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Combining SSH and cDNA microarrays for rapid identification of differentially expressed genes

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ABSTRACT

Comparing patterns of gene expression in cell lines and tissues has important applications in a variety of biological systems. In this study we have examined whether the emerging technology of cDNA microarrays will allow a high throughput analysis of expression of cDNA clones generated by suppression subtractive hybridization (SSH). A set of cDNA clones including 332 SSH inserts amplified by PCR was arrayed using robotic printing. The cDNA arrays were hybridized with fluorescent labeled probes prepared from RNA from ER-positive (MCF7 and T47D) and ER-negative (MDA-MB-231 and HBL-100) breast cancer cell lines. Ten clones were identified that were over-expressed by at least a factor of five in the ER-positive cell lines. Northern blot analysis confirmed over-expression of these 10 cDNAs. Sequence analysis identified four of these clones as cytokeratin 19, GATA-3, CD24 and glutathione-S-transferase μ-3. Of the remaining six cDNA clones, four clones matched EST sequences from two different genes and two clones were novel sequences. Flow cytometry and immunofluorescence confirmed that CD24 protein was over-expressed in the ER-positive cell lines. We conclude that SSH and microarray technology can be successfully applied to identify differentially expressed genes. This approach allowed the identification of differentially expressed genes without the need to obtain previously cloned cDNAs.

INTRODUCTION

Methods to define patterns of gene expression have applications in a wide range of biological systems. One approach to understanding physiological mechanisms is to identify patterns of gene expression associated with varying physiological states. For example, investigators have been interested in examining differential gene expression in different cell types, in cells during different stages of differentiation, under various growth conditions and after introduction of a cloned gene such as a new transcription factor. Various methods to compare patterns of gene expression have been described, including differential hybridization screening (1), subtractive library construction (2), representational difference analysis (RDA) (3,4), differential display (5,6), conventional cDNA array hybridization (7) and serial analysis of gene expression (SAGE) (8,9). A technique called suppression subtractive hybridization (SSH) has recently been described which is based on technology similar to RDA but with modifications to normalize for mRNA abundance (10).

SSH has previously been used to compare patterns of gene expression in breast cancer cell lines discordant for ER expression (11,12). In a study to identify genes differentially expressed in ER-positive cells, RNA from ER-positive MCF7 cells was used as 'tester' and RNA from ER-negative MDA-MB-231 cells was used as 'driver' (11). Individual cDNA clones generated by SSH were used as probes on northern blot to identify differentially expressed genes. Of 48 clones randomly chosen for analysis, 42 had inserts that were used as probes for northern blot analysis and of these, 29 clones were confirmed to be differentially expressed in ER-positive MCF7 cells. Although these experiments were successful, screening for differentially expressed clones with northern blot is tedious and expensive. The emerging technology of cDNA microarray hybridization offers the possibility of providing a rapid, high throughput method to screen an SSH cDNA library for expression in a panel of cell lines.

Microarrays containing cDNA clones have been used to compare patterns of gene expression in which thousands of genes can be examined in a single hybridization (13–15). As usually applied, cloned cDNAs of known genes are placed on the array and these cDNAs are chosen to include the 3′ end of the mRNA. Fluorescent probes are prepared from mRNA using an oligo(dT) primer and reverse transcriptase. We hypothesized that cDNA microarray chips could be used to efficiently screen an SSH library. However, SSH generates small cDNA clones ranging from 50 to ~1000 bp. The cDNA clones generated by SSH can be from any part of the mRNA and are not 3′ selected. One possible advantage of this approach is that differentially expressed genes could be identified without the need to examine previously cloned genes. In addition, the SSH selection step increases the number of differentially expressed genes, thereby decreasing the examination of multiple 'house-keeping' genes. This study was designed to determine the feasibility of combing the technologies of SSH and cDNA microarrays as a means of obtaining differentially expressed genes.

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MATERIALS AND METHODS

Cell culture

MCF7, MDA-MB-231, T47D and HBL-100 cells were obtained from ATCC. All cell lines were maintained in DME supplemented with 10% fetal calf serum as previously described (16).

