Technical Advance

Expression Profiling of Ductal Carcinoma *in Situ* by Laser Capture Microdissection and High-Density Oligonucleotide Arrays

Veronica Luzzi, Victoria Holtschlag, and
Mark A. Watson

*From the Department of Pathology and Immunology and the
Alvin J. Siteman Cancer Center, Washington University School of
Medicine, St. Louis, Missouri*

Gene expression profiling through the use of nucleic acid arrays is a powerful method for the molecular classification of human neoplasms. Laser capture microdissection is an equally useful technique to selectively isolate defined cell populations from heterogeneous histological tissue sections. In this report, we demonstrate how a modest use of laser capture microdissection is sufficient to isolate nanogram quantities of high-quality RNA. Together with the use of several internal standards and microcapillary electrophoresis of input RNA, two rounds of linear molecular amplification have been used to generate sufficient quantities of labeled target for hybridization to high-density oligonucleotide expression arrays. Results demonstrate that the technique is reproducible, generates only modest biasing of the original transcript population, and is comparable to the sensitivity achieved with standard methodology. Using this approach, we have compared the expression profiles of nonmalignant human breast epithelium and adjacent ductal carcinoma *in situ* lesions from breast cancer patients. Several genes, previously implicated in human breast cancer progression, demonstrate differential expression among the microdissected cell populations. (Am J Pathol 2001, 158:2005–2010)

It is increasingly clear that gene expression profiling through the use of nucleic acid array technology will be a powerful approach toward the molecular classification of cancer.^{1–4} It may be argued that examining the expression profile of the complete cellular microenvironment of a solid tumor provides the best biological and clinical perspective of the disease process. In other cases, however, information regarding cell-specific expression sig-

natures may be lost by grossly sampling the heterogeneous cell population that is usually characteristic of solid neoplasms, particularly in such diseases as breast and prostate adenocarcinoma. Laser capture microdissection (LCM) is an effective tool to isolate defined populations of cells from heterogeneous tissue sections.⁵ However, except in a few cases,⁶ the resulting quantity of RNA is insufficient for nucleic acid array-based studies. More recently, a number of techniques have been presented for the amplification of small quantities of total RNA either directly⁷ or from LCM isolated tissue.^{8–10}

In this report, we demonstrate how a modest effort using LCM can yield nanogram quantities of high-quality RNA. To increase standardization across clinical specimens, isolated RNA is quantitated and qualitatively assessed using microcapillary electrophoresis, and staggered internal control transcripts of defined copy number are added to the sample before first-strand cDNA synthesis. Using two rounds of linear amplification, a sufficient quantity of labeled target is generated to hybridize to high-density oligonucleotide arrays (Affymetrix GeneChips; Affymetrix, Santa Clara, CA). Based on expression levels of internal controls, we demonstrate that this procedure is reproducible and results in an analytical sensitivity and precision comparable to standard methodologies that routine use 200 to 1000 times greater input RNA. Using this approach, expression profiling was performed on RNA isolated from nonmalignant mammary ductal epithelial cells and adjacent regions of ductal carcinoma *in situ* from three patients with breast cancer. Several genes previously implicated in breast cancer progression, including the breast tumor marker PS2,¹¹ were differentially expressed in the microdissected cell populations. This study demonstrates the feasibility of

Supported in part by grant no. 016-99 from the Mary Kay Ash Charitable Foundation.

Accepted for publication March 2, 2001.

Address reprint requests to Mark A. Watson, M.D., Ph.D., Dept. of Pathology and Immunology/Box 8118, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. E-mail: watsonm@labmed.wustl.edu.

applying LCM and nucleic acid expression array technology to histologically complex clinical specimens for the purposes of molecularly dissecting human neoplasia.

