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Identification of genes expressed in premalignant breast disease by microscopy-directed cloning

(ductal carcinoma *in situ*/differential display/polymerase chain reaction)

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ABSTRACT **Histopathologic study of human breast biopsy samples has identified specific lesions which are associated with a high risk of development of invasive breast cancer. Presumably, these lesions (collectively termed premalignant breast disease) represent the earliest recognizable morphologic expression of fundamental molecular events that lead to the development of invasive breast cancer. To study molecular events underlying premalignant breast disease, we have developed a method for isolating RNA from histologically identified lesions from frozen human breast tissue. This method specifically obtains mRNA from breast epithelial cells and has identified three genes which are differentially expressed in premalignant breast epithelial lesions. One gene identified by this method is overexpressed in four of five noncomedo ductal carcinoma *in situ* lesions and appears to be the human homologue of the gene encoding the M2 subunit of ribonucleotide reductase, an enzyme involved in DNA synthesis.**

The development of a malignant neoplasm is presumed to involve a series of genetic alterations that confer increasing growth factor independence and metastatic capability on somatic cells. Identifying the molecular events that lead to the initial development of a neoplasm is therefore critical to understanding the fundamental mechanisms by which tumors arise and to the selection of optimal targets for gene therapy and chemopreventive agents. As intermediate endpoints in neoplastic development, some premalignant breast lesions represent important, and possibly rate-limiting, steps in the progression of human breast cancer, and careful epidemiological studies have established the relative risk for breast cancer development for specific histologic lesions (1, 2). In particular, invasive breast cancer develops in the region of the previous biopsy site in 25–30% of patients after diagnosis of noncomedo ductal carcinoma *in situ* (DCIS) (3, 4), providing strong evidence that this premalignant lesion is a determinant event in breast cancer progression (5). While these morphologically defined risk associations have been widely accepted, progress in defining the molecular correlates of these lesions has been hampered by an inability to identify and sample them in a manner which would allow the application of molecular techniques (6). Although mutations in known oncogenes and tumor-suppressor genes have been identified in invasive breast cancer and in high-grade carcinoma *in situ* (comedo ductal carcinoma *in situ*), mutations are rarely present in early premalignant lesions (7–10). To study molecular events in human premalignancy we have developed a method for isolating epithelial cell RNA from histologically identified lesions in human breast biopsy specimens, and we have used this method to clone genes which are differentially expressed in DCIS.§

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MATERIALS AND METHODS

Tissue Characterization and Selective Harvesting. Freshly obtained breast biopsy, mastectomy, or reduction mammoplasty specimens from the Surgical Pathology Laboratories of Vanderbilt University Hospital, Baptist Hospital, Memorial Hospital, and Associated Pathologists in Nashville, Tennessee, were evaluated for inclusion in the study. The specimens were serially sectioned and areas of breast parenchyma, exclusive of adipose tissue, were submitted for frozen section analysis to identify normal breast epithelial elements, noncomedo DCIS, and invasive breast carcinoma. With identification of these entities, a portion of the sample was then submitted for routine paraffin embedding and sectioning, and areas not required for confirmation of the diagnosis were maintained frozen in OCT compound (Miles) at −70°C. For harvesting of selected lesions the samples were remounted in a cryostat and the hematoxylin- and eosin-stained frozen section slides were used to identify and map epithelial elements for directed harvesting by 2-mm punches from the frozen block. We selected lesions for microlocalization that were relatively isolated and homogeneous. Thus we did not utilize cases in which invasive, DCIS, and normal epithelial components were admixed and could not be selectively harvested as a homogeneous sample by a 2-mm punch. Two biopsy and three mastectomy specimens were selected. A total of four reduction mammoplasty specimens were used as negative controls. After harvest, additional frozen sections were obtained to confirm the precision of the harvesting procedure. See Fig. 1.

Immunohistochemical/Histochemical Analysis. To confirm the purely epithelial nature of the DCIS lesions and to identify genes useful in molecular characterization of the samples, Formalin-fixed paraffin-embedded sections of the tissues were stained with Masson's trichrome and were also immunohistochemically analyzed for the presence of cytokeratin (keratins 8 and 18) and vimentin with antibodies obtained from Becton Dickinson and Boehringer Mannheim, respectively. Antibodies were used at recommended dilutions and immunohistochemical staining was performed on an automated Ventana model 320 immunostainer, which utilizes a modified avidin–biotin complex method with diaminobenzidine visualization. After immunostaining, all slides were lightly counterstained with hematoxylin.

