2006). Variation among the results can be attributed to the differences in the resolution of the arrays and the different approaches taken to analyze the data. It is still not clear exactly which target genes on 8q are responsible for cancer progression.

In this report, we have combined high resolution (100 kb) chromosome 8 BAC arrayCGH data with a high-density oligonuclueotide array expression analysis from a panel of breast cancer cell lines to identify common regions of genomic change and correlate the expression of genes located within the consensus regions. BAC clones are designed to keep the insert DNA intact and reduce the potential for recombination by maintaining a low copy number of one or two copies per cell; however, this makes it difficult to generate sufficient amounts of DNA for the arrays. A high throughput amplification protocol using the Phi29 DNA polymerase reaction was developed to prepare enough DNA for the arrays. The Phi29 DNA polymerase reaction was selected because the error rate is 5×10^{-6}, 100 times less than that for Taq polymerase, and it has a 3′-5′ exonuclease proofreading capability (Dean et al., 2002). Prior methods used to amplify BAC DNA included linker-mediated PCR (Pfeifer et al., 1989) and degenerate oligonucleotide primed PCR (Telenius et al., 1992), which use Taq polymerase to replicate the DNA.

The utility of the chromosome 8 array was tested using a breast cancer cell line (SKBR3) with known genomic changes in 8q, and this high resolution analysis was extended to assess the genomic changes in seven more breast cancer cell lines. Expression profiles for these cell lines using high density (>48,000) oligonucleotide arrays were generated to correlate the genomic and expression changes. Finally, RT-qPCR was used to evaluate the expression of selected transcripts in 30 breast tumor samples.

MATERIALS AND METHODS

Cell Lines, Tissue RNA, and Primary Tumor RNA

Human mammary epithelial cells (HMEC) are primary cells derived from normal female donors and were purchased from Cambrex (Walkersville, MD). We chose HMEC as a reference because these cells are derived from normal female donors and are likely to represent "normal" gene expression in mammary epithelial cells. The chromosome XXX (GM04626), XXXXY (GM12013), and normal female (GM10959) lymphoblast cell lines were from the Coriell Cell Repositories (Camden, NJ). Normal male genomic DNA was purchased from Promega (Madison, WI). The breast cancer cell lines, derived from primary tumors (HCC38, HCC1419, BT474) and metastases (SKBR3, T-47D, MDA-MB415, MDA-MB-435S, MDA-MB-436), were obtained from ATCC (Manassas, VA) and maintained according to the recommendations. RNA samples from 30 malignant, invasive ductal breast carcinomas and matching normal RNA for 11 samples were provided by the Midwestern Division of the Cooperative Human Tissue Network, which is funded by the National Cancer Institute (http://www-chtn.ims.nci.nih.gov/regional.html). Total RNA from 10 different human tissues were purchased from Clontech (Mountain View, CA).

Construction of Chromosome 8 BAC array and arrayCGH

The 1463 BAC clones and DNA used to construct the chromosome 8 Human-BAC microarray were a subset of the Human "32K" BAC Re-Array library from the BACPAC Resources (Children's Hospital Oakland Research Institute, Oakland,

TABLE I. CGH and Expression Array Results and Their Validation by qPCR for Representive Genes from Regions of Gain/Loss on Chromosome 8 for SKBR3 Cell Line

					Genomic		Expression	
Region of gain/ loss	RP11 BAC clone	Nucleotide position	Gene symbol	Ref Seq ID	Chr 8 BAC aCGH cal ratio	qPCR fold difference	Oligo array cal ratio	RT-qPCR fold difference
1	RP11-133E24	71613225-71808853	LACTB2	NM_016027	4.94	6.02	14.65	9.65
1	RP11-400D10	75012726-75219332	TCEB1[a]	NM_005648	3.14	2.05	2.36	nd
1	RP11-48D4	77869827-78033463	ZFHX4	NM_030708	5.58	5.50	0.51	nd
2	RP11-795H2	81091081-81295886	TPD52[a]	NM_001025252	3.80	5.46	6.08	10.13
3	RP11-295O15	86862766-86899546	REXO1L1	NM_172239	3.08	3.30	2.08	10.48
3	RP11-529J9	91080056-91289372	DECR1	NM_001359	5.35	7.36	5.97	nd
4	RP11-272K10	95938245-96146446	TP53INP1	NM_033285	0.69	0.15	0.60	0.03
5	RP11-695B4	106496372-106684350	ZFPM2	NM_012082	0.54	0.23	0.28	nd
6	RP11-25B11	114122770-114300771	CSMD3	NM_198123	5.57	2.97	3.98	nd
7	RP11-466M24	116410840-116591978	TRPS1[a]	NM_014112	6.36	5.40	3.41	2.30
7	RP11-764P21	117641578-117867444	EIF3S3[a]	NM_003756	3.42	5.20	5.55	nd
7	RP11-683N15	120160000-120334811	MAL2	NM_052886	4.96	8.45	22.97	17.00
7	RP11-816L5	121438897-121636326	MRPL13	NM_014078	4.59	3.97	12.02	nd
8	RP11-237F24	128760341-128894279	MYC[a]	NM_002467	5.33	6.23	0.45	0.13
9	RP11-76C2	139637734-139788737	COL22A1	NM_152888	0.63	0.41	0.27	nd

Data expressed with reference to HMEC cell line.
[a]Genes previously known to be amplified in SKBR3; nd, no data.

CA, http://bacpac.chori.org/). An additional 48 chromosome X and 46 chromosome 21 BAC clones were also included in the array. The Phi29 DNA polymerase reaction was used to amplify 10 ng of BAC DNA templates, and the yields were approximately 30 μg. The details of DNA amplification, preparation of arrays, and hybridization methods are provided under Supplemental Methods (Supplementary material for this article can be found at http://www.interscience.wiley.com/jpages/1045-2257/suppmat).

FISH Analysis

Metaphase preparations from SKBR3 cultured cells, labeling, and hybridization were performed by standard techniques (Dutra et al., 1996) (see Supplemental Methods for details).

cDNA Preparation and Quantitative PCR

Total RNA was extracted using phenol-chloroform (TRIzol reagent, Invitrogen, Carlsbad, CA), treated with DNase, and the quality of the RNA was assessed using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). cDNA was prepared using the SuperScript First Strand Synthesis kit (Invitrogen).

The $2^{-\Delta\Delta CT}$ method was used to determine the relative gene expression (Livak and Schmittgen, 2001). The *GAPDH* gene was the internal control for all qPCR experiments. For genomic DNA qPCR, the iQ SYBR Green kit was used (Bio-Rad, Hercules, CA). For real-time quantitative PCR (RT-qPCR), TaqMan assays for specific genes were used following manufacturer's instructions (Applied Biosystems, Foster City, CA). The primer sequences used for qPCR, and the IDs for the TaqMan assays, are listed in the Supplemental Table 1.

Both genomic (qPCR) and expression (RT-qPCR) validation data are represented as fold changes relative to that in HMEC cell line.

