Careful Histological Confirmation and Microdissection Reveal Telomerase Activity in Otherwise Telomerase-negative Breast Cancers

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ABSTRACT

Studies of invasive breast cancers consistently identify a subset of tumors without telomerase activity, compromising its utility as a tumor marker. Telomerase-negative tumors may represent a biologically different subset, or the result could be attributed to assay imperfections. To resolve this issue, we tested 105 invasive breast cancers for telomerase activity and found that 23 (22%) tumors were telomerase negative. Careful histological confirmation of an adjacent cryosection and/or microdissection of pure tumor cells reduced this number to 5 (5%). Thus, truly telomerase-negative invasive breast cancers are rare, making this enzyme a potentially very useful tumor marker in breast cancer.

INTRODUCTION

Telomerase is a ribonucleoprotein enzyme active in germ cells, immortal cell lines, and the majority of malignancies examined, but it is inactive in most normal somatic tissues (1). It has been proposed as a potentially useful marker of malignancy. In several studies of primary invasive breast cancers, approximately 75–95% of tumors demonstrate telomerase activity, whereas 5–25% are telomerase negative (2–7). Obviously, telomerase activity as a marker of invasive breast cancer is more useful if it detects 95% of the tumors rather than 75%; therefore, the determination of the real telomerase-negative proportion of invasive breast cancers becomes crucial. Additionally, some studies (2, 3), although not all (4, 5), have found that smaller, lymph node-negative tumors are more likely to be telomerase negative than their larger, lymph node-positive counterparts. Based upon this association between telomerase activity and traditional staging parameters, Hiyama et al. (2) postulated that telomerase activity is acquired during tumor progression to metastasis. If this is true, then telomerase-positive tumors are biologically different (i.e., have the capability for metastasis) from their telomerase-negative counterparts, and this marker may be useful in clinical decision-making. An alternative explanation for telomerase-negative tumors is that a negative result is an artifact of the method of testing, and that the absence of telomerase activity in such tumors represents a false-negative result. Possible reasons for a false-negative result in the TRAP assay include insufficient viable invasive cancer cells examined, poor tissue maintenance, inadequate PCR amplification, or telomerase inhibitors in the surrounding tissue. To determine whether telomerase-negative tumors are truly telomerase negative or represent methodological imperfections, we screened a series of breast cancers for telomerase activity.

MATERIALS AND METHODS

Samples. The tissue samples used in this study were obtained from an existing frozen tumor bank in the Johns Hopkins Breast Cancer Research Program. The database for this tumor bank includes information regarding the gross tumor size and presence or absence of lymph node metastases in the breast cancers. No information regarding distant metastases is available; therefore, formal staging was not performed. Only primary breast cancer specimens were considered for entry into the study. In addition, six specimens of normal breast tissue from reduction mammoplasties and mastectomy distant from the cancer site were included as negative controls. All tissue specimens were obtained from excess clinical specimens, and institutional guidelines for the acquisition and maintenance of such specimens were followed.

Screening. Breast tissue samples stored at −80°C in the Johns Hopkins Breast Cancer Program Tissue Inventory were tested for telomerase activity using the TRAP assay (3). For the
original screening, a small quantity of frozen tissue (approximately equal to 5–10 10-μm sections) was shaved from the specimen into 60 μl of telomerase lysis buffer [1 × TLB = 0.5% (3-[1-cholamidopropyl-dimethyl-ammonio]-1-propane sulfonate), 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.1 mM (4-[(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and 10% glycerol; Ref. 3]. The tissue samples were homogenized by physical disruption using the barrel of a 30-gauge needle and then left on ice for 30 min. The lysate was then centrifuged at 13,000 × g for 25 min at 4°C, and the supernatant was removed. Protein concentration was measured in each extract using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) and adjusted to 0.1 μg/μl TLB. The extracts were flash frozen in liquid nitrogen and stored at −80°C until use. All extracts underwent TRAP assay within 4–6 weeks. The final pathology report from the paraffinized sections obtained at the time of the resection was reviewed for each of the breast tumors and confirmed as to the diagnosis of invasive breast cancer.