Purification of RNA. RNA was isolated from the frozen tissue cores by mincing the cores in 5.6 M guanidinium isothiocyanate and 40% (wt/vol) phenol, centrifuging to remove particulate matter, reducing viscosity by multiple aspirations through a 22 gauge needle, extracting with chloroform, and precipitating with ethanol. The particulate ma-

Abbreviations: DCIS, ductal carcinoma *in situ*; GAPD, glyceraldehyde-3-phosphate dehydrogenase.

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§The sequence discussed in this paper has been deposited in the GenBank data base (accession no. L27636).

terial resistant to guanidinium/phenol extraction appeared white and fibrous and was presumed to represent breast stroma. This particulate material was sparse in DCIS samples but abundant in samples obtained from reduction mammoplasty. To obtain RNA samples that presumably included RNA derived from these stromal cells, this white particulate material was homogenized, washed with phosphate-buffered saline (PBS), treated with collagenase at 37°C for 30 min, sonicated, extracted with phenol/chloroform, and precipitated with ethanol.

mRNA Isolation and Screening. Multiple punches from individual lesions were needed to obtain sufficient RNA for poly(A) selection and library construction. Two hundred micrograms of total RNA was obtained by pooling 20 punches from each normal breast tissue sample (reduction mammoplasties) or 5–8 punches from each DCIS lesion, presumably reflecting the greater cellularity of the DCIS samples. Pooling of 2-mm punches was done only on individual lesions from single patients or on normal tissue from individual patients. Individual samples that did not yield sufficient RNA were not further analyzed (there was no pooling of samples from multiple patients). After assessment of the purity of RNA samples (see Figs. 3 and 4), the RNA was either used directly for differential display (11–13) or used to construct cDNA libraries by standard methods (14, 15).

Differential display was performed according to the published method (11–13), using the following sequences and temperatures. A 0.5-μg sample of poly(A)-purified mRNA was transcribed with 300 units of reverse transcriptase from Moloney murine leukemia virus for 60 min at 35°C along with 2.5 μM $T_{11}CA$ and 20 μM deoxynucleoside triphosphates. After heat inactivation at 95°C for 5 min, a 10-base oligonucleotide (5′-GTTCCTCACT-3′) was added to 5 μM and PCR amplification was performed, employing *Taq* DNA polymerase with the following cycle conditions: 40 cycles were with denaturation for 1 min at 94°C, annealing for 2 min at 42°C, and extension for 1 min at 72°C.

cDNA libraries were constructed in λ phage (Lambda ZAP, Stratagene, La Jolla), using poly(A)-selected mRNA from frozen tissue samples. All unamplified libraries had greater than 50% inserts and contained between 2×10^6 and 7×10^7 phage recombinants with an average insert size varying between 500 and 1000 base pairs (bp). To readily screen the cDNA libraries, plasmid DNA was prepared from the cDNA libraries after helper phage rescue and was screened by two independent methods. Initially, the libraries were differentially screened as unamplified phage libraries with probes prepared by phage T7 RNA polymerase transcription of a DCIS library versus a control library. Thereafter, we employed the rescued cDNA plasmids as templates for low-stringency PCR with a pair of randomly generated 25-bp primers as described by Welsh and coworkers (16–18). Random 25-bp primers were generated by a computer-based algorithm (R. Jotte and J.T.H., unpublished work). Forty cycles of PCR were performed with denaturation for 1 min at

