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Notes

We thank Dr. Charles Bowden for his insightful comments and valuable advice and Fran Stewart for her assistance with statistical analyses.

Manuscript received June 21, 1995; revised October 11, 1995; accepted October 20, 1995.

Telomerase Activity in Human Breast Tumors

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Background: **The activity of the ribonucleoprotein enzyme telomerase is not detected in normal somatic cells; thus, with each cell division, the ends of chromosomes consisting of the telomeric repeats TTAGGG progressively erode. The current model gaining support is that telomerase activity in germline and immortal cells maintains telomere length and thus compensates for the "end-replication problem." *Purpose:* Our objective was to determine when telomerase activity is reactivated in the progression to malignant breast cancer and if knowledge of telomerase activity may be an indicator for the diagnosis and potential treatment of breast cancer. *Methods:* Using a polymerase chain reaction-based telomerase activity assay, we examined telomerase activity in 140 breast cancer specimens (from 140 patients), four phyllodes tumors (from four patients), 38 noncancerous lesions (20 fibroadenomas, 17 fibrocystic diseases, one gynecomastia; from 38 patients), and 55 adjacent noncancerous mammary tissues (from 55 of the 140 breast cancer patients). In addition, 33 fine-needle-aspirated breast samples (from 33 patients) were analyzed. *Results:* Among surgically resected samples, telomerase activity was detected in 130 (93%) of 140 breast cancers. Telomerase activity was detected in 68% of stage I primary breast cancers, in 73% of cancers smaller than 20 mm, and in 81% of axillary lymph node-negative cancers. Moreover, the activity was detected in more than 95% of advanced stage tumors but in only two (4%) of 55 adjacent noncancerous tissues. While telomerase activity was not detected in any of 17 specimens of fibrocystic disease, surprisingly low levels of telomerase activity were detected in nine (45%) of 20 fibroadenomas. Among samples obtained by fine-needle aspiration, 14 (100%) of 14 patients whose fine-needle-aspirated specimen contained telomerase activity and who subsequently underwent surgery were confirmed to have breast cancer. Multivariate analysis of 125 specimens from patients for whom data were available on age at surgery, stage of disease, tumor size, lymph node status tumor histology, and menopausal status indicated that stage classification exhibited the strongest association with telomerase activity (for stage I versus stages II-IV: odds ratio = 1.0 versus 73.4; 95% confidence interval = 2.0-959.0; P = .02). *Conclusion:* Telomerase activity was detected in more than 95% of advanced stage breast cancers. It was absent in 19%-32% of less advanced cancers. Since a determination of any association between telomerase activity and patient survival is not possible at the present time, it remains to be determined whether lack of telomerase activity predicts for favorable outcome. [J Natl Cancer Inst 1996;88:116-22]**

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See "Notes" section following "References."

Breast cancer is the most common malignancy in U.S. women and the second leading cause of cancer death, exceeded only by lung cancer in the United States (*1,2*). In Japan, the incidence of breast cancer is lower than in the United States, although it is gradually increasing (*2*). During the last decade, intense clinical and biological research efforts have been initiated to determine the causes of breast cancer. A number of factors, both endogenous and exogenous, that increase the risk of breast cancer development have been identified (*2-4*). During the previous decade, screening for early detection of breast cancer has increased dramatically (*2,5*). The increased use of mammography has led to early detection of nonpalpable breast cancers, and fine-needle aspiration has been shown to be a useful procedure for the early and accurate diagnosis of these cancers. Tumor size, lymph node status, and histopathologic findings are considered to be good prognostic indicators in breast cancer (*6,7*). Moreover, several molecular changes, such as amplification or overexpression of the c-erbB-2 gene (also known as ERBB2) (*8*), overexpression of the epidermal growth factor receptor (*9*), DNA aneuploidy (*10*), estrogen and progesterone receptor status (*11*), and the diminished expression of BRCA1 (*12*), appear to be involved in breast cancer development. However, the molecular events underlying the development of human breast cancer still need to be elucidated.

Telomeres are specialized structures containing unique $(TTAGGG)_n$ repeats at the ends of eukaryotic chromosomes; these repeats are thought to be important in the protection and replication of chromosomes (*13,14*). Lagging strand DNA synthesis at the very end of linear chromosomes cannot be completed (referred to as the "end-replication problem") and this situation results in the progressive shortening of telomeric repeats with each division (*15,16*). Telomerase contains an RNA component that has a template region complementary to $(TTAGGG)_n$ repeats that permits the de novo synthesis of TTAGGG telomeric DNA onto chromosomal ends (*17-19*). While germline cells expressing telomerase activity maintain telomeric repeats, in somatic cells, progressive erosion of telomeres with each cell division is likely due to the repression of telomerase activity during development (*20-23*). Although the reactivation of telomerase alone may be insufficient for cells to proliferate indefinitely, its expression and the stabilization of telomeres appear to be concomitant with the attainment of immortality in cancer cells (*24,25*). Previously, we (*26*) hypothesized that telomerase reactivation had an important role in the etiology of breast cancer (*26*). To add experimental support to this hypothesis and to evaluate the clinical usefulness of detecting telomerase activity in clinical specimens, we examined telomerase activity in a variety of different types of breast cancer and noncancerous tissue specimens.