**Histological Confirmation and Microdissection.** All telomerase-negative breast cancers identified in the original screening were subjected to serial sectioning on a cryostat to obtain five to nine serial 10-μm sections on five slides, which were maintained at −80°C. One of these (from the middle section) was fixed, stained with H&E, and evaluated by the study pathologist. If a section was judged to have <10% invasive tumor or to exhibit necrosis, all of the slides were discarded, and the tumor was resectioned until an acceptable area for testing was obtained. If an acceptable section was not obtained after three different areas of the gross tumor were cut, the banked tumor specimen was judged not to include viable invasive cancer. Once an acceptable area had been sectioned, the remaining four slides were divided; one was used for TRAP testing of the entire 10-μm section, and the other three were used for microdissection of a pure tumor sample.

For the histologically confirmed testing, one 10-μm cryosection of the frozen tissue sample was scraped off of the slide, homogenized with a 30-gauge needle, and lysed in 40 μl of TLB. For the testing of microdissected samples, an appropriate area of the tumor section was identified and marked on the H&E section by the study pathologist. This area was judged to contain invasive tumor and to be free of contaminating inflammatory cells or necrosis. Microdissection was performed on the adjacent frozen 10-μm section. The slide was quick-thawed, and microdissection was immediately performed using a 30-gauge needle on a 1-ml syringe under a dissecting microscope at ×100 similar to the manner described previously (Ref. 8; Fig. 1). The microdissected sample was lysed in sufficient TLB to contain at least 20 tumor cells per μl of TLB. Preliminary examination of known telomerase-positive breast cancer samples revealed that telomerase activity could be reliably identified in microdissected samples containing at least 50 tumor cells (data not shown). For this reason, all microdissected samples contained at least 100 tumor cells in 5 μl of TLB to be tested. The extraction was performed as described above, and the supernatants were flash frozen and maintained at −80°C. All extracts underwent TRAP assay within 4–6 weeks. **TRAP Assay.** Each assay was run accompanied by an intraassay control, where the tissue lysate was inactivated by heating to 94°C for 10 min or by the addition of 0.1 μg of RNase A and incubation at 37°C for 10 min. Each set of 20 assays included a negative control, which contained the PCR reaction mixture only, no extract, as well as a positive control extract, containing 0.5 μg of protein, from HBL-100 cells (American Type Culture Collection, Rockville, MD), an immortal cell line derived from human breast epithelium. This cell line has inherent telomerase activity. The one-tube telomerase assay was performed as described by Wright et al. (9) with minor modifications. In brief, 5 μl of the cell extract containing either 0.5 μg of protein (whole sections) or the lysate from at least 100 tumor cells (microdissections) were added to a 50-μl reaction mixture containing 20 mM Tris-HCl (pH 8.3), 68 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 0.005% Tween-20, 50 μM deoxyribonucleotide triphosphate, 0.2 μg of TS oligonucleotide (5′-AAATCCGTCGACGAGTT-3′; Operon) end-labeled with γ32P-ATP via the T4 polynucleotide kinase reaction, 5 attograms of an ITAS, and 2.5 units of Taq DNA polymerase. The assay tubes additionally contained 0.1 μg of CX oligonucleotide

![A](https://example.com/image1.jpg) ![B](https://example.com/image2.jpg)

**Fig. 1** Photomicrographs demonstrating the technique of microdissection. **A,** invasive tumor before microdissection; **B,** an adjacent cryosection of the same tumor after microdissection, demonstrating the removal of a pure population of tumor cells.
Table 1  Comparison of the presence or absence of telomerase activity with tumor size and lymph node metastasis in the screened breast cancers

<table>
<thead>
<tr>
<th>Tumor size, cm</th>
<th>Telomerase positive n (%)</th>
<th>Telomerase negative n (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤2</td>
<td>22 20 (91)</td>
<td>2 (9 ± 12)</td>
</tr>
<tr>
<td>&gt;2</td>
<td>80 60 (75)</td>
<td>20 (25 ± 10)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3 2 (67)</td>
<td>1 (33)</td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>31 24 (77)</td>
<td>7 (23 ± 15)</td>
</tr>
<tr>
<td>Present</td>
<td>51 37 (72)</td>
<td>14 (27 ± 12)</td>
</tr>
<tr>
<td>Unknown</td>
<td>23 21 (91)</td>
<td>2 (9)</td>
</tr>
</tbody>
</table>