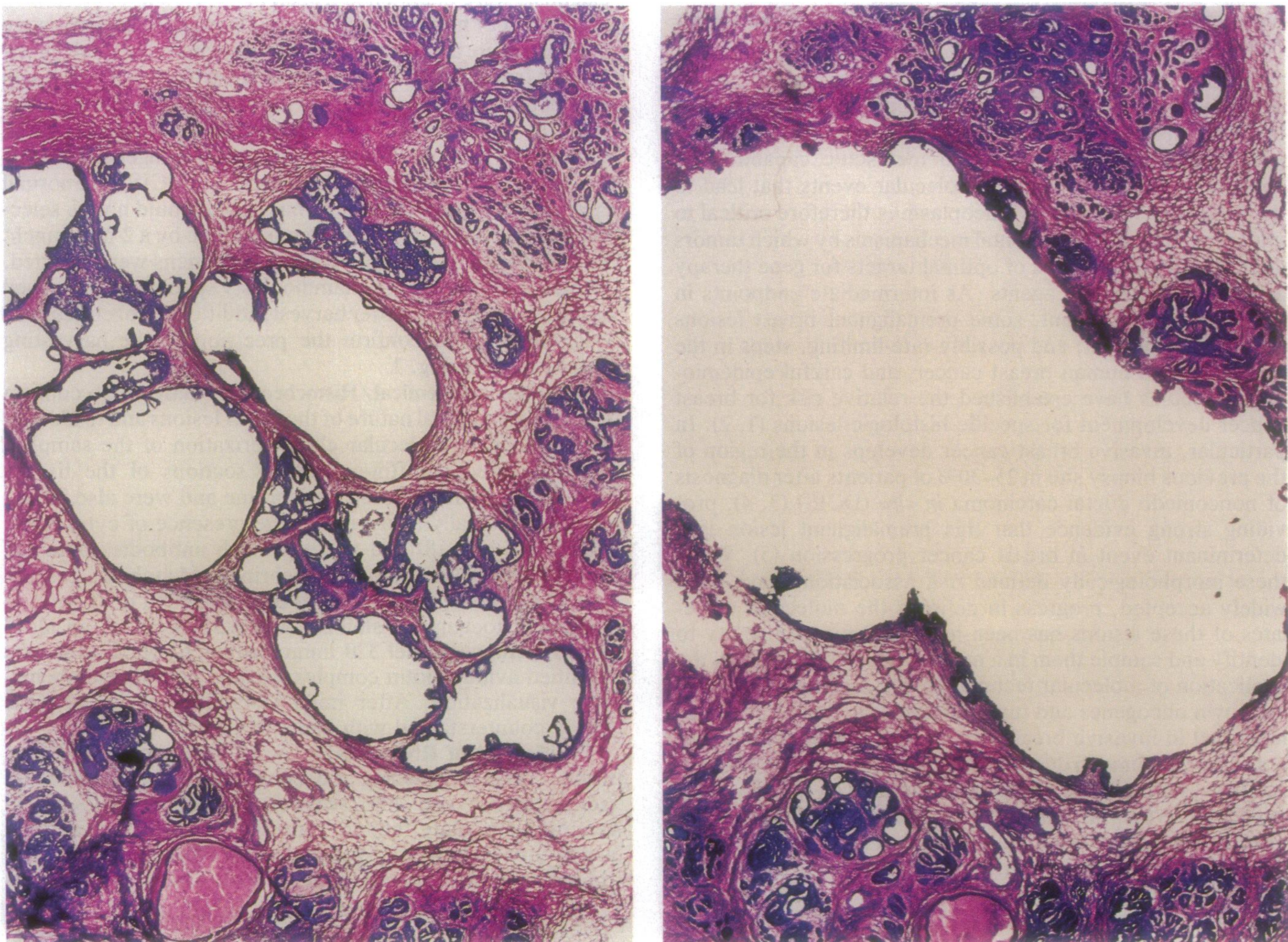

FIG. 1. Selective harvesting of breast epithelial elements. (*Left*) Hematoxylin- and eosin-stained frozen section slide from patient 12, diagnosed as noncomedo DCIS. (×15.) This slide was used to selectively harvest lesional tissue from the frozen block by directed 2-mm punches. (*Right*) After harvest, a second frozen section was obtained to confirm the precise location of the harvest. (×15.)

94°C, annealing for 2 min at 25°C, and extension for 1 min at 72°C.

The amplified samples obtained by either differential display or PCR were then resolved on nondenaturing 8% polyacrylamide gels, which were dried and autoradiographed. Specific bands were then reamplified with the primers used for their generation and purified on nondenaturing 8% polyacrylamide gels, and the gel bands were extracted, subcloned by standard methods, and sequenced.

