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# Telomerase Activity in Normal and Neoplastic Breast<sup>1</sup>

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## ABSTRACT

Telomerase activity is detected in the majority of human tumors and provides a mechanism to escape from proliferative limitations due to telomere loss. Similar to other studies, telomerase activity was detected with a modified telomeric repeat amplification protocol in the majority (76%) of 49 human breast cancer specimens, including most (75%) ductal carcinoma *in situ* specimens. There were no correlations between telomerase activity and tumor stage or estrogen/progesterone receptor status. In four of seven invasive tumors, telomerase expression seemed to be heterogeneous because not all microdissected regions were telomerase positive. Low levels of telomerase activity were also detected in a minority (17%) of breast specimens from patients without evidence of cancer. These findings suggest that telomerase activation can occur early in breast cancer progression and may be periodically down-regulated during subsequent progression.

## INTRODUCTION

Telomere loss is a consequence of the inherent inability of DNA polymerases to completely replicate DNA termini (1, 2). Progressive reductions of telomere sizes have been observed in primary cell lines and with aging (3-5). Telomerase is a ribonucleoprotein that can repair telomeres through the addition of TTAGGG direct repeats (6). Telomerase is expressed in germ cells but is absent or expressed at low levels in somatic tissues (7). Telomerase expression may represent a critical step in tumorigenesis because it overcomes the limitations of catastrophic telomere loss, and its activity is present in most human tumors (7-10), including the majority of breast cancers (7, 11).

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To better characterize when telomerase is expressed during breast cancer progression, we examined a series of normal breast tissues and *in situ* and invasive tumors.

## MATERIALS AND METHODS

**Specimens.** Primary breast cancer tissues ( $n = 49$ ) were frozen at the time of resection and stored at  $-70^{\circ}\text{C}$ . Noncancerous breast tissues ( $n = 23$ ) were obtained from autopsies without evidence of carcinoma. Clinical information was obtained from medical records.

**Telomerase Assay.** Single 5-10- $\mu\text{m}$  cryostat-frozen tissue sections approximately 0.5-1  $\text{cm}^2$  in size were extracted and analyzed by a modified TRAP<sup>3</sup> assay (7, 12). Histological examination verified that at least 50% of all cells were malignant. Approximately 5-10% of each protein extract were assayed for telomerase. PCR products were labeled with [<sup>33</sup>P]dCTP present in the reaction mix. A total of 40 PCR cycles was utilized to enhance the sensitivity of the assay for the small amounts of tumor tissue present in the single tissue slices. This modified TRAP assay (12) fails to detect telomerase from primary human fibroblasts but can detect as few as 10 telomerase-positive tumor cells mixed with 100,000 telomerase-negative primary fetal foreskin fibroblast cells (data not shown). For all telomerase-positive specimens, RNase digestion confirmed the expected loss of telomerase activity. The absence of inhibitors was tested by adding the equivalent of 50 telomerase-positive tumor cells to the negative specimens to demonstrate the expected amplification. Autoradiography was performed with a phosphorescence imaging system (PhosphorImager 445SI; Molecular Dynamics, Sunnyvale, CA).

**Microdissection and Telomerase Analysis.** Single 5-10- $\mu\text{m}$  frozen breast cancer sections were placed onto plastic slides and then cut along with the underlying plastic slides into 8 portions of approximately equal sizes. Microscopic examination of a stained "mirror" frozen section ensured that each microdissected region contained at least 50% tumor cells. Telomerase activity was assayed as above, except 50% of the protein extract was used. Independent microdissections were repeated at least twice. A microdissected region was considered telomerase-negative if no activity was detected or if the region was negative in two of three independent microdissections. For controls, artificial mixtures of a telomerase-positive breast cancer cell line (SKBR3) and telomerase-negative primary fetal foreskin fibroblasts (MRC-5) were frozen and sectioned onto plastic slides. These mixtures were also assayed for telomerase activity, similar to the frozen breast cancers.