(5′-CCCTTACCCTTACCCTTACCCTAA-3′) sequestered under a wax barrier by the HotStart method (Life Technologies, Inc., Gaithersburg, MD). The reaction mixtures were incubated at room temperature for 45 min, allowing the telomerase enzyme to extend the TS substrate. The reaction mixture was then heated to 94°C for 5 min, followed by 30 cycles of PCR at 56°C for 30 s, 72°C for 45 s, and 94°C for 30 s. The PCR products were electrophoresed on a 10%, nondenaturing, 0.5-mm polyacrylamide gel (18 × 16 cm) run in 0.5× TBE buffer at 350 V until the bromphenol blue marker dye had run off. The gels were dried and exposed overnight to Kodak X-OMAT film (Eastman Kodak, Rochester, NY). Results were scored in a binary fashion, with a positive result defined as any banding pattern (laddering) beyond background, and a negative result was scored as no banding beyond background with a positive ITAS control sequence. If the ITAS control sequence was absent in a lane with no banding, the PCR reaction was repeated following phenol/chloroform extraction of the enzyme assay. All negative results were repeated for confirmation when possible. To determine whether tissue degradation was responsible for the persistent telomerase negativity, we performed a 28S rRNA assay on the tissue lysates. The assay consists of a single-step reverse transcription-PCR amplification and quantification of a 91-bp fragment of 28S rRNA from the tissue lysates using the EZ RNA PCR kit (Perkin-Elmer, Foster, CA). All negative results were repeated for confirmation when possible. To determine whether tissue degradation was responsible for the persistent telomerase negativity, we performed a 28S rRNA assay on the tissue lysates. The assay consists of a single-step reverse transcription-PCR amplification and quantification of a 91-bp fragment of 28S rRNA from the tissue lysates using the EZ RNA PCR kit (Perkin-Elmer, Foster, CA).

Results

Screening. Twenty-eight (27%) of the 105 tumors tested were negative initially for telomerase activity by the TRAP assay. Ten of these showed no internal control (ITAS) band, suggesting the presence of PCR inhibitors in the reaction. Following phenol/chloroform extraction, ITAS was positive in all 10 tumors; 5 showed positive telomerase banding pattern, whereas 5 remained negative for telomerase. Thus, 23 (22%) of the 105 tumors lacked detectable telomerase activity. The proportion of tumors exhibiting telomerase activity did not differ significantly by gross tumor size or lymph node status (Table 1).

Six normal breast tissue samples obtained from reduction mammoplasty (5 specimens) and 14 mastectomy samples distant from the primary site (4 specimens) were uniformly negative for telomerase activity (data not shown). Each section was histologically confirmed to contain at least 5% breast epithelium.

Histological Confirmation. The TRAP assay typically involves very small quantities of tissue extract for two reasons: its sensitivity and the tendency for tissues to contain inhibitors of the PCR amplification step. The purpose of the histologically confirmed method of testing is to directly assure that an adequate proportion of the tissue being tested in fact contained viable, nonnecrotic, invasive breast cancer cells, an important step given the gross heterogeneity of breast cancers. One hundred and five tumors were screened. In all cases, the pathological report of the paraffinized tumor sample from the original sectioning confirmed the diagnosis of invasive breast cancer. All 23 telomerase-negative tumors identified in the original screening effort underwent sectioning with histological confirmation of the adjacent 10-μm section (Fig. 2). Seven (30%) did not have sufficient invasive tumor in the initial sectioning to be tested (one necrotic, two in situ carcinoma only, and four without notable tumor cells at all). Further sectioning of the tumor mass provided an adequate sample for testing in three tumors. However, in four tumors, no adequate tumor sample was ever obtained, despite three separate sectioning efforts (one necrotic tumor, two residual in situ carcinoma only, and one without any residual cancer). Once an adequate section containing more than 10% viable invasive carcinoma was obtained in the 19 remaining tumors, TRAP analysis of the entire section was performed. Thirteen (68%) were telomerase positive (Fig. 3, left panel), 6 (32%) were telomerase negative (Fig. 3, middle panel).