Cloning of Probes and Nuclease Protection Assays. The keratin 8 probe employed for nuclease protection assays was obtained by PCR amplification of genomic DNA with the following primers: 5′-GAGTCCTCTGACGTCCTGCCCA-3′ and 5′-GGCAGACGTAGCTGAGGTTTTA-3′, which amplify a 261-bp fragment from the 3′ untranslated region of human keratin 8 (19). The vimentin probe employed for nuclease protection assays was obtained by PCR amplification of genomic DNA with the following primers: 5′-CAGGTTATCAACGAAACTTCTC-3′ and 5′-CTGTAAACTAGATTATGTTGTA-3′, which amplify a 254-bp fragment from the 3′ coding and untranslated region of human vimentin (20). For a control probe to ensure equal loading and recovery of RNA, we used a T7 RNA polymerase-generated probe for human glyceraldehyde-3-phosphate dehydrogenase (GAPD) which protects a 140-bp *Sac* I–*Xba* I fragment (a generous gift from Janice Nigro, Vanderbilt University). The DCIS-1 probe was generated by linearizing the rescued plasmid with *Pvu* II, which generates a 200-bp protected fragment. RNase protection assays were performed with the above-cited probes, using the methods we have reported previously (21).

RESULTS

Tissue samples were obtained from breast biopsies diagnosed as DCIS by specific histopathological criteria (22). Control mRNA was obtained from reduction mammoplasty specimens and from cultured human breast epithelial cells. Because noncomedo DCIS is a microscopic lesion, localization of DCIS was accomplished by light microscopy, and identified regions of DCIS were selectively harvested, employing a 2-mm punch. A similar approach was used to isolate mRNA from lobules of normal breast from reduction mammoplasties. As shown in Fig. 1, the 2-mm punch provided a well-tailored excision. This microlocalization method was performed with extreme care and was absolutely crucial to the success of these studies, as contamination by normal breast epithelial cells, large numbers of stromal cells, or invasive disease would clearly negatively skew the differential screening approach. If the punch biopsy did not in large part excise the DCIS without contamination by other cell types or tissues, then the sample was not used for mRNA isolation. This is demonstrated in Fig. 1, where approximately 80% of the harvested tissue represents DCIS, and the remaining 20% represents stromal elements. After microlocalization harvesting of the frozen tissue, RNA was isolated, purified, and employed in reverse transcriptase PCR or to construct cDNA libraries as described in *Materials and Methods*.

To evaluate the location and extent of stromal cells and extracellular matrix adjacent to DCIS lesions, we performed histologic staining with Masson's trichrome and immunohistochemical staining with antibodies directed against both epithelial (cytokeratin) and stromal fibroblast (vimentin) protein markers. These results are presented in Fig. 2 and demonstrate that DCIS lesions contain abundant cytokeratin, consistent with their epithelial nature. In results not shown, breast stromal fibroblasts express vimentin, but not cytokeratin. This immunohistochemical profile suggested a means to assess the relative purity of our RNA samples by determining the level of cytokeratin (keratin 8) and vimentin expression with RNase protection assays. This method was chosen because it is semiquantitative and can be performed on small amounts of unselected RNA. Vimentin mRNA was identified in the presumed stromal fraction of the normal breast tissue (Fig. 3, compare expression in NL3 with other patient samples), but no expression of vimentin was detected in the DCIS or normal samples obtained with much less vigorous extraction techniques. This finding may be a result of the inherent epithelial/stromal ratio in DCIS, but it is still an indication of the relative purity of the sample and would indicate that our normal epithelial samples also contain epithelial elements predominately. Control studies demonstrated that DCIS, normal breast, and invasive breast cancer samples express cytokeratin mRNA in similar amounts, but that stromal cell RNA does not contain detectable cytokeratin mRNA when assayed by nuclease protection assays (Fig. 3 *Lower*).

cDNA libraries were then constructed in λ phage, using poly(A)-selected mRNA from the following samples: cultured human breast epithelial cells, tissue from three reduction mammoplasty patients, tissue from three DCIS patients, and tissue from one DCIS patient (patient 10) that showed a focus of invasion adjacent to an area of DCIS. To assess the integrity of these libraries and to confirm our ability to clone genes which we would expect to be expressed in the libraries, we performed PCR on rescued plasmid DNA and were able to identify keratin 8 from all libraries (data not shown).