Expression Analysis (Oligonucleotide Microarrays)

Expression arrays were generated from a 48,958 HEEBO (Human Exonic Evidence Based Oligonucleotide) 70mer-oligonucleotide set (Invitrogen). The oligos represent 26,121 Refseq (www.ncbi.nlm.nih.gov) and 24,048 Ensembl (www.ensembl.org) genes based on the Feb. 2005 version of the Ensembl database. Dried oligos were resuspended in 3× SSC solution and spotted on Corning Life Sciences epoxy coated slides. Detailed methodology is provided under Supplemental Methods.

Scanning and Image Analysis

A laser confocal scanner (Agilent Technologies) was used to scan the hybridized Cy3 and Cy5 probes on the slides. The fluorescent intensities at the target locations on the array were measured using the DEARRAY software (Khan et al., 1998). After background subtraction, average intensities at each oligo in the sample hybridization were di-

vided by the average intensity of the corresponding oligo in the reference hybridization. The ratios were normalized on the basis of the distribution of ratios of all targets on the array. Low quality measurements (i.e., copy number data with mean reference intensity less than 3× background fluorescent fluctuation, or spot size less than 20% of normal spot size) were excluded from the analysis and were treated as missing values.

arrayCGH Data Analysis

The genomic alignment of BAC clones was obtained from Children's Hospital Oakland Research Institute (Oakland, CA, http://bacpac.chori.org/), with option of bcgsc V2.0 (hg17 coordinates). ArrayCGH microarray data were normalized by a median centering method over all data points. ArrayCGH segmentation algorithm developed under MATLAB (Natick, MA) was applied to all arrays to extract segmented regions (as shown in Fig. 3B). The segmentation algorithm, a merge/divide based algorithm, is listed as follows: (1) divide each chromosome into 20-kb segments, (2) summarize each segment's statistics: mean (μ), SD (σ), etc, (3) merge two neighboring segments when their mean difference is less than $P = 0.05$ (z-test), (4) split segments when its SD (σ) is greater than $6\sigma_{\text{median}}$ (σ_{median} is derived from moving-window of 10 probes and the median of σ from all windows), and (5) continue the process until no segment can be merged to its neighbor or divided into multiple segments.

Consensus gain or loss regions were obtained from segmented arrayCGH data with an additional condition: at each probe, at least k samples shall have a copy number ratio of c. The results are illustrated in Figure 3B, where the black line is the reference line, and the first red line to the right-side of the baseline presents regions in which at least one ($k = 1$) sample above copy number ratio ($\log_2$-transformed) of $c = 0.3$. The second red line to the right side of baseline for $k = 2$. For lines representing $k \geq 5$, blue color is used instead of red color. Below the reference line, the first green line presents at least one ($k = 1$) sample below copy number ratio ($\log_2$-transformed) of -0.3. Note that within any of these regions, we do not assume that the same cell line stays above the copy-number status; only number of samples were counted, regardless which sample exceeds the limit. Here we chose a copy number ratio of 0.3 (or ratio $c = 1.23$). Although the threshold seems small, it was an averaged ratio over a segment, and it was sufficiently large relative to the noise level of the array. For a given threshold (e.g., 0.3), we calculated all BACs covered in the region (with ID, chromosome, start-position, end-position), oligo-probes (used for expression profiling, see following description) in the region (ID, chromosome, start-position only since the oligo-probe are fixed size in base-pair). For each oligo probe, we also provide significance statistics (t-statistic and P value) for overexpression and underexpression.

Expression Profiling Data Analysis

The expression profiling data were processed with the following steps:

1. Each array was normalized by the Lowess nonlinear normalization method.
2. For two replicated (dye-swapped) arrays, we used a weighted average method to form one averaged profile for each cell-line. The weighted average process was performed for each probe as follows

$$t_{\text{avg}} = \begin{cases} (w_1 t_1 + w_2 t_2)/(w_1 + w_2), & \text{if } w_1 + w_2 \geq 0.2 \\ (t_1 + t_2)/2, & \text{if } w_1 + w_2 < 0.2 \end{cases}$$

   where t_1 and t_2 are $\log_2$ ratios from two replicated hybridization, and w_1 and w_2 are two measurement qualities for two ratio measurements. The aggregated quality is $(w_1 + w_2)/2$.
3. To identify overexpressed and underexpressed genes, a t-test was performed probe-wise over eight samples. Quality weights were not used here, except at the final step if needed (both t-statistic and P value were reported).

Correlation of Copy Number and Expression Changes

To evaluate the relationship between gene expression level and DNA copy number alteration, the Pearson correlation coefficient was calculated (value from −1 to 1) from eight matching DNA and RNA samples (averaged over three DNA hybridizations, and two RNA hybridizations for each sample). Segmented DNA copy numbers were mapped to RNA probes (per gene). For eight samples, the correlation coefficient at 0.05 confidence level was 0.622.

All of the data from the BAC and expression arrays is deposited at GEO (accession number GSE6567) (http://www.ncbi.nlm.nih.gov/geo/).