![Flow diagram depicting the results of histological confirmation, microdissection, and subsequent TRAP analysis of banked breast cancers](image-url)

**Fig. 2** Flow diagram depicting the results of histological confirmation, microdissection, and subsequent TRAP analysis of banked breast cancers that were telomerase-negative on original screening.
Telomerase Activity in Telomerase-negative Breast Cancer

**Fig. 3** Left panel: telomerase activity in one tumor that was telomerase negative in the original screening (1A) but was telomerase positive in the histologically confirmed whole section (1B). In this as in all telomerase-positive sections, the microdissected pure tumor sample also remained telomerase positive (1C). Middle panel: telomerase activity in one tumor that was telomerase negative in the original screening (data not shown) and was very weakly telomerase positive in a histologically confirmed whole section (2B); but it was clearly telomerase positive following microdissection (2C). Right panel: telomerase activity in one tumor that was telomerase negative in the original screening (data not shown) and remained telomerase negative in spite of histological confirmation (3B) and microdissection (3C). Cell lysates of HBL100 breast epithelial cells (0.5 μg of protein) served as a positive control, whereas the negative control reaction contained an equal volume of lysis buffer in lieu of tissue lysate. Inactivation of the telomerase activity in the extracts by heating the reaction to 95°C for 10 min is indicated by + on top of the lanes. The amplification products of 10 attograms of the ITAS is shown.

and right panels) in repeated assays. By using this technique to improve the quality of the tissue being tested for telomerase activity, only 6 (6%) of 105 breast cancers lacked detectable telomerase activity (Fig. 2).

**Microdissection.** Breast tumors often include normal stroma, in situ carcinoma, and fibrosis from previous manipulation such as biopsy. The purpose of the microdissection method of testing is to test a pure sample of tumor cells without stroma, inflammation, or other contaminating cells. The six tumor sections that remained telomerase negative after histological confirmation were microdissected, and a pure tumor sample was tested. One was telomerase positive (Fig. 3, middle panel), whereas five remained telomerase negative (Fig. 3, right panel). Of the five tumors that remained persistently telomerase-negative despite careful histological confirmation and microdissection, two were smaller than 2 cm, two were larger, and one was inflammatory. Two were from patients without axillary lymph node involvement, and three were from patients with involved nodes. Thus, of the 105 tumors screened, the addition of this technique decreased the proportion of telomerase-negative tumors to 5% (Fig. 2).

As a control for the technique of microdissection, nine of the tumors that tested positive after histological confirmation were microdissected, and the pure tumor sample was tested. All remained telomerase positive (data not shown).

**Tumor Heterogeneity.** When possible, a second set of sections from a different region of the 19 screened telomerase-negative tumors was cut and tested for telomerase activity using the same methods described above. In this way, a separate section of viable invasive carcinoma from another area of the tumor bulk was tested in 15 of the tumors. In 11 of these 15 tumors, the second area gave similar results to the previous telomerase assay. Four tumors, however, demonstrated telomerase activity heterogeneity in that the second facet of the tumor mass gave a reproducibly different result from the initial facet tested. As before, a telomerase-negative result was not considered truly negative unless the microdissected sample was tested twice without evidence of telomerase activity. One tumor that demonstrated telomerase activity heterogeneity after careful histological confirmation was found to be telomerase positive consistently after microdissection, suggesting that the difference between the two facets of the tumor related to stromal differences, perhaps due to the presence of telomerase inhibitors. The other three tumors remained heterogeneous at the level of the tumor cells, with a telomerase-positive region and a telomerase-negative region despite microdissection.

**Tissue Degradation.** The five tumors that remained persistently telomerase negative even after histological confirmation and microdissection were tested by a quantitative reverse transcription-PCR assay for a fragment of 28S rRNA. All five tumor lysates, previously used for TRAP assays, had cDNA levels comparable to samples with high telomerase activity, indicating that the tissue itself had not undergone significant degradation.