Materials and Methods

Tissue Processing and LCM

All human tissue specimens were obtained from the Alvin J. Siteman Cancer Center Tissue Procurement Core facility using an institutional review board-approved protocol. A detailed protocol for tissue preparation and LCM is available at <http://pathbox.wustl.edu/~tisscore/protocols.htm>. Embedded frozen-tissue specimens were cut at 6 μm thickness and immediately fixed in 70% ethanol. Cut sections were stored at 4°C in 70% ethanol for up to 72 hours before staining and dissection. We have previously shown that storage of sections in this manner maintains reasonable tissue morphology, facilitates transfer of tissue to transfer film, and provides excellent preservation of cellular RNA (MAW, manuscript in preparation). Subsequently, tissue sections were stained as previously described.¹² In brief, slides were sequentially dipped five times in deionized water, 10 times in Mayer's hematoxylin solution (Sigma, St. Louis, MO), deionized water, 1 $\times$ automation buffer (Biomedex, Foster City, CA), and deionized water. Slides were then dehydrated for 60 seconds each in 70% ethanol and 95% ethanol, stained for 15 seconds in alcoholic eosin (Sigma), rinsed with 10 dips in 95% ethanol, and placed for 60 seconds in 95% ethanol. Finally, slides were further dehydrated by 10 dips in 100% ethanol, two 60-second washes in 100% ethanol, 10 dips in xylene, and two incubations of 3 minutes each in xylene. Slides were air-dried for 5 minutes and stored in a dessicator for no more than 6 hours before dissection. Nonmalignant ductal epithelial cells and foci of carcinoma *in situ* were isolated from the slides using the PixCell II LCM System (Arcturus Engineering, Mountain View, CA). Between 4300 and 6800 laser pulses (30- μm beam diameter; 30 mW power) yielded an estimated average of 15,000 cells.

RNA Extraction

For RNA dilution experiments, total cellular RNA was isolated from a single culture flask of MDA-MB361 human mammary tumor cells using the RNeasy RNA isolation system (Qiagen, Valencia, CA) and following manufacturer's recommendations. RNA was assessed by formaldehyde agarose gel electrophoresis, quantitated by UV absorbance, and diluted to a concentration of 1 $\mu\text{g}/\mu\text{l}$. For microdissected tissue samples, several LCM caps were pooled into a single tube containing 200 μl of denaturing buffer guanidinium isothiocyanate (GITC) and 1.6 μl of βME . Total RNA was then extracted using a modified protocol of the Stratagene RNA microisolation kit (Stratagene, La Jolla, CA) as previously described.⁶ The total RNA obtained from each LCM-dissected tissue was resuspended in 10 μl of RNase-free water. To assess the quality and concentration of the total RNA, 1 μl was

directly analyzed on an RNA LabChip (Agilent, Wilmington, DE) following the manufacturer's instructions.

Internal Controls

Plasmid constructs encoding transcriptional templates for the *B. subtilis* genes *lys*, *phe*, *trp*, and *thr* were obtained from the American Type Culture Collection (Manassas, VA). Purified plasmid DNAs were used to generate *in vitro*, polyadenylated transcripts using the Megascript kit (Ambion, Austin, TX) and following the manufacturer's protocol. RNA was purified using RNeasy spin columns (Qiagen) and quantitated by UV absorbance. Each of the transcripts was mixed to yield a cocktail of 1×10^6 copies/ μl *trp*, 5×10^6 copies/ μl *thr*, 2×10^7 copies/ μl *phe*, and 1×10^8 copies/ μl *lys*. Before synthesis of first strand cDNA, transcript cocktail was added to the RNA sample at a final concentration of 1×10^6 copies of *trp* per 10 μg of total RNA. This corresponds approximately to the four synthetic transcripts being present at 1:2000 (*lys*) to 1:200,000 (*trp*) endogenous transcripts.