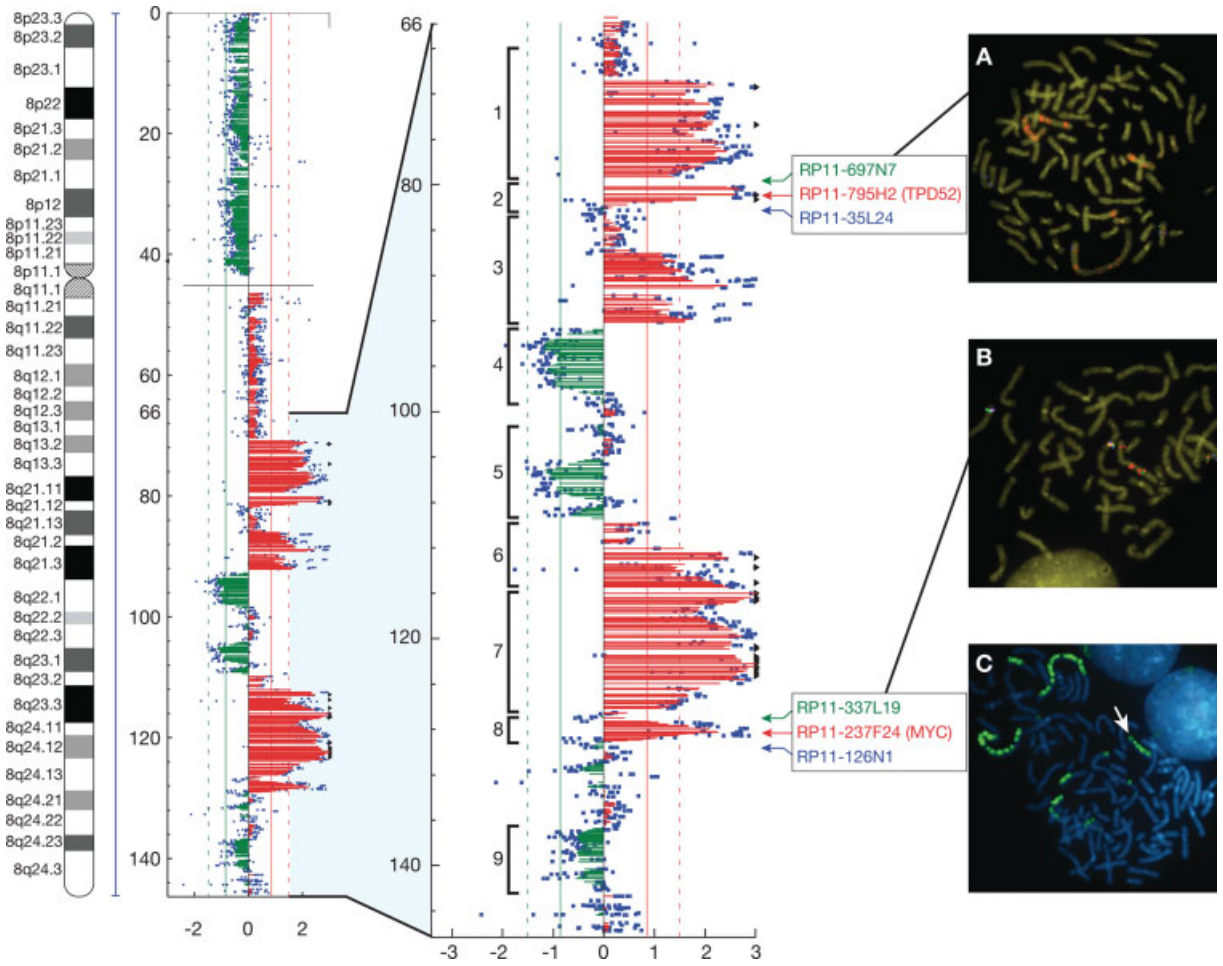

Figure 1. Display of a hybridization using differentially labeled genomic DNA from the breast cancer cell line, SKBR3 (labeled with Cy5, red), versus the reference, human mammary epithelial cells (HMEC) (labeled with Cy3, green). The normalized $\log_2$ ratios (Cy5/Cy3) of the BAC clones are shown. In the left chromosome 8 panel, the red bar represents a positive moving average ratio (1 Mb moving window), or copy number gain, and the green bar represents a negative moving average ratio (copy number loss). The corresponding normalized ratio (premoving averaging) were displayed as blue squares, with low quality ratio as light gray squares. Ratios beyond display range were marked at the side with a black triangle sign. The dashed red and blue lines demarcate 99% confidence intervals of raw ratio, and solid red and blue lines 99% confidence intervals of moving averaged ratio. The expanded view represents the q arm distal to 8q12.3 (66–146 Mb). Nine distinct regions of amplification or deletion were identified (bracketed regions, numbered 1 to 9). SKBR3 metaphase FISH pictures, A and B represent validation of amplification using BAC probes for amplicons #2 and #8 respectively; C is hybridization with chromosome 8 painting probe and the arrow points to the normal chromosome 8.

RESULTS

Preparation and Validation of the BAC Array

We generated a DNA array of 1,463 overlapping BAC clones creating a tiling path along chromosome 8 with a resolution of 100 kb. This clone set is part of the Human "32K" Re-Array library of BAC clones for the entire human genome (Krzywinski et al., 2004). Also included in the array was a set of 48 BAC clones from chromosome X. To evaluate if the array could detect copy number variation, DNA from a series of cell lines with an increasing number of X chromosomes and normal male reference genomic DNA (this pooled reference DNA was used only for X chromosome evaluations), were differentially labeled and hybridized. The BAC clones were printed in triplicate and each experiment was done in duplicate with dye swap. The average normalized fluorescent ratios were: XY versus XY (0.99 ± 0.01), XY versus XX (1.65 ± 0.13), XY versus XXX (2.29 ± 0.26), XY versus XXXXY (2.61 ± 0.32). The mean ratios increase with the corresponding increase in chromosome X copy number and single copy changes can be detected with this array.

Characterization of Genomic Gain/Loss in 8q of the SKBR3 Breast Cancer Cell Line

To test the utility of the chromosome 8 array in high resolution mapping, the SKBR3 breast cancer