**DISCUSSION**

In this study, we have tried to define the true telomerase-negative proportion of invasive breast cancers. This definition carries both biological and clinical implications. If there exists a subset of invasive breast cancers that lack telomerase activity, an obvious question is whether such cancers are inherently less aggressive than their telomerase-positive counterparts. Clinical support for this concept comes from studies by Hiyama et al.
are several potential reasons for a histologically confirmed detected telomerase activity in the pure tumor cell sample. There tive tumors with microdissection by analysis of pure tumor samples following microdissection detected telomerase activity in one sample. Thus, the proportion of invasive breast cancers without telomerase activity was found in this study to be 5%. Microdissection and TRAP analysis of one of four telomerase-negative portions of the telomerase-heterogeneous tumors also detected telomerase activity in the pure tumor cell sample. There are several potential reasons for a histologically confirmed telomerase-negative tumor to be telomerase-positive when a microdissected pure tumor sample is tested. Stromal dilution of the telomerase-positive tumor cells is possible but unlikely given the sensitivity of the TRAP assay and the fact, that to be eligible, the section had to contain at least 10% tumor cells. Inhibition of PCR amplification by Taq inhibitors could also cause a false-negative result, although the detection of the ITAS internal control sequence makes the presence of Taq inhibitors less likely. Finally, telomerase inhibitors in the surrounding tissue could dampen telomerase activity of the tumor cells while allowing the ITAS control sequence to be amplified normally. Such inhibitors, which have been demonstrated in cancer tissues (10), would be unlikely in a pure tumor population, such as a microdissected specimen.

The reason for the absence of telomerase activity in the tumors that remained persistently telomerase negative is unclear. Evaluation of the size and lymph node status of the tumor is notable for the variety of stages represented, arguing against a link with prognosis, although without outcome data this is impossible to truly assess. It is possible that enzymatic degradation occurred in tumors that were originally telomerase positive, e.g., if the tissue was mishandled at the time of collection. However, all five persistently telomerase-negative tumors had robust 28S rRNA levels, arguing against significant tissue damage. Regardless of cause, these tumors appear to represent a very small fraction of the total population of invasive breast cancers.

Heterogeneity in telomerase activity was found in three breast cancers of the 15 tumors with two different areas examined. These tumors had areas that were reproducibly telomerase positive and other areas that were telomerase negative, even in microdissected pure tumor cell samples. This suggests that telomerase activity, like genomic instability, can be heterogeneus from one area of the invasive cancer to another and could be acquired during tumor progression.

This study demonstrates that telomerase activity is nearly ubiquitous in invasive breast cancers. Based on this finding, the previous studies mentioned earlier in which telomerase activity was absent from a substantial proportion of tumors were likely confounded by gross or microscopic tumor heterogeneity, producing a false-negative result in the telomerase activity assay. Future investigators, especially those working in breast cancer, should be skeptical regarding the content of banked tumors and confirm all samples by staining of an adjacent section or similar method of direct histological confirmation. In this study, this technique alone raised the percentage of telomerase-positive tumors to 95%. Microdissection, although identifying the true telomerase-positive nature of a few samples more, is too tedious and contributes too little to be used routinely. It does, however, allow testing of a pure tumor sample and would also be useful in studies of a progression model for the activation of telomerase within tumors and to dissociate the ability of neighboring stromal cells to inhibit telomerase activity.

Based upon the results of this study, which suggest that telomerase activity is present and readily measurable in the vast majority of invasive breast cancers, future directions of exploration might involve the use of this tumor marker as a diagnostic tool. For instance, in the future, telomerase activity in cells extruded in nipple aspiration fluid could provide a valuable adjunct to mammography, especially in young women. In ad-
dition, our data imply that exploration of telomerase positivity or negativity as a predictor of response or outcome is not likely to be fruitful, although quantitative examination of this marker by newly developed methods (11, 12) may in fact prove useful.

REFERENCES
Careful histological confirmation and microdissection reveal telomerase activity in otherwise telomerase-negative breast cancers.


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