RNA isolation

Cell lines were grown to ~80–90% confluence prior to lysis for preparation of mRNA. Approximately 10^7 cells were used for each batch. Cells were lysed and mRNA isolated using the Fast Track 2.0 Kit (Invitrogen) according to the manufacturer's instructions. RNA yield was determined by measuring absorbency at 260 nm.

cDNA microarray

A cDNA microarray chip containing 332 SSH clones was made as previously described (13,14). Briefly, inserts in the pCR II vector (Clontech) were amplified with PCR using primers from sequences flanking the cloning site. PCR fragments were visualized on 1% agarose gel to ensure adequate PCR amplification prior to being robotically printed onto glass slides. One microgram of mRNA from each cell line was used to make cDNA probes for hybridization to the microarray. Probes were made by reverse transcription of mRNA in the presence of either Cy-5 or Cy-3 labeled dUTP (Amersham) using SuperScript II (Gibco-BRL) as previously described (15). Hybridizations and subsequent scanning, visualization and quantitation were performed as previously described (13,14).

Northern blot assay

Different mRNA isolates from the same cell line were pooled to minimize variability from batch to batch. One microgram of each mRNA was electrophoresed on a standard 1.2% formamide agarose gel prior to mRNA transfer to Nytran using the Turboblot System (Schleicher and Schuell). Probes were made from the previously isolated SSH fragments using a random primed DNA labeling kit (Boehringer Mannheim) in the presence of [α-^{32}P]dCTP (Amersham). Blots were hybridized and washed as previously described. All autoradiographs shown are following an overnight exposure to film.

DNA sequencing

Each clone from SSH was subcloned into the vector pCR II that has SP6 and T7 promoter sequences flanking the cloning site. Dideoxynucleotide sequencing was performed using [α-^{35}S]dATP and the Sequenase protocol (USB) with primers that annealed to the SP6 and T7 sequences. Sequencing reaction products were subsequently electrophoresed on 4% acrylamide gels and exposed to film. Homology searches were performed using the BLAST program.

Flow cytometry

Cell lines were grown to confluence prior to harvest for flow cytometry. Cell monolayers were washed twice with phosphate-buffered saline (PBS) and were detached with 0.5 mM EDTA in PBS. Cells were collected by centrifugation and washed once with PBS. Cells were labeled with the CD24 monoclonal antibody ML5 (Pharmingen) at a concentration of 10 mg/ml for 30 min, followed by three washes in PBS. The secondary antibody was an FITC-conjugated goat anti-mouse IgG (Sigma) diluted 1/100, followed by two washes in PBS. Cells were analyzed on a FACScan (Becton Dickinson) using LYSIS software and were gated to include only whole cells.

Immunofluorescence

Cells were grown to confluence on Falcon CultureSlides (Becton Dickinson). Cells were fixed with 4% paraformaldehyde for 10 min, followed by permeabilization with methanol for 2 min. The ML5 monoclonal antibody (Pharmingen) was used as the primary antibody followed by detection with an FITC-conjugated goat anti-mouse IgG (Sigma). Cells were washed three times with PBS following each antibody. Cells were imaged with Zeiss Axioskop (Carl Zeiss) and images were captured using a Zeiss digital camera using Adobe Photoshop.

RESULTS

cDNA microarrays

We have previously described the use of SSH to generate cDNA clones of genes differentially expressed in ER-positive (11) and ER-negative (12) breast cancer cell lines. However, our previous experiments relied on screening cDNA clones with northern blot analysis, which severely restricted the number of clones that could be analyzed. In this study we sought to use cDNA microarray technology to perform a high throughput analysis of cDNA clones with the intent of identifying genes expressed in association with the ER phenotype in breast cancer cell lines.

A set of 332 clones was generated by SSH in which ER-positive MCF7 mRNA was used as 'tester' and ER-negative MDA-MB-231 cDNA was used as 'driver'. This set of cDNA clones should be enriched for genes over-expressed in MCF7 cells compared to MDA-MB-231 cells. The cDNA inserts of each clone were amplified with PCR and spotted on a microarray using robotic printing. Multiple housekeeping genes and randomly selected cDNAs were also printed on the same array to serve as internal controls. The microarrays were subsequently hybridized with cDNA probes labeled with fluorochromes. Probes were prepared from two ER-positive cell lines (MCF7 and T47D) and two ER-negative cell lines (MDA-MB-231 and HBL-100). An example of one such hybridization is shown in Figure 1 in which the MCF7 cDNA probe is labeled with Cy-5 fluorochrome (red) and MDA-MB-231 is labeled with Cy-3 fluorochrome (green). Red and green fluorescence indicates greater relative expression in MCF7 and MDA-MB-231, respectively; yellow fluorescence indicates equal expression. The portions of the array imprinted with SSH generated clones are outlined by boxes. As noted in Figure 1, nearly every red hybridizing clone indicating over-expression in MCF7 was generated by SSH. Randomly selected human cDNAs are unlikely to demonstrate relative over-expression in MCF7 cells.