Comparison of gene expression between samples was performed by differential screening, differential display, or a PCR-based library screening method. Fig. 4 shows the results of PCR amplification of cDNA library samples obtained from DCIS samples, normal breast epithelial cells, and invasive cancer. Although few genes shown in this figure are differentially expressed in the majority of samples with DCIS, the heterogeneity of gene expression in patient samples is seen. Employing these methods, we identified 10 differentially expressed clones, and the three that showed the greatest difference in expression were further characterized by DNA sequencing. Comparison of the sequenced clones with the GenBank data base demonstrated that one clone is homologous to a human gene previously named hbc002C (23) which was cloned from normal human islet cells. Another of the

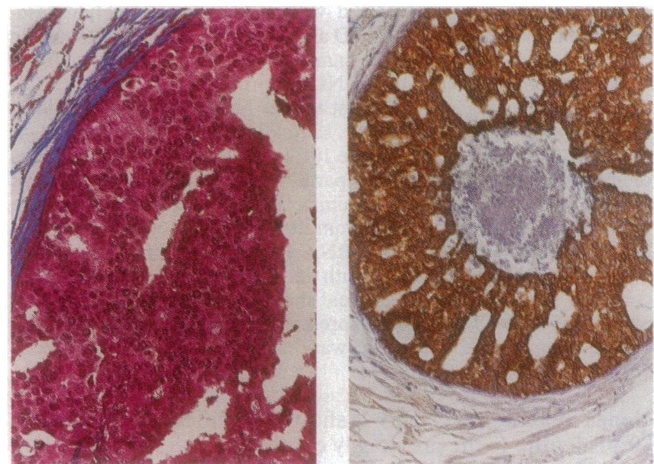

FIG. 2. Histological/immunohistochemical characterization of DCIS. (*Left*) Masson's trichrome stain, illustrating purely epithelial nature of DCIS without intervening stromal elements. Note epithelial elements staining red and stromal elements staining blue. (Patient 10; Formalin-fixed paraffin-embedded tissue; ×90 final magnification.) (*Right*) Cytokeratin immunostain illustrating strong cytokeratin positivity (brown staining) in DCIS and no staining of adjacent stromal cells. (Patient 10; Formalin-fixed paraffin-embedded tissue; ×70 final magnification.)

