Quantitative and Spatial Measurements of Telomerase Reverse Transcriptase Expression within Normal and Malignant Human Breast Tissues

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Abstract
The enzyme telomerase catalyzes the de novo synthesis of telomere repeats, thereby maintaining telomere length, which is necessary for unlimited cellular proliferation. Telomerase reverse transcriptase (TERT), the catalytic domain of telomerase, is the rate-limiting factor for telomerase activity and is expressed in virtually all tumors. Thus, TERT has been proposed as a marker with diagnostic and prognostic potential in breast cancer as well as a basis for breast cancer therapeutics. In these contexts, it is important to define the sites and extent of TERT expression in normal and cancerous human breast tissues. In this study, levels of TERT mRNA were measured within a set of 36 breast carcinomas and 5 normal breast samples by quantitative real-time reverse transcription-PCR, and we subsequently identified and characterized the cells expressing TERT mRNA within these tissues using in situ hybridization. The results show that (a) detectable TERT mRNA expression is specific to the epithelial cells; (b) TERT is expressed in both normal and malignant breast tissues; (c) the pattern and level of TERT expression are heterogeneous, with ~75% of tumors expressing bulk TERT mRNA levels equal to or less than those within normal breast tissue; and (d) tumors expressing above-normal levels of TERT mRNA are more likely to be histopathologic grade 3 (P = 0.002), contain high fraction of cells in S phase (P = 0.004), and have increased levels of MYC mRNA (P = 0.034).

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Introduction
Loss of telomere function is a pivotal mechanism generating genomic instability, a common feature of most, if not all, neoplasms. Telomeres, the nucleoprotein structures forming the tips of chromosomes, protect these ends by preventing them from being treated as double-strand breaks and prohibiting their fusion to other chromosome ends (1). They consist of specific telomere-associated proteins bound to the 3’ termini, loses up to 40 to 200 bases during each cell division (4). This DNA attrition provides an additional function to telomeres: to limit unrestricted cellular proliferation. This is evident in cultured cells where the reduction of telomere DNA is associated with cellular senescence and, eventually, cell death. This loss may be compensated through the activity of telomerase or telomerase-independent recombination mechanisms (called alternative lengthening of telomeres; ref. 5). Telomerase is a reverse transcriptase that synthesizes telomere repeats on the telomere ends (6). The core enzyme is a nucleoprotein formed by two distinct gene products: telomerase reverse transcriptase (TERT), the catalytic protein subunit, and telomerase RNA component, an internal RNA component that provides the template for telomere DNA synthesis. In human cells, telomerase RNA component is ubiquitously expressed, whereas TERT is not. Consequently, telomerase activity is attenuated in most adult somatic cells and is only found in certain renewable cells, such as germ cells, stem cells, and intestinal crypt cells (7-9). Moreover, TERT mRNA reconstitutes telomerase activity when ectopically expressed within cells normally lacking this activity (10), and a recent finding has shown a strong correlation between TERT mRNA levels and telomerase activity within breast carcinomas (11). For these reasons, the expression of TERT mRNA is regarded as the rate-limiting factor of telomerase activity within tissues, including breast carcinomas (12, 13).

The expression of the TERT subunit, and resulting telomerase activity in normal tissues, is attributed only to a limited subset of cells, whereas the activation of the gene in tumors is arguably one of the most common features of malignant transformation, occurring in nearly all tumor types (14). This expression has been shown to be directly influenced by the MYC proto-oncogene (15), which is...
involved in cell proliferation and is commonly up-regulated in cancer cells. The resulting telomerase activity bestows a limitless replicative potential on the cell, a hallmark of cancer (16), and it has been shown that TERT, along with SV40 large T oncprotein and an oncogenic allele of Ras, can generate tumorigenic conversion of normal human epithelial and fibroblast cells. In contrast, cells that did not express TERT did not form tumors (17, 18).

Given the importance of telomerase to the tumorigenic process (i.e., telomere lengthening and cellular immortality), there have been many studies investigating its activity within breast carcinomas and normal breast tissue over the past decade, but relatively few are examining TERT expression within these tissues. In fact, in a recent review, Baykal et al. have warned that the results of telomerase activity measurements within normal breast tissue and cancers should be interpreted with caution due to cellular heterogeneity, experimental bias, and complications of the telomerase activity assay (19).