Three separate mRNA isolations were obtained from each cell line and pair-wise hybridizations were performed in which each ER-positive cell line (MCF7 and T47D) was compared to each ER-negative cell line (MDA-MB-231 and HBL-100). The hybridizations were scanned and quantitative information was obtained for each hybridization. In previous studies, northern blot analysis was performed for clones 1–48 of which 42 had cDNA inserts (11). In these northern blots, mRNA from MCF7 and

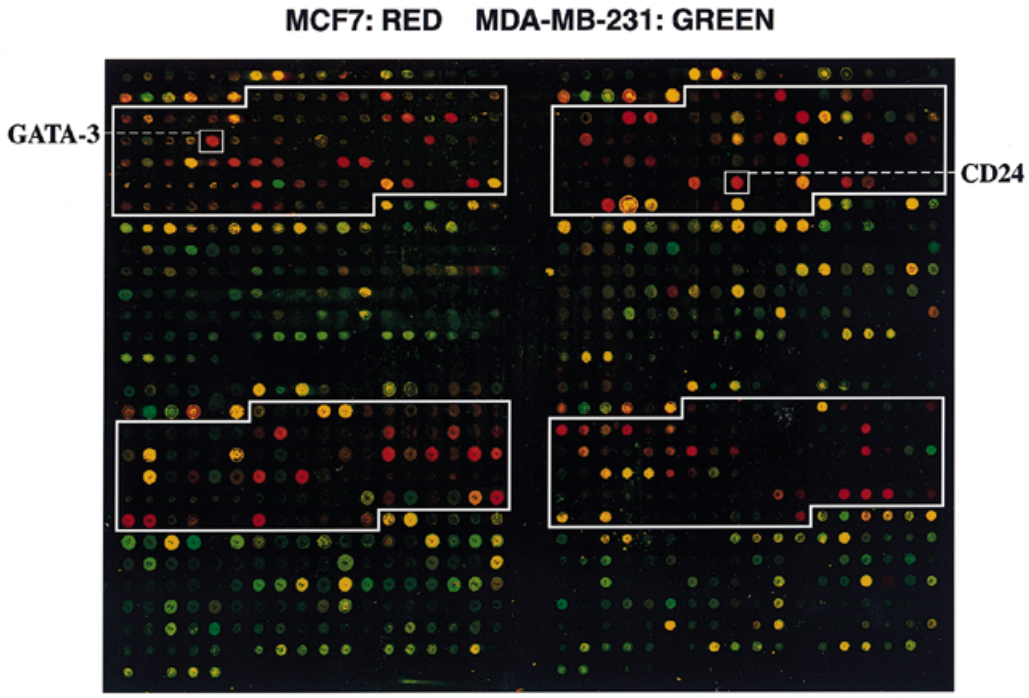

Figure 1. Microarray hybridized with MCF7 and MDA-MB-231. A cDNA microarray was hybridized with fluorescent labeled probes prepared from MCF7 (red) and MDA-MB-231 (green) mRNA. Red color indicates relative over-expression in MCF7 and green color indicates relative over-expression in MDA-MB-231. Yellow color indicates equal expression in both cell lines. The portions of the array imprinted with clones generated by SSH are outlined with boxes. The locations of GATA-3 and CD24 are also indicated.

MDA-MB-231 were hybridized to each cloned insert, and a quantitative value for differential expression was obtained using densitometry. By northern blot analysis, 29 cDNA clones demonstrated differential expression in MCF7 by a factor of five or more. Using the same criteria of differential expression by a factor of five comparing MCF7 and MDA-MB-231, five of the 42 clones were determined to be over-expressed in MCF7 based on microarray data. These data were also analyzed using differential expression ratios of four, three and two. At a ratio of four, six clones were identified by microarray, all of which were also determined to be differentially expressed by northern blot. Lowering the level of differential expression to a factor of three or more, 14 clones would be considered differentially expressed. Thirteen of these 14 clones were also identified as differentially expressed by northern blot. At a ratio of two, 21 clones were identified with microarray of which 17 were differentially expressed by northern blot.