Target Synthesis

A detailed protocol for target synthesis is available at <http://pathbox.wustl.edu/~mgacore/protocols.htm>. For biotin-labeled target synthesis starting from 10 μg of cell line total RNA, reactions were performed using standard protocols supplied by the manufacturer (Affymetrix) and as previously described.¹³ For target synthesis starting from 10 ng of total RNA or LCM-extracted RNA, an initial round of amplification was performed before synthesis of biotin-labeled cRNA. For the first round of amplification, synthesis of first- and second-strand cDNA was performed using the standard protocol provided by the manufacturer (Affymetrix) and as previously described.¹³ However, instead of proceeding to use the double-stranded cDNA in the biotin-labeled *in vitro* transcription reaction, the cDNA was resuspended in 8 μl of RNase-free water and used as a template to transcribe unlabeled antisense RNA (aRNA) using T7 RNA polymerase and the Megascript kit (Ambion). The reaction was incubated for 4 hours at 37°C and the resulting aRNA was purified using RNeasy spin columns (Qiagen). Eluted aRNA was precipitated by adding 0.1 volume of 7.5 mol/L ammonium acetate, 0.02 volumes of 5 mg/ml linear acrylamide (Ambion), and 2.5 volumes of 100% ethanol, and resuspended in 10 μl of RNase-free water. A second round of amplification was initiated by using the aRNA as template. After annealing aRNA with 0.7 $\mu\text{mol/L}$ random hexamers (Pharmacia, Piscataway, NJ) for 10 minutes at 70°C, the mixture was chilled on ice and extended in a 20- μl reaction containing 4 μl of 5 $\times$ first-strand reaction buffer, 2 μl of 0.1 mol/L dithiothreitol, 1 μl of 10 mmol/L dNTPs, and 1 μl of Superscript II (Life Technologies, Rockville, MD). After a 1-hour incubation at 42°C, 1 μl of 2 U/ml of RNase H was added, incubated for 20 minutes at 37°C, and inactivated at 95°C for 5 minutes. The resulting first-strand cDNA was annealed to 100 pmol of high performance liquid chromatography (HPLC)-puri-

Table 1. Comparison of Target Hybridization Signal from 10 μ g versus 10 ng of Input RNA

Sample	10 ng No. 1	10 ng No. 2	10 μ g
Scaling factor	5.6	5.8	2.6
"P" genes	538	428	685
SADV/LAR/AC			
Lys-3'	89,149/7.6/P	108,406/7.6/P	48,427/8.3/P
Phe-3'	9,475/4.3/P	8,475/2.6/P	10,109/4.3/P
Thr-3'	5,345/3.2/P	1,831/1.4/P	1,638/2.3/P
Trp-3'	18/-0.5/A	-190/-0.8/A	-57/-0.6/A
GAPDH-3' (M33197)	117,373/6.3/P	131,802/6.1/P	49,198/4.9/P
ISGF3A-3' (M97935)	2,654/2.8/P	2,907/2.4/P	4,431/5.4/P
TFRR-3' (M11507)	131/0.4/A	408/0.6/A	8,567/5.6/P
"P" versus "A" ($n = 1879$)			
10 ng No. 1	—	159 (8%)	70 (4%)
10 ng No. 2	62 (3%)	—	59 (3%)
10 μ g	208 (11%)	303 (16%)	—

Abbreviations: SADV, scaled average difference value; LAR, log average ratio; AC, absolute call.

fied T7T₂₄ primer (Genset, La Jolla, CA) for 10 minutes at 70°C. Then, second-strand cDNA synthesis was performed by adding 90 μ l of RNase-free water, 30 μ l of 5 $\times$ second-strand reaction buffer, 3 μ l of 10 mmol/L dNTPs, 10 U DNA ligase, 40 U DNA polymerase, and 2 U of RNase H. After incubating the second-strand cDNA reaction for 2 hours at 16°C, 20 U of T4 DNA polymerase were added, followed by incubation at 16°C for 10 minutes. The second-strand cDNA synthesis was stopped by adding 10 μ l of 0.5 mol/L ethylenediaminetetraacetic acid. Double-stranded cDNA was purified by phenol:chloroform:isoamyl alcohol extraction using phase-lock-gel (Eppendorf, Westbury, NY), precipitated with 0.5 volumes of 7.5 mol/L ammonium acetate, 2 μ g of glycogen, and 2.5 volumes of 100% ethanol, and resuspended in 22 μ l of RNase-free water.

Biotin-Labeled cRNA Transcription and Gene-Chip Hybridization

Biotinylated cRNA target was generated from both amplified and nonamplified cDNAs using the Bioarray high-yield transcription kit (Enzo, New York, NY) following the manufacturer's protocol. After a 5-hour incubation at 37°C, the final biotin-labeled cRNA product was purified using RNeasy spin columns (Qiagen) and eluted in 40 μ l of RNase-free water. The concentration of biotin-labeled cRNA was determined by UV absorbance. In all cases, 25 μ g of each biotinylated cRNA preparation was fragmented, assessed by gel electrophoresis, and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix Hu6800SubD GeneChip arrays for 16 hours. GeneChips were washed and stained using the instrument's standard Eukaryotic GE Wash 2' protocol, using antibody-mediated signal amplification.