**Detection of Estrogen and Progesterone Receptors.** Estrogen and progesterone receptor expression was detected from frozen tumor sections using standard immunohistochemi-

<sup>3</sup> The abbreviations used are: TRAP, telomeric repeat amplification protocol; DCIS, ductal carcinoma *in situ*.

Table 1 Telomerase activity and tumor stage

Stage	No.	Positive (%)
DCIS	12	9 (75)
I	9	7 (78)
II	20	17 (85)
III	6	2 (33)
IV	2	2 (100)
Total	49	37 (76)

cal techniques and criteria (13). The receptor status was available for 35 of the tumors.

## RESULTS

Single frozen sections of breast cancer from 49 patients were analyzed by a modified TRAP assay. Telomerase activity was detected in the majority (76%) of breast carcinomas (Table 1). There were no obvious correlations between tumor stage and the detection of telomerase. Telomerase activity could also be detected in the majority (75%) of 12 DCIS specimens. There were no apparent correlations between telomerase activity and tumor estrogen or progesterone receptor expression (Table 2).

Telomerase activity could also be detected in a small proportion of normal breast tissues obtained from autopsies without evidence of carcinoma (Fig. 1 and Table 3). Interestingly, telomerase activity was found in normal breast tissues taken during the luteal phase of the menstrual cycle (three of nine specimens), whereas all eight normal breast tissues taken during the follicular phase were telomerase-negative. A single breast specimen taken during pregnancy was also telomerase-positive, although tissue from a lactating breast was telomerase-negative. To estimate the levels of telomerase activity, serial 10-fold dilutions of the crude extracts were performed before the TRAP assay. Relatively low telomerase levels were present in the four positive normal tissues, whereas higher levels were present in the tumors (Fig. 1).

The proportion of telomerase-positive tumors was less than in previous studies (7, 11), suggesting that the current approach of using single thin tissue sections instead of larger whole tissue masses may be less sensitive for the overall detection of telomerase activity. It may be more difficult to detect telomerase activity from the small tissue specimens, or telomerase expression may be heterogeneous and therefore could be missed with small specimens. To control for the first possibility, artificial mixtures of a telomerase-positive breast cancer cell line and telomerase-negative primary fibroblasts were frozen, and single cryostat sections (approximately 2 mm<sup>2</sup> and placed on plastic slides before extraction) were assayed (Fig. 2). Telomerase activity could still be detected from as few as 1000 cells, even when the proportion of the breast cell line was as low as 5%.

Given this ability to detect telomerase activity from small tumor regions even when the majority of cells are telomerase-negative, the telomerase-positive tumor sections were further subdivided into eight approximately equal regions, with each region containing predominately (>50%) tumor cells (Fig. 3). Homogenous expression of telomerase was present in all regions of three tumors. However, for four tumors, some regions were consistently telomerase-negative (Fig. 3).

Table 2 Telomerase activity and tumor receptor status<sup>a</sup>

Receptor status	Telomerase+	Telomerase-
ER+/PR+	15	3
ER+/PR-	2	1
ER-/PR+	2	1
ER-/PR-	10	1

<sup>a</sup> ER, estrogen receptor; PR, progesterone receptor.