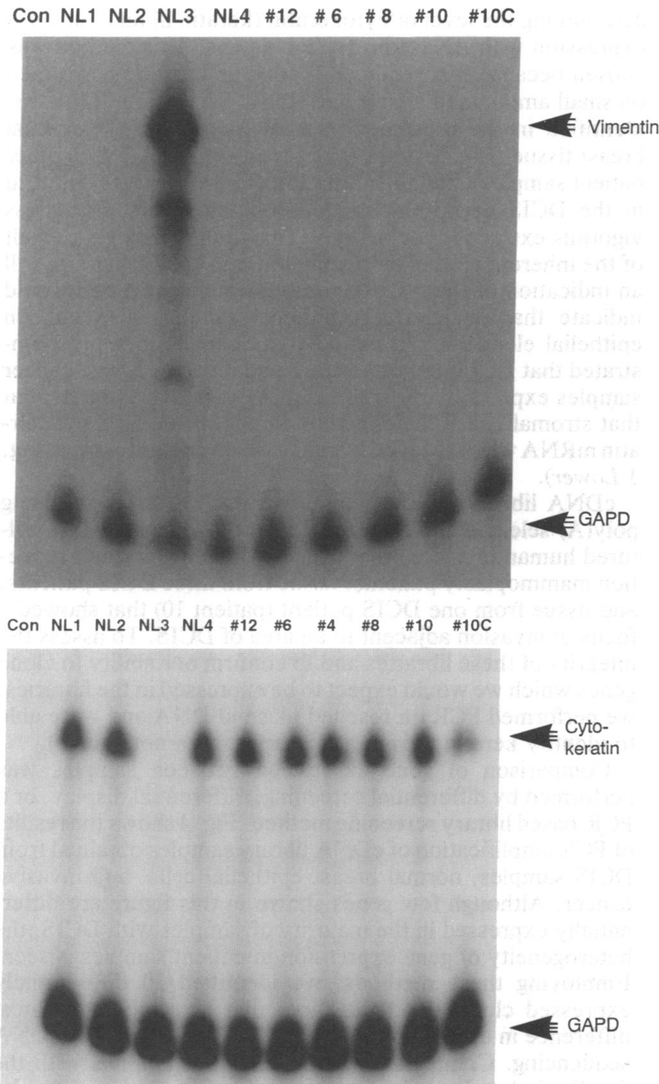

FIG. 3. Molecular characterization of RNA samples. Expression of vimentin (*Upper*) and keratin 8 (*Lower*) mRNA in tissue mRNA samples was analyzed by RNase protection assay (21). One microgram of mRNA was hybridized with ^{32}P-labeled T7 polymerase-generated RNA probes for GAPD and either vimentin or cytokeratin (keratin 8). The following RNA samples were probed: NL1, cultured human breast epithelial cells; NL2, normal breast tissue; NL3, fibrous stromal fraction of breast tissue; NL4, a second sample of normal breast tissue. In addition, samples from DCIS patients 12, 4, 6, 8, and 10 were probed. Sample 10C is RNA obtained from a focus of invasive cancer present in a separate area of the block from patient 10. Con is a control sample containing tRNA. Note that the NL3, the presumed stromal component from normal tissue, does not contain detectable levels of keratin 8 mRNA and contains large amounts of vimentin mRNA, consistent with its mesenchymal character. In contrast, the remaining samples show high levels of keratin 8 expression and no vimentin expression, consistent with their epithelial origin. Note that there was insufficient RNA available to analyze patient 4 in *Upper*.

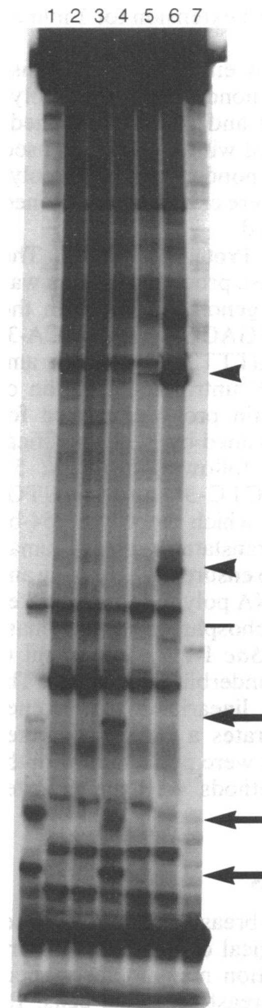

FIG. 4. Differential display of cDNAs obtained from patient tissue samples and controls. Rescued cDNA library samples were used as templates for low-stringency PCR with the primers 5′-GATGAGTTCGTGTCCGTACAACTGG-3′ and 5′-GGTTATCGAAATCAGCCACAGCGCC-3′; 40 cycles were performed with denaturation for 1 min at 94°C, annealing for 2 min at 25°C, and extension for 1 min at 72°C. The samples correspond to those in Fig. 3: lane 1 is from patient 12; lanes 2 and 3 are from separate phagemid rescues of NL1 to show reproducibility; lane 4 is from patient 8; lane 5 is from patient 10, and lane 6 is from sample 10C. Lane 7 is control λ phage vector without cDNA inserts. Arrows mark cDNAs which are differentially expressed in DCIS samples versus normal, and arrowheads mark cDNAs which are differentially expressed in the invasive cancer. The bar marks a cDNA which is expressed in normal breast cells at higher levels than in DCIS or invasive cancer.

clones (here termed DCIS-1) showed significant (greater than 80%) identity to the previously cloned hamster gene encoding the M2 subunit of ribonucleotide reductase (24). Although human M2 has been cloned (25), comparison of the hamster and human cDNA sequences with our clone indicates that DCIS-1 is homologous to an alternatively spliced polyadenylylated form of the human ribonucleotide reductase which has not been described previously (GenBank accession no. L27636).

Because the DCIS-1 clone was identified by cloning methods which included selection and amplification, it was important to confirm that the gene was differentially expressed in the original unselected, unamplified, RNA samples. To verify that DCIS-1 is differentially expressed in DCIS versus normal breast epithelial cells, we performed a nuclease protection assay to analyze expression levels in tissue samples. The results (Fig. 5) demonstrate that DCIS-1 is expressed 4- to 30-fold higher in DCIS samples than in normal breast epithelial cells obtained from tissue or culture. The DCIS sample from patient 8 does not exhibit elevated expression of the DCIS-1 gene, suggesting that expression of this gene is not universal in DCIS, and this may reflect the