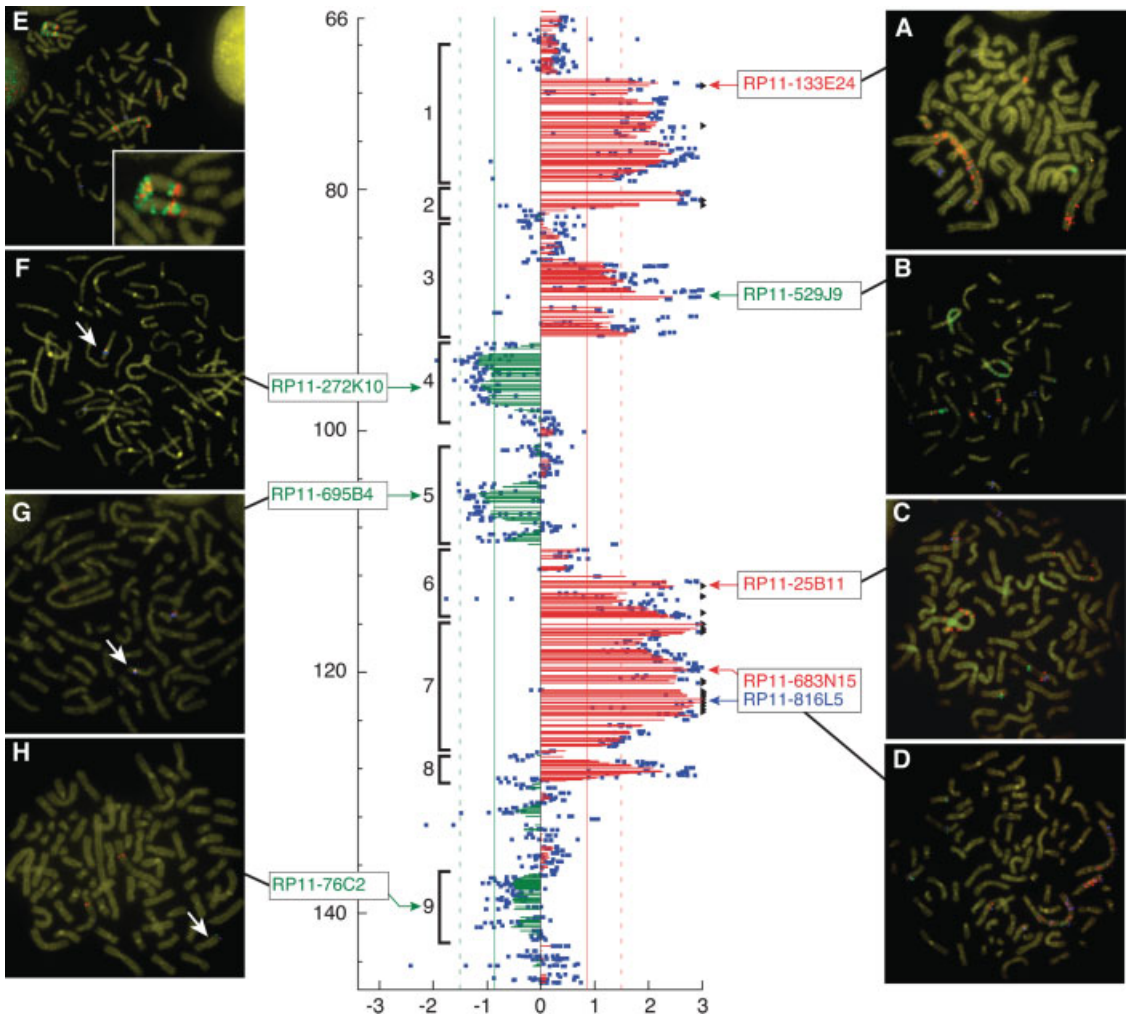

Figure 2. FISH analysis of BAC clones containing amplified or deleted genes in the SKBR3 cell line. A. Amplicon #1 (70.5–79.5 Mb) BAC clones for the gene *LACTB2* (RP11-133E24) and those flanking the peak, RP11-347D13 and RP11-697N7, B. Amplicon #3 (85.1–92.6 Mb) BAC clones for the gene *DECR1* (RP11-529J9) and those flanking the peak, RP11-35C05 and RP11-731D23, C. Amplicon #6 (110–115 Mb) BAC clones for the gene *CSMD3* (RP11-25B11) and those flanking the peak RP11-15N22 and RP11-442F21, D. Amplicon #7 (115.1–127.0 Mb) BAC clones for the genes *MAL2* (RP11-683N15) and *MRPL13* (RP11-816L5) in the 120–121 Mb region of the amplicon #7 are highly amplified: The BAC clone (RP11-2K18) located between peaks 6 and 7 (~115 Mb) is included as a reference, E. BAC clones from the 8q21 [RP11-603J12 (green) near *TPD52* gene] and 8q24 [RP11-237F24 (red) *MYC* gene] hybridized together along with a probe BAC clone RP11-455L12 (blue) from the intervening unamplified region, F. Deletion Peak #4 (93.1–98.9 Mb) BAC clone for the gene located *TP53INP1* (RP11-272K10) shows a signal only in the normal appearing chromosome 8 (arrow): The clones flanking the region RP11-731D23 and RP11-455L12 are also included, G. Deletion Peak #5 (103.8–108.7 Mb) BAC clones for the gene *ZFPM2* (RP11-695B4) and flanking clones, RP11-150P21 and RP11-15N22 are shown, H. Deletion Peak #9 (136.8–140.7 Mb) BAC clones for the gene *COL22A1* (RP11-76C2) and flanking clones, RP11-141J23 and RP11-557A20.

line was selected because it contains two regions of amplification located at 8q21 and 8q24 previously identified by lower resolution CGH (Rummukainen et al., 2001; Savinainen et al., 2004). Normal HMEC reference genomic DNA and SKBR3 genomic DNA were labeled and hybridized to the chromosome 8 array. Figure 1 displays the hybridization results, the $\log_2$ of the normalized fluorescent ratios for each BAC clone. The expanded view of Figure 1 represents the q arm distal to 8q12.3 (66–146 Mb). Nine distinct regions of amplification or deletion were identified (bracketed regions, numbered 1 to 9). Separate peaks were identified where flanking clones on either side of the peak had a calibrated ratio of approximately 1.0.

Twenty-five BAC clones were selected to delineate further the boundaries of these regions by metaphase fluorescence in situ hybridization (FISH) analysis (Figs. 1 and 2), and selected genes in each region were chosen for further validation of the extent of gain/loss by qPCR (Table 1). The increased resolution of the array more precisely defined the boundaries of the amplicons, and the

genes within them. The BAC clone RP11-795H2 containing the *TPD52* gene and the clones RP11-697N7 and RP11-35L24 that flank the amplicon #2 were used as probes in a multicolor FISH analysis with SKBR3 metaphase spreads, and multiple signals indicate that the clone containing the *TPD52* gene is amplified (Fig. 1A). The *TPD52* gene was identified in the peak of a 2.4 Mb amplicon (#2) that is located between a larger amplicon that contains the *TCEB1* gene and another previously undetected peak in the 8q21 region. Similarly, the BAC clone RP11-237F24 that contains the *MYC* gene and the clones RP11-337L19 and RP11-126N1 that flank amplicon #8 were used as probes (Fig. 1B). The *MYC* gene is located at the peak of a 2.9 Mb amplicon that is separate from a larger peak (amplicon #7) that contains the *TRPS1* and *EIF3S3* genes in the 8q24 region. A chromosome 8 painting probe (Vysis) was used to identify the chromosome 8 abnormalities (Fig. 1C). The SKBR3 cell line contains one normal appearing chromosome 8 (arrow), several regions of chromosome 8 that have translocated to other chromosomes, and one very large chromosome 8 with homogeneously staining regions. A tetraploid cell is shown and there are duplicate copies of the indicated changes. This result is consistent with previous reports describing the spectral karyotyping of SKBR3 (Davidson et al., 2000; Kytola et al., 2000). The chromosome 8 array detected known amplified genes in the 8q region of SKBR3 including *TCEB1*, *TPD52*, *TRPS1*, *EIF3S3*, and *MYC*, and the average normalized ratios for the BAC clones containing those specific genes and validation of those results by qPCR are in Table 1. The primers used for this and other qPCR reactions are listed in Supplemental Table 1.

The arrayCGH data revealed many novel regions of copy number gain/loss in SKBR3. The 8q21 region was resolved into three separate amplicons (#1–#3). The genes with the highest level of amplification in the 8q21 region include *LACTB2* and *ZFHX4* in amplicon #1, and *MMP16* and *DECR1* in amplicon #3. Validation using FISH analysis shows that the BAC clones RP11-133E24 containing the *LACTB2* gene and the clone RP11-529J9 containing the *DECR1* gene are amplified (Figs. 2A and 2B). The 8q24 region was resolved into three independent amplicons (#6–#8). Several highly amplified genes included *CSMD3* (amplicon #6), *MAL2* (amplicon #7), and *MRPL13* (amplicon #7), and the amplification of clones containing these genes are shown in Figures 2C and 2D. The highly amplified BAC clones from the 8q21 or 8q24 region are observed on the very long homogeneously staining region chromosome 8. Interestingly, when a BAC clone from the 8q21 region (RP11-603J12, near the *TPD52* gene) was hybridized together with a BAC clone from the 8q24 region (RP11-237F24, *MYC* gene), we observed that the signals from both clones were clustered together in three separate locations on the long chromosome 8 (Fig. 2E).

Three new regions of copy number loss were identified at 8q22, 8q23, and 8q24. Three BAC clones containing the specific genes located at the peaks of the deleted regions, *TP53INP1* (deletion peak #4), *ZFPM2* (deletion peak #5), and *COL22A1* (deletion peak #9), were chosen for validation by FISH (Figs. 2F–2H). The signal for each of the three BAC clones that contains a deleted gene (*TP53INP1*, *ZFPM2*, or *COL22A1*) is only seen in the normal appearing chromosome 8. Additional FISH experiments were done using a single colored probe for each of the three BAC clones that contain one of the deleted genes. About 20 cells were counted for each experiment, and on an average, one signal was seen for each of the deleted genes. Table 1 lists the average normalized calibrated ratios for the BAC clones containing those genes and the genomic DNA qPCR validation of those results.