Due to telomerase’s role during tumorigenesis as well as its proposed utility as both a diagnostic and prognostic tool in breast cancer (ref. 11; reviewed in ref. 19) and a target for cancer therapy (20-22), it is important to define the sites and extent of TERT expression. The goal of the present study was to determine, within breast tissues, how TERT mRNA levels within individual cells and clusters of cells relate to bulk measurements of TERT mRNA within the tissue. In the present study, we measured levels of TERT mRNA within a set of breast carcinomas and normal breast samples by quantitative real-time reverse transcription-PCR (QRT-PCR) and subsequently identified and characterized the cells expressing TERT mRNA using in situ techniques.

We show that TERT mRNA expression is specific to the epithelial cells in both normal and malignant tissues, and the pattern and level of this TERT expression are heterogeneous. In addition, we show that ~75% of tumors expressed bulk TERT mRNA levels equal to or less than those within normal breast tissue and that the tumors within the group expressing above-normal levels of TERT mRNA were more likely to display an aggressive phenotype.

**Results**

**Real-time RT-PCR**

TERT mRNA was detected within all breast samples (normal and tumor) using QRT-PCR. Normalized levels of mRNA ranged from 0.03 to 22.42 arbitrary units (AU) within the 36 breast carcinomas and from 0.29 to 2.09 AU within the 5 tumor-adjacent, histologically normal breast tissues (Fig. 1). The 95% confidence interval of TERT mRNA levels within the sample of normal breast tissues was 1.11 ± 0.99 AU (Fig. 1, shaded area). Twenty-three of 36 (64%) tumors expressed levels within this range, 10 (28%) had levels that were above this range, and 3 (8%) tumors had levels that were below this range.

The characteristics of the 10 breast tumors that had bulk TERT levels (measured by QRT-PCR) above the 95% confidence interval of the normal breast tissues were 1.11 ± 0.99 AU (Fig. 1, shaded area). The distribution of tumor sizes within these tissues. In fact, in a recent review, Baykal et al. have warned that the results of telomerase activity measurements within normal breast tissue and cancers should be interpreted with caution due to cellular heterogeneity, experimental bias, and complications of the telomerase activity assay (19).

FIGURE 1. Distribution of TERT mRNA within breast tissue. TERT mRNA was measured within 36 breast tumors using QRT-PCR and is expressed as relative AU. Tumors are arranged along the abscissa by increasing TERT level and are labeled as tumor (T) 1 to 36. The histopathologic grade of each tumor is shown as shaded bars and is denoted above its corresponding sample number as either grade 1 (hatched samples B & 20), 2 (white), or 3 (black). TERT mRNA was measured within five independent histologically normal samples, and the 95% confidence interval (95% CI) of this normal set is indicated by the shaded area. The distribution of TERT levels within the tumors is displayed as a quantile box plot. The box represents the interquartile range (25-75th percentile) and the line through this box is the median value (0.525). The bottom and top bars of the whisker indicate the minimum and maximum values, respectively, whereas the next set of bars indicates the 10th and 90th percentiles, respectively.

The tumors with above-normal TERT levels were all high grade (P = 0.002, Fisher’s exact test), contained a greater fraction of high S-phase tumors (P = 0.004, Fisher’s exact test), and had increased levels of MYC mRNA (P = 0.034, Wilcoxon rank sum test). The distribution of tumor sizes within this group, however, was not different than the group of tumors with normal TERT levels (P = 0.768, Wilcoxon rank sum test). The three tumors with a below-normal TERT mRNA level did not have any significantly distinguishing characteristics.

Because of the direct activation of TERT transcription by MYC, we investigated the possible relationship between MYC and TERT mRNA levels within the tumors. Although linear regression identified a positive association between MYC and TERT mRNA levels (P = 0.018), MYC was not predictive of TERT (indicated by low r²), indicating a more complex
regulatory mechanism of TERT expression. This is consistent with the findings of Bieche et al. (23), who identified an association between MYC and TERT overexpression, but inconsistent with those of Kirkpatrick et al. (11), who did not find a similar association.

Telomerase lengthens the telomeres by de novo synthesis of TTAGGG repeats on the telomere ends. Without knowledge of the complex model of telomere length regulation (24, 25), it is easy to speculate that tumors with the highest levels of TERT mRNA, and presumably telomerase activity, would also contain the longest telomeres. This however, was not the case. Telomere content, a proxy for telomere length (26, 27), was intense in the WI-38 cells ectopically expressing TERT mRNA (Fig. 2B), whereas TERT fluorescence was absent in the TERT-negative cell line, WI-38 (Fig. 2C), but was intense in the WI-38 cells ectopically expressing TERT mRNA (Fig. 2D). Both TERT and GAPDH signals were abolished by elevating the wash temperatures to 75°C, which is above the Tm of both probes in the ISH buffer (data not shown).

