Analysis of Telomerase Activity Levels in Breast Cancer: Positive Detection at the in Situ Breast Carcinoma Stage

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Abstract
Telomerase activity has been implicated to be associated with most human malignant tumors, including breast cancer. To evaluate possible associations with well-known prognostic factors in breast cancer, we performed a semiquantitative analysis of telomerase activity levels using the very sensitive PCR-mediated telomeric repeat amplification protocol. Telomerase activity was detected in 99 of 104 breast cancer samples analyzed (95.2%), whereas no activity was detected in 10 of 10 adjacent nonmalignant breast tissues. Analysis of five breast fibroadenoma samples revealed telomerase activity in one (20%). In contrast to previous observations, we observed that 100% of stage I breast tumors were positive for telomerase activity. More interestingly, we detected telomerase activity in six of six ductal carcinoma in situ samples (i.e., stage 0). In our semiquantitative analysis of levels of enzymatic activity, we found no statistically significant correlation at the \( P < 0.05 \) level between telomerase levels and lymph node metastasis status, estrogen and progesterone receptor status, tumor size, S-phase fraction, and ploidy. The only statistically significant correlation was found with patient age (rho = −0.3; \( P = 0.03 \)). We observed no statistically significant difference in the telomerase activity levels of early tumors (stages 0 and I) versus more advanced lesions (stages II to IV). Nevertheless, stage IV tumors displayed a tendency for higher telomerase activity levels. In summary, no clear association was observed between telomerase levels and known breast cancer prognostic indicators. However, telomerase detection by the telomeric repeat amplification protocol method, due to its high sensitivity, may be of value in early breast cancer diagnosis and detection, because our data indicate that telomerase reactivation appears to constitute a relatively early event in breast carcinogenesis.

Introduction
The specialized synthesis of telomeric repeats, TTAGGG, in vertebrates, was demonstrated to be performed by the ribonucleoprotein telomerase using its RNA component as a template (1–3). It has been postulated that telomerase activity is associated with acquisition of an immortal phenotype in vitro, and it was shown that most immortal cell lines express this enzyme (reviewed in Ref. 4). Using a very sensitive PCR-based TRAP assay,\(^3\) it has been demonstrated that most human adult somatic tissues do not show evidence of active telomerase (5). Activity of this enzyme, however, has been observed in germ line cells, bone marrow, activated peripheral blood lymphocytes, and possibly stem cells (reviewed in Ref. 4). Interestingly, telomerase activity was detected in numerous human cancer types, suggesting that tumor cells may need reactivation of this enzyme to remain viable (4, 6–14).

Breast cancer is one of the tumor types in which telomerase activity has been demonstrated (12). Furthermore, it has been suggested recently that telomerase detection could have prognostic implications in breast cancer (5, 12). Exactly when in the process of breast carcinogenesis reactivation of telomerase occurs remains to be determined.

Although numerous somatic mutations affecting various genes have been described in sporadic breast cancer, it still remains to be determined which anomalies could be considered causative of breast carcinogenesis. Certain specific aberrations, such as ERBB2 or EGFR amplification and overexpression and P53 mutation, have been postulated and explored as of possible aid in determining breast cancer prognosis (15–18).

In this report, we analyze possible associations of telomerase with well-known prognostic factors in breast cancer to further evaluate whether telomerase detection and quantification could have potential impact on breast cancer prognosis.

Materials and Methods
Unselected breast cancer samples were obtained from the Cooperative Human Tissue Network (about one-half of the samples) and from the Department of Pathology, University of Texas M. D. Anderson Cancer Center. Breast cancer samples and adjacent nonmalignant breast tissues were obtained from 104 patients, frozen, and stored at −80°C until use. We also analyzed a group of five breast fibroadenomas obtained from Cooperative Human Tissue Network. For most of the breast cancer samples, information about estrogen and progesterone

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\[^3\] The abbreviations used are: TRAP, telomeric repeat amplification protocol; ITAS, internal telomerase assay standard; DCIS, ductal carcinoma in situ.
receptor status, lymph node metastasis status, S-phase, ploidy, tumor stage, and age of patients were available. Tumor staging was performed according to the guidelines of the American Joint Committee on Cancer (19).