The data for hybridization comparisons for the entire set of 332 cDNA clones were also analyzed. At a differential ratio of three, 76 of the 332 cDNA clones (23%) were considered differentially expressed based on the two-cell line comparison (MCF7 versus MDA-MB-231). The hybridization comparisons were considered for all four cell lines with the requirement that a clone be differentially over-expressed in the two ER-positive cell lines compared to the two ER-negative cell lines. At a differential expression ratio of three, 22 cDNA clones (7%) were differentially expressed. When the differential ratio was increased to a factor of five or more, 10 cDNA clones (3%) were considered differentially expressed. These 10 cDNA clones were subsequently analyzed by northern blot.

Northern blot

Northern blot analysis was used to confirm the results of microarray hybridizations. Clones 87, 99, 129, 154, 173, 189, 191, 198, 219 and 236 all demonstrated consistent over-expression in ER-positive cell lines by a factor of five or more. Figure 2 shows the results of northern blots probed with each cloned insert. Northern blots hybridized with probes for ER and actin are shown as controls. As seen in Figure 2, each clone hybridizes to an mRNA with over-expression in ER-positive cell lines (MCF7 and T47D) compared to ER-negative cell lines (MDA-MB-231 and HBL-100). These results confirm that the microarray hybridization at a ratio of differential expression of a factor of five or more identified genes over-expressed in ER-positive cells.

Each of these cloned inserts was sequenced and the sequence was compared to known sequences in the GenBank database. These results are summarized in Table 1. Clones 99, 154 and 191 were identified as expressed sequence tags (ESTs) that map to a single cDNA encoding a protein with unknown function (accession no. AA442829). Clone 236 was identified to another EST (accession no. T08550). Clones 173 and 189 did not match a previously reported sequence; however, the size of the mRNA and the pattern of hybridization makes it likely that these two clones are from the same gene.

Among the known genes, none had previously been reported to be differentially expressed in ER-positive compared to ER-negative breast cancer cell lines. Clones 87, 129, 198 and 219 were from the genes cytokeratin 19, GATA-3, CD24 and glutathione-S-transferase μ-3, respectively. CD24 is a membrane antigen previously reported to be involved in differentiation of hemato-

poietic and neural cells (17–19). One previous report examined CD24 expression in a group of cancer cell lines one of which was derived from a breast cancer (20). However, no association with ER expression has been reported. Further experiments were performed to determine if CD24 protein was expressed in these breast cancer cell lines.

Table 1. Differentially expressed clones

Clone	Sequence identity	mRNA size (kb)	Fold	Over-expression
			N[a]	M[a]
87	Cytokeratin 19	1.4	5	13
99	EST[b]	1.0	5	17
129	GATA-3	2.0	8	14
154	EST[b]	1.0	4	14
173	unknown[c]	0.9	16	13
189	unknown[c]	0.9	9	53
191	EST[b]	1.0	4	11
198	CD24	2.0	6	19
219	Glutathione-S-transferase μ-3	0.8	8	15
236	EST	1.5	10	24

[a]N, northern blot; M, microarray. Fold over-expression for northern blots based upon densitometry from MCF7 mRNA compared to MDA-MB-231 mRNA using β-actin levels for normalization. Fold over-expression for microarray data based upon ratio of fluorescence for MCF7 probe compared to MDA-MB-231 probe.

[b]Clones 99, 154 and 191 were each identical to separate ESTs that have been found to map to a single expressed cDNA.

[c]The sequences of clones 173 and 189 did not match any other sequence in the GenBank database. However, based upon the similarity of their mobility in RNA gels and banding patterns, we believe these clones are separate isolates of the identical expressed mRNA.

Characterization of CD24 expression

Flow cytometry utilizing a commercially available antibody to CD24 was performed on the ER-positive and ER-negative cell lines. As seen in Figure 3, CD24 is expressed in the ER-positive cell lines MCF7 and T47D. As expected, there is no CD24 expression detected in the ER-negative cell line MDA-MB-231. There was a slight shift in fluorescence noted in HBL-100 even though no CD24 mRNA was detected by northern blot.