Data Analysis

The images from the scanned chips were processed using Affymetrix Microarray Analysis Suite 4.0. In the first

set of validation experiments that used duplicate RNA samples, the image from each GeneChip was individually scaled such that the average intensity value for all arrays was adjusted to 1500. Scaled average difference value, log average ratio, and absolute call data from each GeneChip were exported to flat text files and used for numerical analysis. For analysis of clinical specimens, the GeneChip image of the carcinoma *in situ* sample was normalized to the corresponding image of the nonmalignant epithelium sample, across all probe pair sets. Difference call, fold change, average difference value, and absolute call data from each of the three specimen pairs analyzed were exported to flat text files. The complete set of data may be downloaded from the site: <http://pathbox.wustl.edu/~mgacore/>.

Results and Discussion

To determine whether LCM-derived material could be used in oligonucleotide array expression studies, we first investigated the reliability of an approach using transcription-based linear amplification of RNA,^{7,8,10,14} starting with 10 ng of total RNA. This quantity was chosen because we have previously shown that 10 ng of high-quality RNA can be obtained with minimal LCM dissection (MAW, unpublished data). Ten μ g of total RNA isolated from the human breast carcinoma cell line, MDA-MB361 was used for the generation of biotin-labeled aRNA using standard protocols.¹³ This generally yielded 50 to 70 μ g of labeled target, in keeping with previously published reports.¹³ Two additional aliquots of 10 ng each from the same RNA preparation were used to generate labeled targets, using a single round of transcriptional amplification before the synthesis of biotinylated target. This protocol generally yielded 24 to 32 μ g of labeled target, representing an approximate 50,000-fold amplification. Before the first round of cDNA synthesis, all RNA samples were spiked with a set of four *in vitro*-generated polyA transcript controls at defined copy numbers per mass of total cell line RNA. Probe sequences for these transcripts are represented on all Affymetrix GeneChips so that measurement of their hybridization signal to the array can provide a rough estimate of the molecular

and analytical precision, linearity, and sensitivity of the experiment.

Table 1 provides a summary of results from this first experiment. The scaling factor is a coefficient used to multiply signal intensities of each probe set so that the average signal intensity over the entire chip reaches a common target intensity (in this case 1500 units). Roughly speaking, it is a measure of total hybridization signal intensity. Although the total mass of biotinylated target hybridized to the array was comparable for all three samples, the hybridization signal (as reflected by the scaling factor) was approximately twofold lower in samples derived from 10 ng of total RNA as compared to that from 10 μ g. Another means to score the assay sensitivity is to examine the number of genes scored as detected ("P") versus nondetected ("A") within each sample. For the 10- μ g sample, 685 of 1879 (36%) probe sets were scored "P" whereas in the two 10-ng samples 538 (29%) and 428 (23%) were scored "P". Looking at individual transcript controls, high copy number transcripts (ie, *lys*) showed comparable levels of signal intensity that, because of the approximate twofold difference in scaling factors used, resulted in scaled average difference intensity values that were approximately twofold higher in amplified RNA versus nonamplified RNA arrays. Moderately abundant transcripts (ie, *phe*) showed very similar scaled average difference values for all three samples whereas lower abundant transcripts (ie, *thr*, *trp*) began to demonstrate a much higher degree of variability. Furthermore, using the standard protocol, ratios of *lys*, *phe*, and *thr* were maintained at an almost perfect fivefold decrement as would be expected from their corresponding starting copy number of transcripts. Samples that experienced an additional round of amplification, however, showed some skewing of this linearity. The endogenous transcripts for glyceraldehyde-6-phosphate dehydrogenase and ISGF3A, a general transcription factor, demonstrated comparable behavior. The transcript for transferrin receptor, however, was detected with 10 μ g of starting RNA, but could not be detected in either sample of amplified RNA. The number of discordant "P" and "A" calls is also summarized in Table 1. Most importantly, the number of discrepant calls between the two amplified samples is relatively low (3 to 8%) and comparable to the variability observed between identical sets of 10- μ g RNA samples (MAW, unpublished data). As expected, the highest discordance rate was between transcripts scored as "P" in the 10- μ g sample, but "A" in the amplified RNA samples. In summary, these data suggest that although detection sensitivity is compromised to some degree and there is some nonlinearity in comparing amplified versus nonamplified material, the protocol provides reproducible results from a single additional round of transcript amplification when starting with 1000-fold less input RNA. Although we sought to determine the minimum amount of RNA necessary to achieve a reasonable hybridization signal, we also suspect that some issues of sensitivity and linearity can be better preserved when greater than 10 ng of starting material is used.