The TRAP assay allows the detection of in vitro telomerase products (5, 20). We used a modified version of this assay. In brief, telomerase adds TTAGGG repeats to the 3' end of TS primer (5'-AATTCCGTCGAGCAGAGTT-3'; Refs. 5 and 21). The number and amount of the repeats added is dependent upon telomerase activity. In a second step, telomerase products are amplified using the CX primer (5'-CCCTTACCCCTACCTTACCCCTTACCCA-3') and Taq DNA polymerase. As positive control, a cell extract from a sample with known telomerase activity (a rat mammary tumor line) was used. As negative control, lysis buffer was substituted for cell extract (20). To compare the level of telomerase activity in different tumor samples, we used a semiquantitative analysis based on the use of an internal standard (ITAS), which amplifies from the same primers (22). This internal standard, which consists of a 150-bp DNA product, allows identification of false-negative tumor samples that could contain Taq polymerase inhibitors.

Cell extracts were obtained from 10–50-mg samples of the tumors, and telomerase assay was performed according to a method described previously (5, 20), with minor modifications (22, 23). In a standard procedure, we used 2 μl of tissue extract (protein concentration, 0.5 μg/μl per assay). The CX primer, ITAS, and Taq DNA polymerase (7 units/assay) were added to each sample at a "hot start" after 5 min incubation at 90°C. Because telomerase has an RNA component, 5 μl of the tumor cell extract from the same samples and the positive cell extract were incubated with 1 μl of RNase A (1 mg/ml) as an additional experimental control.

Aliquots (10 μl) of the PCR mixture were analyzed on 8% non-denaturating, 0.4-mm acrylamide gels (20 × 40 cm) run in 0.5× TBE buffer until the xylene cyanol had migrated 17 cm from the origin. The gels were then dried and exposed for 20 h to hyperfilm MP films (Amersham Corp., Arlington Heights, IL). Following autoradiography, each gel was analyzed after overnight exposure using a Molecular Dynamics Phosphorlmager (Sunnyvale, CA). This scan was used to perform the measurements of the telomerase ladder amplification intensity. Thus, area integration of all peaks (except the first band from the bottom) were normalized to the signal from the internal standard and then, after background subtraction, expressed as relative to the positive control signal that was run with each experiment. The first band from the bottom was not included, because it usually incorporates background from primer-dimer formation (8, 13). The method described is only semiquantitative, but it is sufficient for the comparative analysis of the tumors relative to the same positive-control cell extract.

Analysis of the levels of telomerase activity and other clinico-pathological characteristics was performed using non-parametric Spearman rank correlation and t test.

Results and Discussion

To confirm the linearity of the TRAP assay, we performed a dilution experiment of the telomerase-positive breast cancer cell line MDA-MB-157 (Fig. 1). The activity of the telomerase ladder was normalized to that of the internal standard (ITAS; Ref. 22), expressing the results relative to the activity found in the 100-cell equivalent sample. Linearity of activity with cell number was observed (Fig. 1), in agreement with observations published previously (22).

Telomerase activity was then analyzed and detected in 99 of the 104 breast cancer samples (95%). It was undetectable in 10 of 10 adjacent nonmalignant breast samples (Fig. 2). As expected, the enzymatic activity detected is abolished if samples are pretreated with RNase A (Fig. 2).

Interestingly, we detected telomerase activity in six of six DCIS samples analyzed (Fig. 3A; stage 0 in Fig. 4A). Four of these DCIS samples were classified as high nuclear grade and showed high telomerase activity, whereas the two samples classified as low nuclear grade showed very weak telomerase activity (Fig. 3A). These findings suggest that the reactivation of telomerase activity occurs relative early in breast carcinogenesis. We also analyzed a small group of breast fibroadenomas for telomerase activity and observed one positive tumor of five tested (20%).

Several clinical, histological, and biological indicators of prognosis are commonly used to determine the therapeutic management of breast cancer patients. Marker combinations are usually more accurate than single markers (18, 24). A major goal of our study was to evaluate the role of telomerase detection as a possible additional prognostic indicator. Our overall incidence of telomerase-positive breast cancer samples (95.2%) is similar to that reported previously by Hiyama et al. (Ref. 12; 93%). However, whereas Hiyama et al. (12) found 68% of stage I breast carcinomas to be positive for telomerase activity, we found 100% of the 17 stage I breast carcinomas to be positive. The few tumors negative for telomerase activity were found to be advanced-stage (II and III) rather than early-stage tumors, as can be observed in Fig. 4A, in which breast cancer samples are grouped by tumor stage. Furthermore, we did not find any correlation between the very few tumors (five samples) found to
Fig. 2. TRAP assay from human breast cancers (T) and matching adjacent nonmalignant tissues (N) with internal standard (ITAS). To show assay specificity, tumor samples were RNase A pretreated (+RNase A).