Indirect immunofluorescence was used to further characterize the expression of CD24. As expected for a cell surface molecule, MCF7 and T47D cells demonstrated strong fluorescence on the cell membrane while no fluorescence was detected in MDA-MB-231 cells or in the negative controls (Fig. 4). HBL-100 cells had a unique fluorescence pattern where signal was detected in a punctate pattern that was not present in the negative control (Fig. 4d and h). The immunofluorescence was not clearly associated with the cell surface or with any specific subcellular organelle. HBL-100 cells are transformed normal mammary epithelial cells and were not derived from a primary breast tumor. There are a number of possibilities to explain this finding; however, in light of this unusual pattern of fluorescence and the fact that no CD24 mRNA was detected, this result is likely due to cross reactivity with an antigen expressed in HBL-100. These data confirm that CD24 protein is over-expressed in the ER-positive cell lines compared to the ER-negative lines.

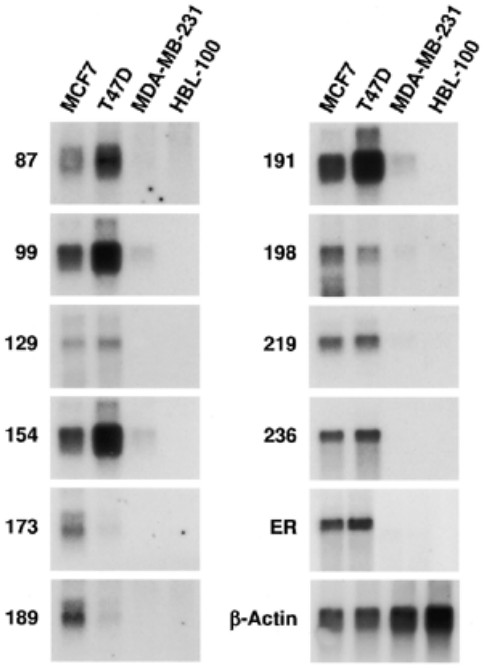

Figure 2. Northern blots of selected clones. Northern blots were performed with RNA from cell lines as indicated. Each blot was probed with inserts from the SSH clones as numbered, ER or actin. These results demonstrate over-expression of each of the 10 cDNA clones in MCF7 and T47D compared to MDA-MB-231 and HBL-100.

DISCUSSION

The recently developed technique of SSH has allowed the isolation of cDNA clones differentially expressed comparing two RNA populations (10). We have previously used SSH to identify genes expressed in breast cancer cells in association with ER expression (11,12). However, our previous experience screening SSH generated clones by northern blot analysis proved to be tedious and inefficient. In this study we have successfully combined the technologies of SSH and cDNA microarrays to develop a high throughput screening procedure to identify genes differentially expressed in association with the ER phenotype.

The use of cDNA arrays for genetic screening has the obvious advantage of allowing the analysis of multiple clones with a single hybridization. However, conventional arrays necessitate a manual comparison of two hybridizations to identify differentially expressed genes. Microarray technology offers several advantages over conventional cDNA arrays. First, the use of different fluorescent tags allows a direct comparison of the relative mRNA abundance in two RNA populations. Second, the small size of the array allows hybridizations in decreased volumes with less probe than conventional technology. Computerized scanning of the array provides a quantitative value of the relative abundance comparing two cell types (based on color). Screening clones generated by SSH resulted in a smaller array enriched for differentially expressed genes. In addition, the use of the array to screen cDNA clones generated by SSH allowed the identification