We next applied this amplification strategy to RNA isolated from LCM tissue. Three surgically resected hu-

Figure 1. LCM and RNA isolation. Representative field of normal ductal epithelium before (A) and after (B) cell procurement by LCM. Regions of normal epithelium and adjacent regions of DCIS were individually dissected from three different tumor tissue specimens in a similar manner. RNA isolated from microdissected tissue was analyzed by capillary electrophoresis (Agilent RNA LabChip). C: A pseudo-electrophoretogram of several of the RNA samples isolated. One μ l (10%) of each RNA sample was assessed. **Lane 1:** RNA ladder; **lane 2:** DCIS 1 (4.3 ng/ μ l); **lane 3:** DCIS 2 (6.2 ng/ μ l); **lane 4:** nonmalignant epithelium 2 (8.3 ng/ μ l); **lane 5:** nonmalignant epithelium 3 (4.0 ng/ μ l); **lane 6:** DCIS 3 (6.0 ng/ μ l). **Lanes 7-9:** Positive control RNA isolated from one complete 5- μ m section of specimen 1 (71 ng/ μ l), specimen 2 (73 ng/ μ l), and specimen 2 duplicate (120 ng/ μ l), respectively. Note that the band intensity in each lane is scaled individually to that lane, rather than over the entire gel. In samples with degraded or limiting amounts of total RNA (eg, **lane 5**), decreased signal-to-noise ratio is responsible for the large number of background bands present in each lane.

man breast cancer specimens were chosen that contained predominant components of nonmalignant ducts and carcinoma *in situ* localized within a single histological section. Figure 1 shows representative LCM procurement of nonmalignant ductal cells. Approximately 5000 30- μ m laser firings were used to retrieve ~15,000 to 20,000 cells of each specified type. To determine the quality and

Table 2. Genes Differentially Expressed in Microdissected Nonmalignant Ductal Epithelial Cells *versus* Carcinoma *in Situ*

Gene	Accession	Fold change (DCIS <i>versus</i> NL)		
		Patient 1	Patient 2	Patient 3
Lys-3'	X17013	NA	NA	-1.1
Phe-3'	M24537	NA	NA	1.2
Thr-3'	X04603	NA	NA	-1.6
GAPDH-3'	M33197	-1.1	1.5	-1.4
Beta-actin-3'	X00351	1.3	-1.3	-1.3
ISGF3A-3'	M97935	-1.4	-1.1	1.1
c-fos	V01512	2.5	2.5	-4.1
Lactoferrin	X53961	(19.1)	(10.3)	-16
26S proteasome-associated pad1 homologue	U86782	(2.6)	(-1.4)	2.6
pS2	X52003	4.9	(-1.1)	20.8
SIX1	X91868	(2.9)	(-1.7)	(31.1)
Total genes scored "increased" in DCIS <i>versus</i> NL		46	79	105
Oxytocin receptor	X64878	-4.7	1.1	-4.1
Hevin like protein	X86693	-3.6	1.1	-2.5
Total genes scored "decreased" in DCIS <i>versus</i> NL		69	16	76

Abbreviations: DCIS, ductal carcinoma *in situ*; NL, normal.

quantity of resulting RNA, a 1- μ l aliquot (10%) of each sample was analyzed by microcapillary gel electrophoresis with fluorescence detection (Figure 1C). In most cases, RNA retrieved from LCM tissue was of high quality, as judged by 2:1 band intensity of 28S and 18S ribosomal bands. Typical yields ranged from 40 to 80 ng of RNA, or ~12 ng of RNA per 1000 30- μ m laser pulses. In addition to assessing RNA quality, quantitation of total LCM RNA allowed for the addition of polyA transcript standards at defined copy number per total mass of input RNA. Approximately 50 ng of RNA from patient-matched nonmalignant and malignant cells were amplified and used to generate labeled targets as described above.