Fig. 3. Detection of telomerase activity in human breast cancers. A, telomerase activity in DCIS (LG, low nuclear grade; HG, high nuclear grade). B, activity in lymph node metastasis negative (LN−) and positive (LN+) tumors. Note that lymph node-negative tumors have the same activity levels as lymph node-positive tumors (ITAS, internal standard).

be negative for telomerase activity and any of the known prognostic indicators.

In our semiquantitative analysis of levels of enzymatic activity, we found no statistically significant correlation at the $P < 0.05$ level between telomerase levels and tumor size, lymph node metastasis, estrogen and progesterone receptor status, S-phase fraction, and ploidy, (Fig. 4 and Table 1). Axillary lymph node status is generally accepted as one of the best prognostic indicators for breast cancer recurrence (24). We observed as much telomerase activity in lymph node-negative tumors as in tumors positive for lymph node metastasis (Fig. 4E). Interestingly, we only found statistically significant correlation with patient age (Fig. 4C; Table 1). It appears that levels of telomerase activity are higher in tumors from younger patients. However, although $P = 0.03$, the Spearman rank correlation analysis indicates that since the...
rho value is $-0.3$ (Table 1); approximately only 9% of the variability in telomerase levels is explained by age. As mentioned earlier, we observed that all stage 0 and stage I tumors express telomerase activity. We did not observe a statistically significant difference in telomerase activity levels of early tumors (stages 0 and I) versus more advanced lesions (Fig. 4A and Table 1). However, as can be observed in Fig. 4A and Table 1, stage IV tumors appear to show a tendency for
higher telomerase levels. The small number of stage IV tumors precludes further speculation on this observation.

Although patient follow-up was not analyzed in our study, the lack of a clear association between telomerase activity levels and proven prognostic indicators in breast cancer indicates that analysis of the levels of this enzyme by means of the PCR-mediated TRAP assay in human breast cancer may have limited value as a prognostic tool. This appears in contrast to preliminary observations by another laboratory (25). It is unclear at this point whether the hypothesis that a worse prognosis should correlate with higher telomerase levels is incorrect, or alternatively that the lack of correlation observed is the result of the methodological approach. It is then possible that future development of non-PCR-mediated methodologies for measurement of telomerase activity may be better suited for studies of prognosis. On the other hand, because we detected telomerase activity at preinvasive stages of breast cancer, the highly sensitive TRAP assay may be of value in early breast cancer detection and diagnosis, as recently suggested by Hiyama et al. (26). Our findings are in agreement with observations of other neoplasias in which telomerase activity was detected at preinvasive stages of tumor development (13, 14). For instance, telomerase activity has been demonstrated in premalignant prostatic hyperplasias (13), and recently telomerase activity has been also observed in colon adenomas (14). We have also reported a significant increase in telomerase activity at premalignant stages using a mouse multistage carcinogenesis model (23).

As mentioned previously, we also observed that one of five breast fibroadenomas analyzed showed telomerase activity. This incidence appears lower than that reported previously (45%; Ref. 12). Despite the difference in incidence, both studies indicate that some breast fibroadenomas show reactivation of telomerase. It is unclear at this point the putative role for telomerase reactivation in this benign neoplasia.

In summary, telomerase activity was observed in almost all breast cancer samples, regardless of tumor stage. No telomerase activity was detected in the normal breast samples tested, and telomerase activity was detected in 20% of fibroadenomas. No correlation was found between telomerase detection or level of activity and known breast cancer prognostic indicators, which appears to limit the potential value of evaluating the level of this putative biomarker in managing patients with invasive breast cancer. Nevertheless, the final conclusion on this issue will only be resolved after considering the correlation between patient outcome and telomerase levels. Probably the most important conclusion from our study is that telomerase reactivation appears to be an early event in breast carcinogenesis. This factor highlights the potential for using telomerase detection as a possible aid in early tumor detection.

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**Note Added in Proof**


**References**

Analysis of telomerase activity levels in breast cancer: positive detection at the in situ breast carcinoma stage.


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