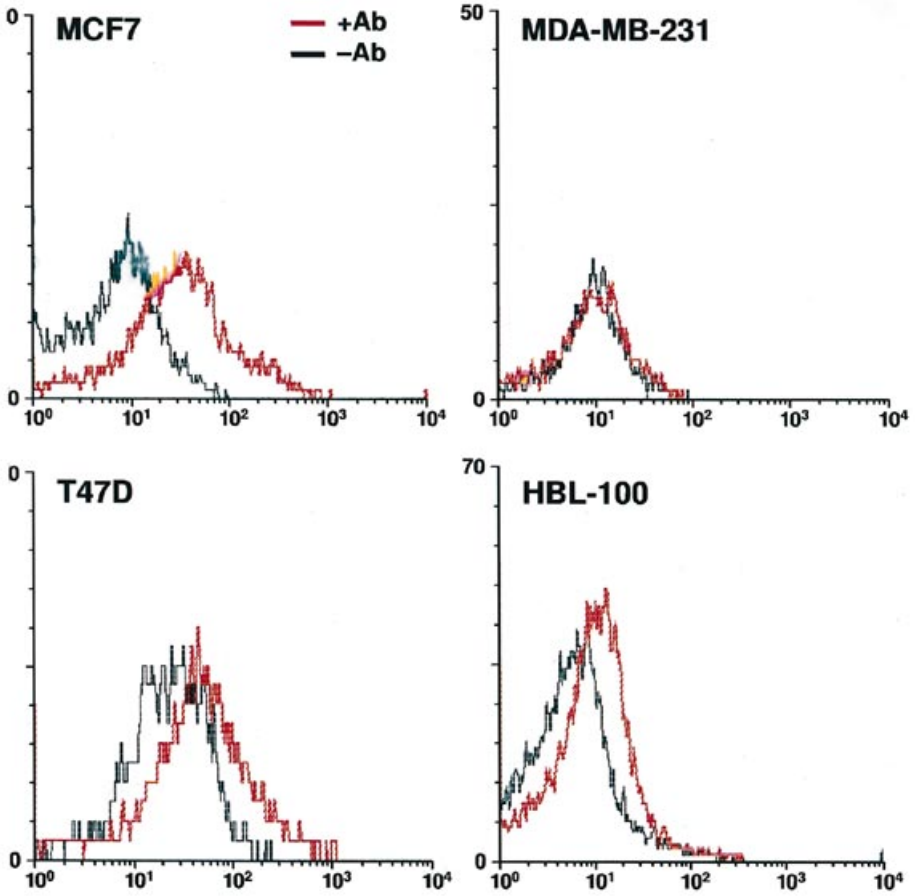

Figure 3. Flow cytometry with CD24 antibody. Flow cytometry was used to examine CD24 protein expression in the four cell lines as indicated. The two curves represent fluorescence with CD24 antibody compared to control (secondary antibody only). A shift in the curve indicates expression in MCF7 and T47D. The slight shift noted in HBL-100 is likely due to cross reactivity (see text for details).

of previously unknown genes—a finding that would not have been possible using conventional arrays of previously cloned genes.

Microarrays may have some disadvantages compared to the more tedious technique of northern blot screening. The SSH clones placed on the array included 48 cDNA clones that had previously been screened by northern blot analysis. Microarray screening failed to identify some genes that were known to be differentially expressed by northern blot. Of particular interest were clones 19 and 35, both of which were derived from cytokeratin 18. By northern blot, both clones demonstrated differential hybridization in MCF7 by a factor of 7–8-fold (11). Microarray hybridization correctly identified clone 19 but not 35. Clone 19 contains a 292 bp insert from the cytokeratin 18 cDNA positions 499–790 and clone 35 contains a 95 bp insert from nucleotides 791–885. This result suggests that smaller inserts may provide less reliable information. Clones from less abundant mRNAs may also fall within the 'noise' of the hybridization signals. For example, microarrays failed to detect clones 6 and 8 which had previously been reported to be differentially expressed by northern blot (11). However, these mRNAs were of low abundance and clones 6 and 8 had insert sizes of 111 and 358 bp, respectively. Therefore, cDNA insert size and mRNA abundance may be factors in reliably identifying differentially expressed genes. In addition, microarray technology, as previously reported, utilized cloned cDNAs that include the 3′ end of the cDNA with probes prepared using oligo(dT) primer. Short SSH clones from the 5′ end of cDNAs are less likely to give reliable hybridization signals than full-length cDNAs. Improvements in hybridization conditions to improve signal to noise ratio and refinements in statistical analysis of the data will improve the ability to consistently identify low copy number mRNAs and clones with small inserts. The use of cDNA probes generated with random primed oligonucleotides rather than oligo dT would also likely improve detection of clones.