Evaluation of Expression Changes in the SKBR3 Cell Line

To evaluate the global expression changes in SKBR3 RNA, an oligonucleotide array (HEEBO) consisting of over 48,958 oligonucleotides representing 26,121 genes was used. RNA from the same reference (HMEC) and SKBR3 were reverse transcribed into cDNA and differentially labeled and hybridized to the array. The expression level did not always correlate with genomic amplification. The *MYC* gene was highly amplified at the genomic level (avg cal ratio 5.33); however, it was underexpressed (avg cal ratio 0.45) and this is consistent with earlier observations (van Duin et al., 2005; Yao et al., 2006). Several of the highest amplified genes did correlate with overexpression and these include *LACTB2*, *DECR1*, *CSMD3*, *MAL2*, and *MRLP13* (Table 1). Also, the genes *TP53INP1*, *ZFPM2*, and *COL22A1* located in the deleted regions were underexpressed. Selected genes were validated by RT-qPCR using TaqMan assays specific for those genes and the results are included in Table 1. Expression changes in the chromosome 8 genes in SKBR3 are provided in Supplemental Table 2.

TABLE 2. List of Differentially Expressed Genes for Five Amplification (1A-5A) and Three Deletion (2D, 3D, 5D) Regions that Show Consistent Gain (or Loss) with at least 0.3 (or −0.3) $\log_2$ Ratio Respectively

Region (consensus)[a]	Probe ID[b]	Genomic start[c]	t-test *P*-value[d]	Average expr. Ratio	Corr, Coef. ρ[e]	RefSeq ID	Gene symbol	Gene title
Common gain region: regions with gain at least 0.3 ($\log_2$ ratio)								
1A (4 out of 8)	[g]**hHR027022**	**86,741,900**	**0.019**	**1.368**	**−0.113**	**NM_172239**	**REXO1L1**	**RNA exonuclease 1 homolog (S. cerevisiae)-like 1 (GOR)**
	hHC015399	87,145,338	0.000	0.552	0.544	NM_033126	PSKH2	protein serine kinase H2
	hHC008596	87,235,426	0.000	1.176	0.231	NM_152565	ATP6V0D2	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit
	hHC002210	87,295,634	0.001	1.267	0.381	NM_138817	SLC7A13	solute carrier family 7. (cationic amino acid transporter. y+ system)
2A (4 out of 8)	hHC010402	88,953,604	0.000	1.302	0.630	NM_152418	WDR21C	WD repeat domain 21C
	hHR008461	89,113,411	0.002	1.415	0.284		C8orf57	Chromosome 8 open reading frame 57
	hHR010557	89,795,999	0.000	1.278	−0.076		LOC441364	LOC441364
	hHR009844	90,838,398	0.001	1.322	0.531		LOC441367	LOC441367
	hHC007656	91,009,143	0.002	1.128	−0.368	NM_004337	C8orf1	chromosome 8 open reading frame 1
	hHE042637	91,728,217	0.000	0.804	−0.046		TMEM64	Transmembrane protein 64 splice variant
	hHC001965	**92,075,959**	**0.000**	**1.363**	**−0.137**	**NM_018710**	**TMEM55A**	**hypothetical protein DKFZp762O076**
	hHA033721	92,479,047	0.001	1.104	0.321	NM_052832	SLC26A7	solute carrier family 26, member 7
3A (7 out of 8)	**hHC014070**	**125,534,037**	**0.001**	**2.099**	**0.664**	**NM_017956**	**TRMT12**	**tRNA methyltranferase 12 homolog (S. cerevisiae)**
	hHC003793	**125,632,555**	**0.000**	**0.468**	**0.604**	**NM_014751**	**MTSS1**	**metastasis suppressor 1**
	hHR008962	126,027,490	0.000	1.207	−0.314		LOC157381	hypothetical protein LOC157381
	hHC008153	126,060,556	0.000	1.288	0.353	NM_152412	NM_152412	zinc finger protein 572
	[g]**hHC008255**	**126,448,252**	**0.018**	**1.304**	**0.540**	**NM_173685**	**NSMCE2**	**non-SMC element 2 homolog (MMS21, S. cerevisiae) (FLJ32440)**
4A (7 out of 8)	**hHC005993**	**126,822,635**	**0.000**	**0.289**	**0.045**	**NM_002467**	**MYC**	**v-myc myelocytomatosis viral oncogene homolog (avian)**
5A (5 out of 8)	hHC014980	140,699,682	0.001	1.227	0.615	NM_016601	KCNK9	potassium channel, subfamily K, member
	hHC009175	141,015,148	0.001	1.220	0.324	NM_020237	C8orf17	chromosome 8 open reading frame 17
	hHC016683	**141,611,167**	**0.014**	**1.479**		**NM_012154**	**EIF2C2**	**eukaryotic translation initiation factor 2C, 2**
Common loss region: regions with loss at least −0.3 ($\log_2$ ratio)[f]								
2D (3 out of 8)	[h]hHA036881	18,837,844	0.000	0.483	0.405	NM_015310	PSD3	pleckstrin and Sec7 domain containing 3
	hHA038317	22,115,316	0.000	0.489	0.464	NM_001199	BMPI	bone morphogenetic protein 1
	hHR019615	22,934,555	0.000	0.372	0.135	NM_003842	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b
	hHR028113	25,352,696	0.000	0.472	0.195	XM_497020	LOC441399	similar to Kctd9 protein
	hHC007225	26,284,106	0.000	0.455	0.500	NM_002717	PPP2R2A	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha
	hHC014229	**26,571,267**	**0.002**	**0.399**	**0.514**	**NM_001386**	**DPYSL2**	**dihydropyrimidinase-like 2**
	hHA036197	26,679,491	0.000	0.466	0.410		ADRA1A	adrenergic, alpha-1 A-, receptor
	hHA035406	29,262,020	0.000	0.456	0.366	NM_001394	DUSP4	dual specificity phosphatase 4
	[i]**hHC020586**	**32,741,061**	**0.000**	**0.182**	**−0.329**	**NM_004495**	**NRG1**	**neuregulln 1**
3D (1 out of 8)	hHC027525	97,916,493	0.000	0.118	0.353	NM_016134	PGCP	plasma glutamate carboxypeptidase
5D (2 out of 8)	**hHC010726**	**134,318,614**	**0.001**	**0.099**	**0.499**	**NM_006096**	**NDRG1**	**N-myc downstream regulated gene 1**

[a]Each region's genomic coverage is illustrated in Figure 3.
[b]Probe IDs in bold are genes validated by qPCR.
[c]Genomic start position mapped from UCSC g17 build.
[d]*P*-value were derived from t-test of 8 expression profiles. Probes were selected from Benjamini-Hochberg corrected ρ-value < 0.01.
[e]Pearson correlation coefficient, ρ, is computed gene-wise from 8 matching expression and aCGH arrays. The upper limit of 95% confidence interval for ρ is 0.622.
[f]Significant genes in loss regions were further selected with average expression ratio less than 0.5.
[g]Biological merits/representative for a given region.
[h]Mulitple significant probes for a same gene.
[i]Part of gene overlaps with the consensus region.