Table 2 shows relative gene expression values (fold change) between nonmalignant epithelial cells and corresponding carcinoma *in situ* from each of the three patients. In the one patient sample pair where they were included, internal control transcripts (*lys*, *phe*, *thr*) varied by no more than 1.6-fold between the independently amplified nonmalignant and malignant samples. Endogenous housekeeping control genes (glyceraldehyde-6-phosphate dehydrogenase, β -actin, and ISGF3A) also demonstrated no more than 1.5-fold differences between a single patient sample pair as well as between all three patient pairs.

As compared to control transcripts, many genes were scored as "increased" or "decreased" expression between the nonmalignant and carcinoma *in situ* samples from each patient pair. From a total of 1069 genes that were expressed in at least one of the three patient sample pairs analyzed, a total of 115, 95, and 181 changes in gene expression were identified in each of the three patient pairs, respectively. Table 2 lists several genes that demonstrated concordant changes in expression of >2.5-fold between the nonmalignant and *in situ* carcinoma samples in at least two of the three patients. Fold change values in parentheses represent estimates, as one of the measured hybridization signals fell below the background level. Not surprisingly, no one gene showed

consistent changes in expression among all three patient pairs. Given the reasonable analytical and molecular reproducibility of the assay as demonstrated (Table 1) and the consistency of internal controls and housekeeping genes, these differences likely represent the inherent biological variability that can be expected when analyzing expression profiles between patients.⁴

Despite the small number of patient samples and gene probes used in the present study, two distinct expression profiles were apparent. Lactoferrin, a marker of estrogen stimulation in breast epithelium,¹⁵ was up-regulated in ductal carcinoma *in situ* (DCIS) from patients 1 and 2, but relatively down-regulated, relative to nonmalignant epithelium in patient 3. In contrast, PS2, a well-characterized estrogen-responsive marker of breast tumor progression¹¹ and SIX1, a homeobox protein frequently up-regulated in metastatic breast cancer,¹⁶ were only overexpressed in patient 3, and to a lesser extent, patient 1. Two other genes, hevin and oxytocin receptor, were also down-regulated in DCIS *versus* the normal duct epithelium of patients 1 and 3, but not patient 2. Hevin/SC1, a SPARC-like extracellular matrix-associated glycoprotein important for cell adhesion and migration, is frequently down-regulated in prostate adenocarcinoma as well as several other tumor types.¹⁷ The peptide hormone oxytocin can inhibit growth of breast cancer cells and expression of the corresponding oxytocin receptor has been well characterized in both nonmalignant and malignant mammary epithelium.¹⁸ Intriguingly, transcription of the oxytocin receptor is also regulated by *c-fos*,¹⁹ a second gene down-regulated in one, but not all three, non-malignant/malignant specimen pairs.

The functional significance of these and other findings from this data set will require additional studies using a larger cohort of specimens, additional numbers of probe pair sets, and more sophisticated bioinformatics approaches. Of more immediate importance, this study demonstrates how LCM can be easily coupled to high-density oligonucleotide array technology to obtain ex-

pression profiles from discrete cell populations in histologically complex clinical specimens. Specifically, we have found that careful preparation and staining of tissue sections, dissection of a large (but not burdensome) number of cells, qualitative and quantitative assessment of isolated cellular RNA, and inclusion of a set of internal transcript controls to monitor assay fidelity can greatly contribute to the success of this approach. Whether the molecular profile of an isolated sample of specific tumor cells or the more comprehensive tumor microenvironment will provide the best clinical diagnostic tool remains to be determined. In any event, the approach described here will be of obvious benefit to more precisely dissect the pathobiology and molecular evolution of disease processes like human breast cancer in complex tissue specimens.