Despite possible limitations, the ability to screen large numbers of clones offers advantages over northern blot screening and provides information of biological interest. The 10 clones identified with over-expression in the ER-positive compared to ER-negative cell lines included four previously identified genes: cytokeratin 19, glutathione-S-transferase μ-3, GATA-3 and CD24. Cytokeratin 8 and 18 have previously been reported to have an association with the ER-positive phenotype (21). Cytokeratin 19 has recently been reported to be co-expressed with cytokeratins 8 and 18 and appears to be associated with tumor

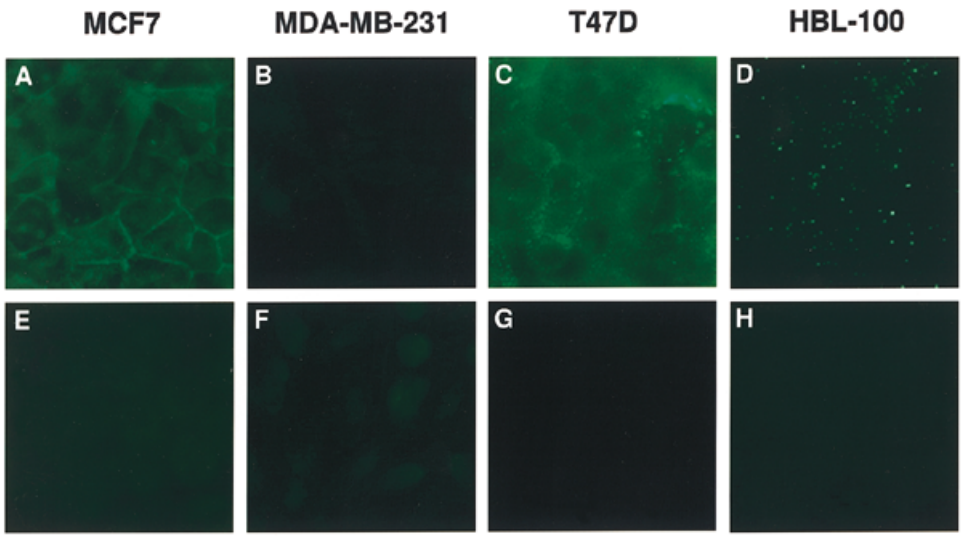

Figure 4. Immunofluorescence with CD24 antibody. Immunofluorescence with CD24 antibody (**A**–**D**) and negative control (**E**–**H**) with secondary antibody only. Cell lines are indicated. Membrane fluorescence is noted in MCF7 and T47D. MDA-MB-231 do not demonstrate expression. HBL-100 demonstrate a punctate staining (D), indicating cross reactivity with unknown antigen (see text for details).

grade (21,22). The co-expression of these cytokeratins may reflect common mechanisms of transcriptional regulation. Studies of glutathione-S-transferase expression in breast cancer indicate that expression may be important for development of drug resistance (23,24). Genetic studies have also linked inheritance of certain polymorphisms to breast cancer susceptibility (25,26). We have recently demonstrated a striking association between GATA-3 and ER expression in breast cancer cell lines and primary breast tumors (27). GATA-3 is highly expressed in T lymphoid cells and is believed to play a role in T-cell development and expression of Th2 associated genes (28,29) and the α-chain (30) and δ-chain (31,32) of the T-cell receptor. Studies in MCF7 cells grown in the presence or absence of estradiol indicated that GATA-3 is not ER responsive (27). It is interesting that CD24 was identified in this study since this gene is also a lymphocyte differentiation antigen (17–19). In addition, CD24 is normally expressed in neurons (33) and keratinocytes (34). CD24 is an extracellular glycoprotein that is attached to the cell membrane via a glycosyl phosphatidylinositol anchor and is associated with intracellular tyrosine kinases (35). Binding of CD24 with monoclonal antibodies can stimulate tyrosine phosphorylation and increase intracellular calcium levels (36). CD24 has been reported to be associated with the tyrosine kinase c-fgr in small cell lung cancer and lyn in an erythroleukemia cell line (37). In hepatocellular carcinoma, CD24 over-expression correlates with p53 mutation and a poorly differentiated phenotype (38). The finding of CD24 expression in the ER-positive cell lines indicates that the pattern of expression of this gene may correlate with ER expression in primary breast cancers.

Our results demonstrate the novel application of SSH and cDNA microarrays for identifying differentially expressed genes. Despite the limitations of cell line models, this approach has provided interesting results that can be translated into an examination of primary breast cancers. The next step will be to extend these techniques to characterize patterns of gene expression in primary breast cancers. The use of microarrays should allow a rapid analysis of gene expression in a panel of primary cancers. A recently published report similarly demonstrated the use of RDA and cDNA microarray hybridization with RNA derived from two Ewing's sarcomas (39). We anticipate that in the future, the genetic pattern of gene expression will serve to refine histological information to provide a better characterization of breast cancer phenotype.

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