## DISCUSSION

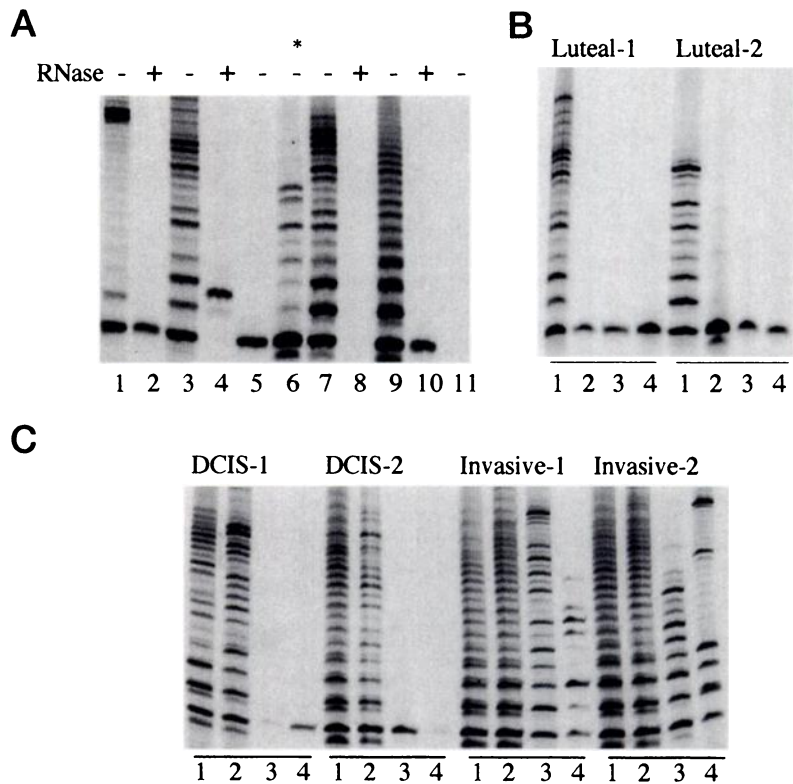
Telomerase activity was detected from the majority of human breast cancer specimens. Telomerase activation can occur early in breast cancer tumor progression because it was detected in most DCIS specimens. However, the overall frequency of telomerase detection was less in this study compared to prior studies (76% versus >90%; Refs. 7 and 11). The TRAP assay is highly sensitive (7) and can be positive even if the majority of tumor cells fail to express telomerase. Although a minority of cancers are telomerase negative and possibly maintain their telomeres through alternative mechanisms (7, 9), the current study indicates that another possible explanation for this discrepancy may be the heterogeneous expression of telomerase in tumor tissues. Because of this heterogeneity, overall telomerase-positive tumors may seem to be telomerase negative if, as in this study, only small tumor regions are examined. Of note, telomerase activity can be detected from >90% of fine needle breast cancer aspirations, which also sample relatively small numbers of cells but typically from many different tumor regions (11).

Telomerase tumor heterogeneity could represent an artifact due to lack of sensitivity or degradation of active telomerase. This seems unlikely because control studies demonstrated the ability to detect telomerase activity from small tumor regions containing only a minority (5%) of telomerase-expressing cells. In addition, telomerase seems to be a relatively stable enzyme because extracts are resistant to multiple freeze-thaw cycles, and activity can be detected in tissues left at room temperature for 24 h (14).<sup>4</sup>

Although most human somatic tissues are telomerase-negative (7), low levels of telomerase are detected in peripheral blood mononuclear cells (15, 16) and are rarely detected in normal breast adjacent to breast carcinoma (7, 11). Occult tumor cells may account for the detection of telomerase activity in normal breast (7, 11). However, in this study, telomerase activity could be detected in normal breast tissue of women without evidence of cancer. By dilution analysis, the telomerase activity present in the normal tissues was lower than that present in the tumor tissues, suggesting that telomerase was expressed by only a small number of the normal cells. Although the numbers are small, the detection of telomerase during the luteal but not the follicular phases of the menstrual cycle suggests that telomerase activity may be regulated. This regulation may be similar to the telomerase up-regulation observed in lymphocytes and hematopoietic cells after mitogenic stimulation (17, 18). A single breast

<sup>4</sup> Unpublished observations.