Within the normal breast tissues, there was strong expression of TERT within the cuboidal epithelial cells of the breast lobule and interlobular ducts. Cell types were verified by immunohistochemistry using epithelial-specific (epithelial membrane antigen) and fibroblast-specific (vimentin) antibodies. This is seen in Fig. 3, where the GAPDH probes generated fluorescence throughout the section (Fig. 3A) and TERT probes localized to the epithelial cells (Fig. 3B). In this photo of a representative specimen of normal breast tissue, the cell types, evident by their morphology, were confirmed by immunohistochemistry specific for vimentin (Fig. 3C) and epithelial membrane antigen (Fig. 3D).

The heterogeneous expression within normal breast tissue is evident in Fig. 3E-G, which display the 4,6-diamidino-2-phenylindole (DAPI; blue) channel, TERT ISH (green) channel, and merged images from the terminal lobular unit (Fig. 3E-G) or ductal system (Fig. 3H-J) of another representative normal breast specimen. Again, the TERT fluorescence is strong and specific to the epithelial cells and is absent in the surrounding fibrous areas. However, not all epithelial cells exhibit the same TERT-specific fluorescent intensity, and some of these cells completely lack detectable levels of TERT (Fig. 3G and J, arrows).

It is important to note that the histologically normal samples were derived from sites 2 to 5 cm from the visible tumor margin. It is unclear whether these tissues are truly "normal," as we cannot rule out the possibility that TERT expression may occur as a result of proximity to the tumor by factors either released from the tumor or in response to the tumor. Additionally, we cannot rule out the possibility that TERT is expressed within these cells because they are precursors to tumor cells. To verify expression levels within truly "normal" breast, we measured TERT mRNA within breast RNA originating from a 27-year-old female sudden death case. The level of TERT mRNA in this sample was 1.83 AU and is consistent with measurements from the "histologically normal" tissues proximal to the tumor. Histologic sections from this case were not available for in situ analysis.

Within the set of infiltrating ductal carcinomas, TERT expression was heterogeneous and, as was the case for the normal tissues, found only within epithelial cells or tumor cells of epithelial origin. This is shown in Fig. 4G, where fibroblast cells, identified by immunohistochemistry and lacking TERT staining, are surrounded by cancerous cells expressing TERT. Epithelial-specific expression is also shown in Fig. 4C and F-J, where the TERT-positive cells are bordered by dark fibrous areas. Again, the cell type identity was confirmed by immunohistochemistry (data not shown).

**FIGURE 2.** Specificity of the TERT ISH assay. TERT and GAPDH mRNA were analyzed by ISH in WI-38 normal lung fibroblast cells, which do not express TERT mRNA (0.00 AU by QRT-PCR), and in WI-38 cells retrovirally expressing TERT mRNA, named "WI-38/TERT," which expressed a high level of TERT mRNA (287.56 AU by QRT-PCR). The probes and type of cells used in each experiment are indicated in the left and on top, respectively. GAPDH, used as a positive control, is detectable within both cell types (A and B); whereas TERT-specific fluorescence is not visible within WI-38 cells (C) but is visible in the WI-38/TERT cells (D). ISH probe-specific fluorescence appears green (Alexa Fluor 488), whereas nuclear counterstaining appears blue (DAPI). Magnification, ×200.

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The intensity of TERT staining differed between tumors, and there was also intratumor variation among the TERT-positive cells within tumors. The levels varied from barely detectable (Fig. 4A and B) to intense (Fig. 4E); however, most tumors contained TERT-positive cells whose maximum staining intensities were comparable with those of normal epithelial cells (Fig. 4C, D, and F-J). Figure 4 also shows the variation of TERT expression between adjacent epithelial cells within the tumor section (Fig. 4B-D) as well as between proximal groups of epithelial cells within the same tumor section (Fig. 4E-H).

Tumor samples 1 and 2 (from Fig. 1) are shown in Fig. 4A and B. These tumors expressed TERT at barely visual levels throughout the section and had the lowest QRT-PCR–defined TERT mRNA levels of all breast specimens. Conversely, the tumors with the highest QRT-PCR–defined TERT mRNA levels, tumors 35 and 36 (from Fig. 1), are shown in Fig. 4G-H and I-J. These tumors also contained an expanded epithelial population but had heterogeneous ISH signals that were more intense than those in tumors 1 and 2 and were comparable with levels observed within the normal breast specimens.