References

1. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, Coller H, Loh ML, Downing JR, Caligiuri MA, Bloomfield CD, Lander ES: Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* 1999, 286: 531–537
2. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JL, Yang L, Marti GE, Moore T, Hudson Jr J, Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO, Staudt LM: Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000, 403:503–511
3. Bittner M, Meltzer P, Chen Y, Jiang Y, Seftor E, Hendrix M, Radmacher M, Simon R, Yakhini Z, Ben-Dor A, Sampa N, Dougherty E, Wang E, Marincola F, Gooden C, Lueders J, Glatfelter A, Pollock P, Carpten J, Gillanders E, Leja D, Dietrich K, Beaudry C, Berens M, Alberts D, Sondak V: Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* 2000, 406:536–540
4. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D: Molecular portraits of human breast tumors. *Nature* 2000, 406:747–752
5. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA: Laser capture microdissection. *Science* 1996, 274:998–1001
6. Sgroi DC, Teng S, Robinson G, LeVangie R, Hudson Jr JR, Elkahoul AG: In vivo gene expression profile analysis of human breast cancer progression. *Cancer Res* 1999, 59:5656–5661
7. Wang E, Miller LD, Ohnmacht GA, Liu ET, Marincola FM: High-fidelity mRNA amplification for gene profiling. *Nat Biotechnol* 2000, 18:457–459
8. Luo L, Salunga RC, Guo H, Bittner A, Joy KC, Galindo JE, Xiao H, Rogers KE, Wan JS, Jackson MR, Erlander MG: Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nat Med* 1999, 5:117–122
9. Leethanakul C, Patel V, Gillespie J, Pallente M, Ensley JF, Koontongkaew S, Liotta LA, Emmert-Buck M, Gutkind JS: Distinct pattern of expression of differentiation and growth-related genes in squamous cell carcinomas of the head and neck revealed by the use of laser capture microdissection and cDNA arrays. *Oncogene* 2000, 19: 3220–3224
10. Ohyama H, Zhang X, Kohno Y, Alevizos I, Posner M, Wong DT, Todd R: Laser capture microdissection-generated target sample for high-density oligonucleotide array hybridization. *Biotechniques* 2000, 29: 530–536
11. Gillesby BE, Zacharewski TR: pS2 (TFF1) levels in human breast cancer tumor samples: correlation with clinical and histological prognostic markers. *Breast Cancer Res Treat* 1999, 56:253–265
12. Goldworthy SM, Stockton PS, Trempus CS, Foley JF, Maronpot RR: Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. *Mol Carcinog* 1999, 25:86–91
13. Mahadevappa M, Warrington JA: A high-density probe array sample preparation method using 10- to 100-fold fewer cells. *Nat Biotechnol* 1999, 17:1134–1136
14. Van Gelder RN, von Zastrow ME, Yool A, Dement WC, Barchas JD, Eberwine JH: Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc Natl Acad Sci USA* 1990, 87:1663–1667
15. Yang N, Shigeta H, Shi H, Teng CT: Estrogen-related receptor, hERR1, modulates estrogen receptor-mediated response of human lactoferrin gene promoter. *J Biol Chem* 1996, 271:5795–5804
16. Ford HL, Landesman-Bollag E, Dacwag CS, Stukenberg PT, Pardee AB, Seldin DC: Cell cycle-regulated phosphorylation of the human SIX1 homeodomain protein. *J Biol Chem* 2000, 275:22245–22254
17. Nelson PS, Plymate SR, Wang K, True LD, Ware JL, Gan L, Liu AY, Hood L: Hevin, an antiadhesive extracellular matrix protein, is down-regulated in metastatic prostate adenocarcinoma. *Cancer Res* 1998, 58:232–236
18. Sapino A, Cassoni P, Stella A, Bussolati G: Oxytocin receptor within the breast: biological function and distribution. *Anticancer Res* 1998, 18:2181–2186
19. Hoare S, Copland JA, Wood TG, Jeng YJ, Izban MG, Soloff MS: Identification of a GABP alpha/beta binding site involved in the induction of oxytocin receptor gene expression in human breast cells, potentiation by c-Fos/c-Jun. *Endocrinology* 1999, 140:2268–2279