**Fig. 1** A, examples of telomerase-positive and -negative specimens with (+) and without (-) RNase pretreatment. The samples are a telomerase-positive gestational breast (Lanes 1 and 2), a positive luteal-phase normal breast (Lanes 3 and 4), a negative follicular-phase normal breast (Lane 5 with Lane 6\* demonstrating positivity after telomerase-positive cells were added to demonstrate the lack of assay inhibitors), a positive DCIS specimen (Lanes 7 and 8), a positive invasive cancer (Lanes 9 and 10), and lysis buffer only (Lane 11). B, serial 10-fold dilutions with subsequent TRAP analysis of two telomerase-positive normal breast tissues obtained during the luteal phase. Only the undiluted crude extracts are telomerase positive. Undiluted, Lane 1; 1:10, Lane 2; 1:100, Lane 3; and 1:1000, Lane 4. The telomerase activities of the undiluted extracts could be abolished by RNase pretreatment (for example, see Lane 4 of A). C, serial 10-fold dilutions with subsequent TRAP analysis of two telomerase-positive DCIS specimens and two telomerase-positive invasive breast cancers. Relatively greater levels of telomerase activity are present in the invasive tumors compared to the DCIS and normal specimens. The telomerase activities of the undiluted extracts could be abolished by RNase pretreatment (for examples, see Lanes 8 and 10 in A).



**Fig. 2** Telomerase activity was detected in artificial single frozen section mixtures of the SKBR3 cell line and primary human fibroblasts. Telomerase activity was detected when as few as 5% SKBR3 cells were present (+) but was not detected (-) when SKBR3 was absent (0%). The total number of cells is approximately constant between the specimens.

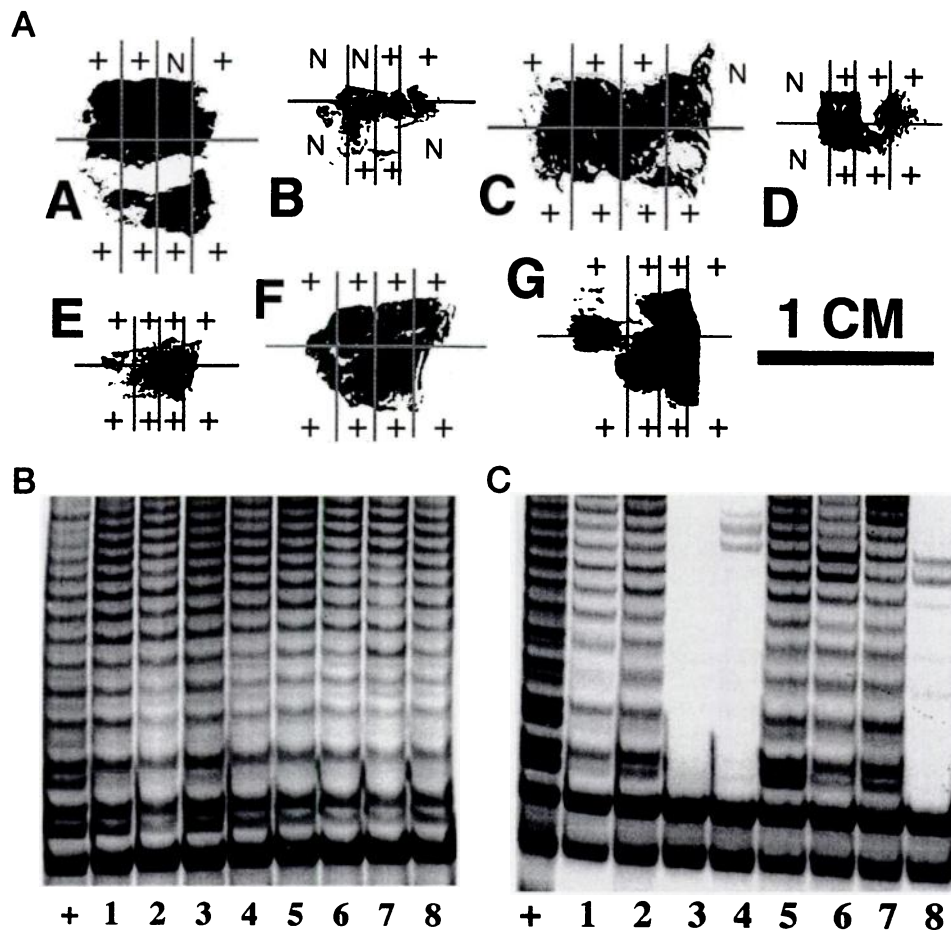
**Table 3** Telomerase activity in autopsy breast specimens without cancer