BAC ArrayCGH on an Additional Seven Breast Cancer Cell Lines

It was of interest to determine if the genomic and expression changes observed in SKBR3 were present in other breast cancer cell lines. Genomic DNA from seven breast cancer cell lines and the reference (HMEC) genomic DNA were differentially labeled and hybridized to the chromosome 8 array. The BAC array hybridizations were done in triplicate including one dye swap, and the average of the three experiments for each cell line is shown in Figure 3A. A segmentation analysis was performed on the averaged data for each cell line; an example segmentation plot is shown in Supplemental Figure 1. A consensus gain/loss plot of the eight cell lines (including SKBR3) was generated using a $\log_2$ threshold value of 0.3 or calibrated ratio of 1.2 (Fig. 3B). We chose a ratio of 0.3 based on the duplicated target variation (mean SD about 0.22 from triplicate printing location) and 0.72 ($\log_2$ ratio of 1.65) for one copy gain. The consensus plot shows a region of 8p deletion and two amplification peaks in 8q (8q21 and 8q24) as well as additional consensus regions. In the consensus plot, five regions of amplification (1A-5A) and five regions of deletion (1D-5D) were designated along the length of the chromosome 8.

Expression Analysis on Breast Cancer Cell Lines

To correlate the genomic changes with expression changes, RNA was extracted from all of the cell lines for global expression analysis in the same manner as used for the SKBR3 cell line. The oligonucleotide microarray hybridizations were performed in duplicate including a dye swap, and the display of the average of two hybridizations (after Lowess normalization) for each cell line for chromosome 8 is shown

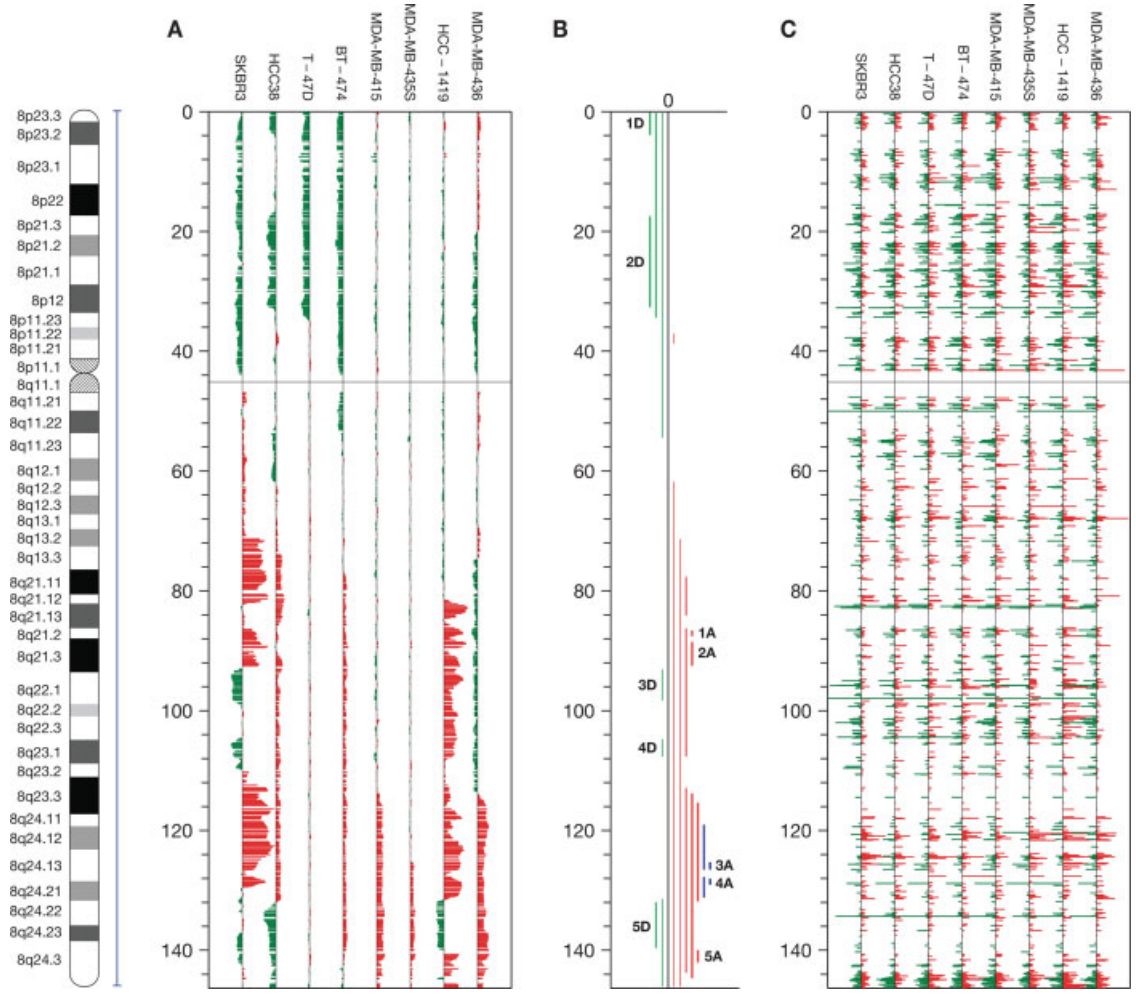

Figure 3. Genomic and expression changes in breast cancer cell lines. A. Display of the average of three chromosome 8 BAC arrayCGH experiments for each of the 8 breast cancer cell lines, B. Consensus plot of segmentation analysis generated using a threshold value of $\log_2$ 0.3 (cal ratio of 1.2), C. Display of the chromosome 8 expression analysis for each cell line using the oligo arrays (HEEBO). The red signals are positive and green signals are negative ratios, relative to the expression in HMEC cell line.