**Discussion**

We investigated the bulk measurements of TERT mRNA levels measured by QRT-PCR and compared them with in situ TERT expression within normal breast tissues and breast tumors. Within these breast specimens, we identify the types of cells expressing TERT and characterize the pattern of TERT expression. The principal findings of this study are (a) TERT expression is restricted to epithelial cells, (b) TERT is expressed in both normal and malignant tissues of the breast, (c) the pattern and level of TERT expression are heterogeneous in both tumor and normal tissues, and (d) tumors with above-normal TERT mRNA levels are most likely to have an aggressive phenotype.

The ISH analysis permits direct visualization and comparison of TERT mRNA expression at the cellular level. Using ISH, we found that TERT mRNA expression is confined to the epithelial cells within both normal breast and cancerous cells of breast tumors, which is consistent with previously published studies (13, 28). This most likely reflects telomerase activity within these cells, which cannot be analyzed using in situ techniques. Telomerase activity has been described previously.
in normal breast specimens, albeit most studies report that only a few normal breast samples contain activity (19, 29). However, we report here intense TERT mRNA staining within eight normal breast specimens. These findings are consistent with those of Liu et al., who showed higher amounts of TERT within normal specimens compared with ductal carcinoma in situ (28). Telomerase activity within the normal breast has been attributed to activated infiltrating lymphocytes or other contaminating cell types (19, 29, 30), but our study shows that normal breast epithelium expresses TERT mRNA and is the likely source of telomerase activity.

The expression of TERT mRNA within tumor epithelia is expected, as telomerase activity has readily been detected within carcinomas in previous studies (14, 29). Using a more quantitative method, QRT-PCR, we have identified large differences in bulk TERT mRNA levels as illustrated by the skewed distribution of TERT levels within the tumor samples (Fig. 1). The source of this intertumor variation was investigated by ISH. We found that tumors with the lowest bulk TERT mRNA levels (as measured by QRT-PCR) also had ISH-defined levels that were barely detectable. The remaining tumors had higher levels of fluorescence, and the intratumor heterogeneity was more apparent. Additionally, tumors with an above-normal, bulk TERT level also had large fractions of cells with intense TERT-specific fluorescence. These observations show that the large variability in bulk TERT mRNA levels, as measured by QRT-PCR, can be explained by differences in the fraction of TERT-expressing cells within the samples rather than large variations in the cellular levels of TERT mRNA.

Although the ISH assay is qualitative, we noticed that the maximal fluorescence exhibited by the tumor samples with the highest bulk TERT levels did not appear any greater than the maximum fluorescence contained within the normal samples. In fact, the normal specimens contained subsets of cells that always exhibited intense TERT fluorescence, although the bulk TERT levels were equivalent to the lowest two-thirds of tumors. These normal specimens, however, have substantially fewer epithelial cells within each section compared with the tumor sections and thus fewer cells to contribute to the bulk TERT measurement (Figs. 3 and 4). We can therefore surmise that a subset of the epithelial cells within the normal tissue express higher cellular levels of TERT mRNA than the average tumor epithelial cell to have bulk TERT mRNA levels equivalent to those within the majority of tumors. Telomerase activity, however, is typically ascribed to breast tumors and not to normal breast tissue, although the published results from multiple studies vary widely (reviewed in ref. 19). It is possible that the nature of normal breast tissue (mostly fatty tissue, containing few epithelial cells) prevents a precise evaluation of telomerase activity within these tissues. TERT expression within the normal epithelial cells suggests that these normal cells do have telomerase activity and may explain why primary cell cultures derived from normal breast tissue show telomerase activity. The presence and relative abundance of these TERT-expressing epithelial cells within normal breast tissue lead us to speculate that tumors of the breast arise from these TERT-expressing epithelial cells.