Specimen	No.	Positive (%)
Follicular phase	8	0 (0)
Luteal phase	9	3 (33)
Gestational	1	1 (100)
Other <sup>a</sup>	5	0 (0)
Total	23	4 (17)

<sup>a</sup> Includes two cases of fibrocystic disease and one case each of infant, lactating, and pubertal breast specimens.

specimen undergoing maximal physiological mitogenic stimulation (*i.e.*, pregnancy) also expressed low levels of telomerase. Down-regulation of telomerase is also observed with maturation of cell lines (19, 20). It seems unlikely that hormonal changes alone substantially influence telomerase expression in breast cancer because there were no obvious correlations between tumor estrogen or progesterone receptor status and the detection of telomerase.

Telomere shortening and telomerase activation occur during tumor progression (10). The current detection of telomerase in only portions of a tumor may reflect an initial lack of telomerase expression with subsequent expression after the progression of different tumor subclones (21, 22) or early expression with periodic repression during tumor progression. The detection of telomerase activity in most early breast tumors (DCIS) suggests the latter alternative. Telomerase is not immediately required for viability because anti-



**Fig. 3** A, single sections from seven telomerase-positive invasive cancers were cut into eight approximately equal regions. The lines show the approximate divisions made in the cancers. Each of the regions was separately analyzed by the TRAP assay. Telomerase activity could not be detected in all portions of four positive breast cancers (A–D). In three cancers (E–G), all portions were telomerase positive. Consistently telomerase-negative regions are indicated by N, with positive areas indicated by +. B, example of an invasive tumor with a homogenous distribution of telomerase expression. Telomerase activity was detected from all eight microdissected portions (Lanes 1–8) of cancer E. A telomerase-positive control (+) is also displayed. C, example of an invasive tumor with a heterogeneous distribution of telomerase expression. In this microdissection, telomerase activity is absent from three of eight regions (Lanes 3, 4, and 8) of cancer A. The higher molecular weight bands in Lanes 4 and 8 are considered PCR artifacts because they do have the characteristic positive ladder pattern and did not disappear with RNase treatment. Repeat analysis demonstrated that only region 3 was consistently telomerase negative (two of three independent microdissections).

sense expression of its RNA component leads to telomere loss, but cell death does not occur until after 20 divisions (23). Human telomerase is highly processive compared to the murine enzyme (24), and repair with the addition of multiple telomeric repeats may subsequently permit residual normal mitotic activity in the absence of telomerase activity. In support of this mechanism, two human breast cancer cell lines isolated from pleural effusions demonstrated telomere shortening during early passage but were immortal and eventually did maintain telomere length (25). Studies of telomere sizes could help resolve whether or not telomerase expression is correlated with telomere size. Unfortunately, the DNA recovered from the small tissue sections was insufficient for Southern blot telomere analysis (data not shown).

Breast cancer progression involves a series of morphological and genetic alterations (26). Telomerase activity is detectable at low levels in some normal breasts and is frequently

expressed early in breast cancer progression. Telomerase activation also seems to occur relatively early in the tumor progression of human head and neck squamous cell carcinoma (27), gastric and colorectal cancer (28), hepatocellular carcinoma (29), and murine dermal papillomas (30). In addition, precursors E6-expressing human keratinocytes expressed telomerase but were not immortal (31). The observed telomerase tumor heterogeneity may reflect epigenetic mechanisms rather than distinct clonal heterogeneity because identical genetic alterations are typically found throughout invasive breast cancers (32). Telomerase is expressed in the majority of breast cancers, suggesting that a large number of divisions accompanied by telomere shortening often precede clonal expansion (10, 22). However, the detection of low telomerase levels in normal breast tissues is also consistent with the alternative that some of the cells selected for clonal expansion may already express telomerase (33).

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