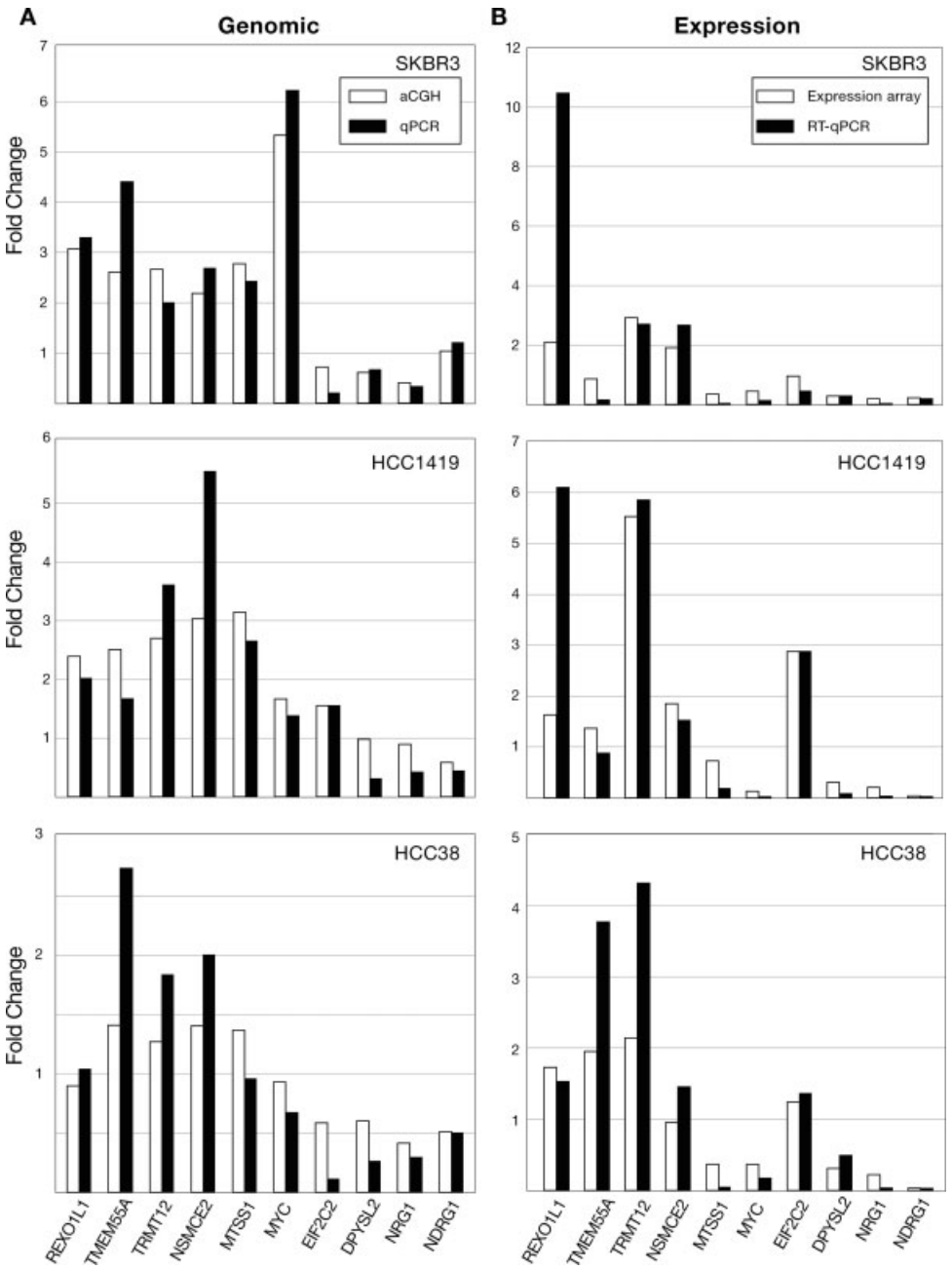

Figure 4. Chromosome 8 arrayCGH results (A) and expression analysis (B) for the SKBR3, HCC1419, and HCC38 breast cancer cell lines and qPCR validations for selected genes located in several of the consensus regions (Table 2, the genes indicated in bold). All of the data indicate the fold changes over the HMEC cell line. The data from the array (open bars) is compared with that of qPCR or RT-qPCR (filled bars) for the changes in DNA and RNA respectively.

in Figure 3C. A list of the genes with significant difference in expression in several consensus regions is included in Table 2. We also listed all significant differentially expressed genes in the eight cell lines in Supplemental Table 3 with Benjamini–Hochberg corrected *P* value less than 0.01. Several genes from the consensus regions were selected for verification by qPCR in three of the breast cancer cell lines (Fig. 4). The results from the genomic qPCR assay were consistent with the BAC array data for the 10 genes tested. The results of the RT-qPCR assay were consistent with the oligo microarray data for most of the genes.

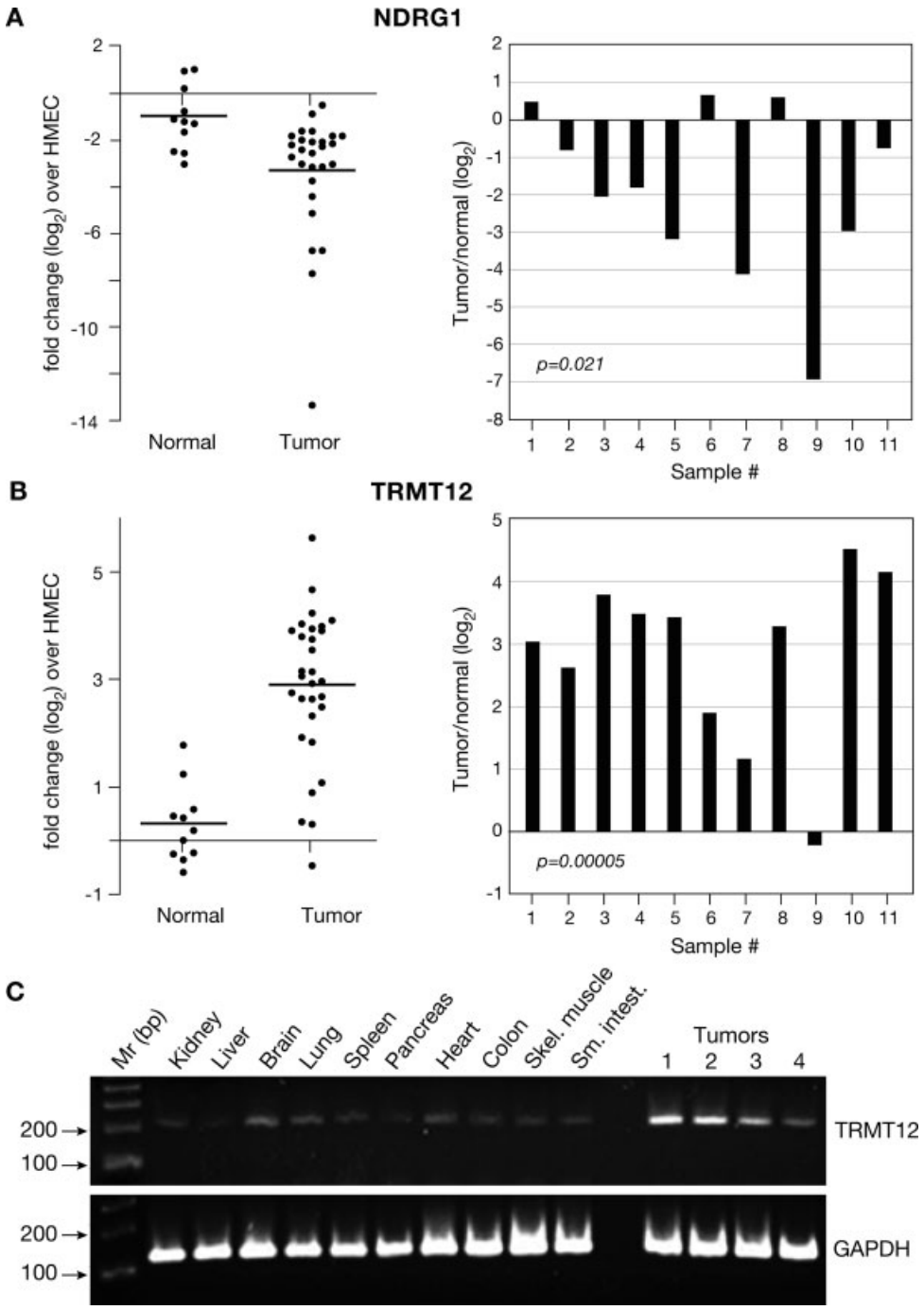

Figure 5. Expression of *NDRG1* (A) and *TRMT12* (B) by RT-qPCR in breast tumor RNA from 30 tumors and matching normal RNA from 11 tissues. Each dot represents the fold change (log2 values) in expression in normal and tumor samples over that in HMEC RNA (left panel). The line indicates the mean expression value over HMEC: for *NDRG1* (normal 0.76 fold, tumor 0.25 fold) and for *TRMT12* (normal 1.41 fold, tumor 10.89 fold). Each bar in the right panel represents the fold change (log_2values) in tumor versus normal for the 11 individual tissues. The *P* values indicate that the expression changes in tumor versus normal is significant. C) An ethidium bromide stained gel showing the results of RT-PCR for *TRMT12* expression in RNA from 10 different human tissues. RNA samples from 4 tumors are also included, and there is increased expression in tumors.