Within our study, telomerase (TERT) was expressed within all tumors as well as within all histologically normal tissues. This finding was confirmed and further evaluated by ISH analysis. We find that the bulk levels of TERT within normal breast are equivalent to those found within the majority of tumors and can be explained by increased cellular expression.
within normal breast epithelia. There was, however, a set of tumors that expressed above-normal bulk levels of TERT, and we show that these tumors had a high fraction of intensely fluorescent TERT-positive cells. They also had a more aggressive phenotype, as they were more likely to be higher grade, have a large population of cells in S phase, and express higher levels of MYC. This indicates that telomerase expression either is a consequence of tumor progression or more likely, given the relationships among telomerase, telomeres, and genomic stability, is providing a selective advantage to the tumor cell. This may explain telomerase’s prevalence within so many different tumor types, including breast carcinoma.

Materials and Methods

Breast Tissue Samples

Thirty-six anonymous, frozen breast tumor specimens were obtained from the University of New Mexico Cancer Research and Treatment Center Solid Tumor Facility. In most instances, tumor size, lymph node status, and the fraction of cells in S phase (based on flow cytometry cell cycle analysis) were included within clinical histories provided with each specimen. Anonymous, unpaired, histologically normal (tumor adjacent) breast specimens, obtained from sites 2 to 5 cm from the visible tumor margin, were provided by the University of New Mexico Department of Pathology. Normal breast RNA, originating from a 27-year-old female sudden death case, was purchased from Clontech (Palo Alto, CA).

Cell Culture

MCF-7 mammary epithelial cells, WI-38 normal lung fibroblast cells, and PA317 mouse embryonic cells were obtained from American Type Culture Collection (Rockland, MD) and were propagated as recommended. WI-38/TERT cells were created by infecting WI-38 cells with a pBABEpuro retrovirus containing the TERT coding sequence and were selected by puromycin resistance (courtesy of Geron, Menlo Park, CA).

Preparation of Tissue Sections and RNA Isolation

Serial frozen sections of breast samples, 10 μm in width, were mounted on Colorfrost slides (VWR, West Chester, PA) and stored at −70 °C. Specimens were either stained with H&E and examined by a board-certified surgical pathologist, who assigned a histopathologic grade to the tumor and analyzed the tumor size, lymph node status, and the fraction of cells in S phase (based on flow cytometry cell cycle analysis) were included within clinical histories provided with each specimen. Anonymous, unpaired, histologically normal (tumor adjacent) breast specimens, obtained from sites 2 to 5 cm from the visible tumor margin, were provided by the University of New Mexico Department of Pathology. Normal breast RNA, originating from a 27-year-old female sudden death case, was purchased from Clontech (Palo Alto, CA).

Table 1. QRT-PCR Primer/Probe Sequences and Final Concentrations

<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>TERT (ref. 8; 300 nmol/L forward/600 nmol/L reverse)</td>
<td>5'-CGGAAGAGTGCTGCTGGAGCAGAA</td>
<td>5'-GGATGAAAGCGGAGTGCTCGGA</td>
</tr>
<tr>
<td>MYC (300 nmol/L forward/300 nmol/L reverse)</td>
<td>5'-CTGTCACTTACAGAGGAGGAG</td>
<td>5'-AGGATCCAGACTGCTGA</td>
</tr>
<tr>
<td>TBP (ref. 23; 600 nmol/L forward/900 nmol/L reverse)</td>
<td>5'-ACAGTACACCCGACGCCTGGAC</td>
<td>5'-TTTCTTGTGCCCCATGGAC</td>
</tr>
<tr>
<td>TERT Taqman probe (250 nmol/L)</td>
<td>5'-6FAM-TGTGCACAGGAGCCAAGAGAAGA-TAMRA</td>
<td>5'-6FAM-TGTGCACAGGAGCCAAGAGAAGA-TAMRA</td>
</tr>
<tr>
<td>MYC Taqman probe (250 nmol/L)</td>
<td>5'-6FAM-CACCCACGCGAGCCTGGACAG-TAMRA</td>
<td>5'-6FAM-CACCCACGCGAGCCTGGACAG-TAMRA</td>
</tr>
<tr>
<td>TBP Taqman probe (250 nmol/L)</td>
<td>5'-6FAM-CCAACACCCGCGAGCCTGGACAG-TAMRA</td>
<td>5'-6FAM-CCAACACCCGCGAGCCTGGACAG-TAMRA</td>
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Quantitative Real-time RT-PCR