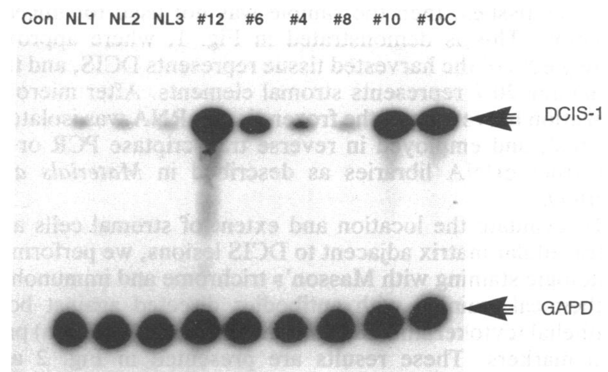

FIG. 5. Expression of DCIS-1 mRNA in tissue mRNA samples analyzed by RNase protection assay. The samples and their order are identical to those in Fig. 3, but they were hybridized with the GAPD probe and the DCIS-1 probe.

prognostic heterogeneity of these lesions: some progress to invasive cancer, but many do not. Further study will be necessary to determine if expression of DCIS-1 or other DCIS marker genes is correlated with progression to invasive cancer in these patients.

DISCUSSION

We have presented a method of microscopy-directed cloning which combines histopathologic microdissection with selective mRNA purification, permitting analysis of differential gene expression from complex tissues containing both epithelial and stromal components. This method was applied to a subset of premalignant breast lesions and identified three cDNAs which were differentially expressed in human DCIS. One of these genes, ribonucleotide reductase M2 subunit, represents a known control point for DNA synthesis, providing an intriguing link between gene expression and altered biologic behavior of DCIS.

Although the inherent difficulty in selectively harvesting stromal and epithelial cell populations from tissue samples was a major concern prior to the initiation of our studies, analysis of epithelial and stromal marker gene expression in our DCIS RNA samples indicates there was no significant cross-contamination of epithelial cells with stromal cells and vice versa. These results suggest that it may be unnecessary to microdissect and amplify RNA from smaller samples such as single cells to obtain relatively pure populations of RNAs from tissue specimens. Although we employed this method to study the epithelial component of DCIS, our method could also be used to selectively enrich for stromal components surrounding DCIS lesions for studies of epithelial–stromal interactions.

The development of a method to identify genes that are differentially expressed in DCIS provides an opportunity to molecularly characterize this premalignant condition. *DCIS-1*, a gene that we have identified by a microscopy-directed cloning method, appears to be the human homologue of the gene encoding the M2 subunit of ribonucleotide reductase. This subunit of ribonucleotide reductase is amplified in conditions of ribonucleotide starvation, and the enzyme has an important role in deoxynucleotide synthesis. The mouse M2 subunit is dramatically induced by serum and appears to be an immediate early gene product (26–28). Although it is intriguing to speculate that the increased expression of ribonucleotide reductase is a consequence of an increased proliferative rate of DCIS cells, proliferation rates for noncomedo DCIS are in fact low (29), suggesting a more complex relationship between increased ribonucleotide reductase expression and the biologic behavior of DCIS than merely an effect on growth rates. The availability of marker genes for DCIS will allow the study of gene regulation in premalignancy and will assist in determining the role of specific transcriptional or post-transcriptional events responsible for the observed differential gene expression in DCIS.

The results demonstrate that a microscopy-directed cloning approach can be employed to identify genes which are differentially expressed in premalignant breast disease. The availability of RNAs which are highly enriched for genes expressed in premalignant epithelial cells will facilitate analysis of pathologic tissue samples by either (*i*) direct analysis of RNA for differential display studies or (*ii*) analysis of stable permanent cDNA banks obtained from individual patient samples. Direct analysis of gene expression by differential display is advantageous because much less RNA is required, and the lack of sample processing prior to PCR means that expressed genes will be represented more accurately. In contrast, cDNA libraries may exhibit somewhat distorted representation, but they allow preparation of stable reagents from patient histopathologic specimens which may be analyzed indefinitely and readily provided to other investigators. Both approaches should facilitate analysis of expression during DCIS induction for either presumed candidate genes (genes linked to hereditary breast cancer or known oncogenes) or novel differentially expressed genes identified by PCR-based cloning methods. This method should also be directly applicable to a variety of pathologic conditions previously intractable to molecular analysis due to the small size of the lesions.

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