Expression of *NDRG1* and *TRMT12* in Breast Tumor Samples

The expression changes observed in the cell lines were further evaluated in breast tumor samples. RNA from 30 invasive ductal breast carcinomas and matching normal RNA for 11 of these tumors were used for expression analysis. RT-qPCR was performed to measure the expression of

a given gene in the tumor samples compared with the expression in the HMEC reference sample, and the fold change was calculated relative to the expression of the *GAPDH* housekeeping gene. First, we tested the *NDRG1* gene (134.3 Mb) reported to be underexpressed in various types of tumors including breast tumors (Bandyopadhyay et al., 2004; Ando et al., 2006). The *NDRG1* gene was located in 8q24, consensus region 5D (Fig. 3B, Table 2). RT-qPCR indicated that 93% of the tumors had a 0.5 or less fold change in the expression of *NDRG1* relative to its expression in HMEC. (Fig. 5A, left panel). The expression in 8 out of 11 tumors was lower compared with their matched normal RNA samples (Fig. 5A, right panel).

In the consensus amplified region 3A, seven out of the eight cell lines showed amplification at the genomic level in the region that extends from 125.3 Mb to 126.5 Mb. The *TRMT12* (tRNA methyltransferase12) gene in this region showed the highest expression ratio across all of the cell lines, and the correlation between the copy number and expression changes among these eight cell lines was significant (Table 2). *TRMT12* expression was greater than 2 fold increased in 87% of the tumors compared with its expression in HMEC (Fig. 5B, left panel). In all but one of the 11 matching pairs of samples, the tumors showed higher expression than the corresponding normal tissues, and the range in overexpression was 2- to 23-fold. To assess the expression of *TRMT12* in different tissues, cDNA from 10 different tissues were prepared and tested by RT-PCR. The results show that the *TRMT12* gene is ubiquitously expressed (Fig. 5C).

DISCUSSION

A high resolution BAC arrayCGH for chromosome 8 was developed as a tool to identify regions of genomic DNA amplification and deletion in tumor cells. By using a tiling path that extends along the length of the chromosome, we hoped to identify genomic changes that may have been missed with lower resolution arrays. A breast cancer cell line (SKBR3) with known genomic amplifications in chromosome 8 was used to test the array. The two amplicons identified by low resolution arrays were further resolved into six distinct amplicons and three novel regions of deletion in 8q. FISH analysis was used to validate the regions of copy number gain/loss. We observed that two discontinuous amplified regions that were far apart appear to be closer in the metaphase spread, thus a combination of high resolution arrayCGH and FISH techniques would be useful to study the organization of the amplified DNA and develop a better understanding of the mechanisms of genomic amplification/deletion in tumor cells. The analysis was expanded to generate high resolution profiles of copy number changes for a total of eight breast cancer cell lines. The increased resolution allowed detection of submegabase size amplicons consistently altered across the cell lines. Global expression analysis allowed us to evaluate the expression changes associated with common regions of gain/loss. Our analysis identified the genes known to be amplified in breast cancer cell lines including *MYC*, and genes known to be in the regions commonly deleted and underexpressed (*NRG1* and *NDRG1*). It is interesting to note that amplification of genes such as *MTSS1* and *MYC* was associated with decreased expression, implying that copy number changes need not result in overexpression. *MTSS1* (125.6 Mb) appears to be amplified in seven out of eight cell lines in our analysis, thus regulation of expression of the genes in the amplified regions is worth exploring. *MTSS1* has been reported to be regulated by methylation of its promoter (Utikal et al., 2006), and it remains to be seen whether a similar (or other) mechanism plays a role in reduced expression in breast cancer.

Several genes were amplified and overexpressed among almost all of the cell lines, and this was regardless of whether the cell line was derived from a primary tumor or from a metastatic site. Such genes may play a role in the underlying mechanism of cancer development. One of the genes amplified in 5 out of 8 cell lines, *C8orf17* (*MOST-1*) has been reported to be amplified/overexpressed in high grade cancers of prostate and breast (Tan et al., 2003), and thus our current findings support this observation. The gene that was amplified in almost all of the cell lines (7/8) and consistently overexpressed was *TRMT12*. We validated this observation in breast tumors and found that *TRMT12* is overexpressed in 10/11 tumors in comparison with their matched normal tissues. The *TRMT12* gene is homologous to the yeast *TRM12* gene (Kalhor et al., 2005). The yeast *TRM12*, also known as *TYW2*, is a tRNA methyltransferase that catalyzes the third step in the biochemical pathway to form wybutosine (yW). In phenylalanine tRNA ($tRNA^{Phe}$), the 37th residue is a guanosine that is posttranscriptionally modified to yW. The 37th position is located immediately 3′ to the anticodon, and the modification present in yW stabilizes the codon-anticodon interaction and functions to maintain the correct reading frame (Noma et al., 2006). Disruption of the yW pathway

can result in hypomodified $tRNA^{Phe}$ where yW is replaced with methyl-1-guanosine at the 37th position (m^1G37). More impotantly, when yW is replaced by m^1G37, −1 frame shifting can occur at a particular heptanucleotide sequence located in a given mRNA sequence (Carlson et al., 1999). More than 25 years ago, it was observed that $tRNA^{Phe}$ from mouse neuroblastoma cells and other rodent tumor cells contains methyl-1-guanosine at position 37 instead of yW, yet its significance in tumorigenesis is still unknown (Mushinski and Marini, 1979; Kuchino et al., 1982). A corollary to this is a recent report showing overexpression of *DUS2*, dihydrouridine synthase located on chromosome 16 (Kato et al., 2005), whose overexpression in lung cancers validates an observation made 30 years back that $tRNA^{Phe}$ from tumors have supernumerary dihydrouridine residues (Kuchino and Borek, 1978). It would be interesting to establish whether or not the human *TRMT12* has a similar function as yeast *TRM12*, and if it does, how altered expression of *TRMT12* affects breast cancer cells. It is possible that overexpression of the *TRMT12* gene could lead to a disruption of the yW biochemical pathway resulting in hypomodified $tRNA^{Phe}$ which could ultimately lead to translational errors in the cancer cells.

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