cDNA was synthesized by random decamer primed reverse transcription using the Retroscript reverse transcription kit (Ambion) and the manufacturer’s standard protocol. Negative controls contained RNase-free water substituted for reverse transcriptase. The mRNA levels of TERT, MYC, and TATA-binding protein (TBP) were measured within breast specimens and the MCF-7 mammary epithelial cell line, WI-38 normal lung fibroblast cell line, and WI-38/TERT retrovirus cells using the Taqman real-time PCR assay (Applied Biosystems, Foster City, CA). TERT (Genbank accession no. NM_003219, bp 1,784-1,928) or MYC (Genbank accession no. V00568 bp 1,270-1,404) was amplified in parallel with the internal control, TBP (Genbank accession no. M55654, bp 861-949), and in quadruplicate, from equal starting amounts of cDNA (1 μL reverse transcription reaction). The TERT primers amplify a sequence element, spanning the splice junction between exons 2 and 3, which is present in the full-length transcript and all known splice variants. The sequences and optimal final concentrations of forward primers, reverse primers, and Taqman probe for the three systems are described in Table 1.

Amplification of TERT, MYC, and TBP cDNA was done using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and the manufacturer’s standard amplification protocol. The relative levels of mRNA were calculated from standard curves generated by coamplification of plasmid standards. The TERT and MYC mRNA levels were normalized to TBP mRNA levels and are expressed as relative AU.

In situ Hybridization

Digoxigenin-labeled antisense probes for TERT and GAPDH were created by in vitro transcription of T7 promoter–tailed PCR products using a nucleotide labeling mix containing digoxigenin-11-UTP (Roche, Penzberg, Germany). The GAPDH T7 antisense probe template was available from a commercial source (pTRI-GAPDH-Human, Ambion), and the TERT antisense probe template was generated using the forward primer 5'-TTTTATGTCACCGGAGACCAAC and the T7-tailed reverse primer 5'-TAATACGACTCACTATAAGAGCTGCTCAGATTCACAAAAGC. PCR cycling variables were identical to those used in QRT-PCR. RNA probe concentrations were measured
with the Ribogreen fluorescent RNA quantitation assay (Molecular Probes, Eugene, OR), and the labeling efficiency was evaluated by comparison to digoxigenin-labeled control RNA (Roche).

Frozen tissue sections or cultured cells were fixed in 4% paraformaldehyde and pretreated with 3% hydrogen peroxide and 0.3% Triton X-100. Specimens were hybridized overnight at 42°C with 200 ng/mL TERT probe, 200 ng/mL GAPDH probe, or RNA hybridization mixture alone (DakoCytomation, Carpinteria, CA). Sections were washed at 65°C in 1 x high stringency wash solution (DakoCytomation) and the digoxigenin-labeled probes were detected using anti-digoxigenin peroxidase-conjugated antibody (Roche). The sections were subsequently treated with biotinyl-tyramide (Genpoint, DakoCytomation), a peroxidase substrate that leads to the localized deposition of biotin. Fluorescent signal was generated by further incubating these sections with 10 ng/mL Alexa Fluor 488–conjugated streptavidin (Molecular Probes). Two negative controls were used for each assay: (a) serial sections were incubated without an antisense probe and (b) serial sections were hybridized with probe but were withheld biotinyl-tyramide amplification reagent during the detection step. Sections were counterstained with nuclear dye, DAPI (Molecular Probes). Images were captured using a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Oberkothen, Germany) and photographed with a CCD camera (Hamamatsu, Bridgeport, NJ). Hybridizations were done in duplicate and repeated on serial sections to confirm results.

Immunohistochemistry

Cells within tissue sections were characterized by immunohistochemistry. Frozen breast sections were fixed in acetone, washed in PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, 2 mmol/L KH2PO4), and incubated overnight with mouse monoclonal anti–epithelial membrane antigen IgG (1.65 mg/L), mouse anti-vimentin IgG (2.75 mg/L; DakoCytomation), primary antibodies, or antibody diluent lacking a primary antibody (negative control). Sections were washed in PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, 2 mmol/L KH2PO4), and incubated without an antisense probe and (b) serial sections were hybridized with probe but were withheld biotinyl-tyramide amplification reagent during the detection step. Sections were counterstained with nuclear dye, DAPI (Molecular Probes). Images were captured using a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Oberkothen, Germany) and photographed with a CCD camera (Hamamatsu, Bridgeport, NJ). Hybridizations were done in duplicate and repeated on serial sections to confirm results.

Statistical Analysis

All statistical analyses were done using JMP version 5.1 software (SAS Institute, Cary, NC). Exact Ps for 2 x 2 frequency tables were calculated by the Fisher’s exact test. Distributions between groups were tested by the Wilcoxon rank distribution.

References

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