Recently, a highly sensitive polymerase chain reaction (PCR)-based telomerase assay called the TRAP (Telomeric Repeat Amplification Protocol) assay was developed for the detection of telomerase activity (*25*). By this method, telomerase activity has been found in most tumor tissues examined (covering a large variety of tumor types). Among these tumors, telomerase activity was detected in 94% of neuroblastoma (*27*), 80% of lung cancer (*28*), 93% of colorectal cancer (*29*), 85% of hepatocellular carcinoma (*30*), and 85% of gastric cancer (*31*). In addition, in cultured cells, 98 of 100 immortal and none of 22 mortal cell populations expressed telomerase activity (*25*). Thus, telomerase activity appears to be repressed in somatic cells and tissues, but it is reactivated in most immortal cells and human cancers. The results of these studies suggest that telomerase activation occurs during the development of various malignant tumors and that, in almost all instances, telomerase activity may ultimately be required to maintain tumor growth. Although it is not known at what stage in cancer development telomerase is reactivated, clinical interest is now focused on whether the detection of telomerase activity may be a useful diagnostic tool in clinical specimens, especially in cytologic materials.

In the present study, we measured telomerase activity and telomere length in benign and malignant breast disease samples in order to evaluate a putative role of telomerase in breast carcinogenesis. In addition, we examined telomerase activity in fine-needle-aspirated samples to determine if telomerase activity can be detected in these specimens and if this activity can be used as a diagnostic or prognostic indicator of breast cancer (which may increase the value of cytologic diagnosis).

Patients and Methods

Tissue Samples

A total of 140 breast cancer tissues (from 140 patients) and 55 adjacent noncancerous breast tissue samples (from 55 of the 140 breast cancer patients) were obtained at the time of surgery. In addition, four phyllodes tumors (one malignant, one borderline, and two benign tumors), 20 fibroadenomas, 17 specimens of fibrocystic disease, and one gynecomastia sample were obtained from 38 separate patients. Among these samples, 64 breast cancers, five fibroadenomas, two cases of fibrocystic disease (mastopathy), and one malignant phyllode tumor were obtained from patients who underwent surgery at Parkland Memorial Hospital, Dallas, TX. The remaining tissues were obtained from patients who underwent surgery at Hiroshima University Hospital, Hiroshima, Japan. Tumor sizes were determined after surgery, and tumor samples were stored at –80 °C until use. Institutional guidelines for the use of patient materials were followed in both the United States and Japan. Written informed consent was obtained from all patients. The breast cancers of patients in both the United States and Japan were staged according to the International Union Against Cancer tumor–node–metastasis (UICC-TNM) classification (*32*).

After we received written informed consent, fine-needle-aspirated samples were obtained from 33 women who underwent cytologic examination for diagnosis of their breast tumors. The fine-needle aspiration was performed as follows: The needle, attached to a syringe, was inserted into the breast tumor. The tumor cells were obtained by applying mild suction. The needle was moved back and forth several times, and then the negative pressure was released. Fine-needle aspiration was performed twice on most samples. One aspirate was examined cytologically, while the other was deposited in a phosphate-buffered saline (PBS) solution. This latter sample was washed two times with PBS, and the number of cells was counted with a hemocytometer. The remainder of the sample was centrifuged at 1500*g* for 5 minutes, and the pellet was stored at –80 °C until use.

Telomerase Assay

Extracts of tissue specimens and assays of telomerase activity were done as described earlier (*25,33*). Briefly, frozen breast tissue samples of 50-100 mg were homogenized in 200 µL of 3-[(3-cholamidopropyl)dimethyl-ammonio]-1 propanesulfonate (CHAPS) lysis buffer. After 25 minutes of incubation on ice, the lysates were centrifuged at 16 000*g* for 20 minutes at 4 °C, and the supernatant was rapidly frozen in liquid nitrogen and stored at –80 °C. The concentration of protein was measured by use of the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL), and an aliquot of extract containing 6 µg of protein was used

for each TRAP assay unless otherwise indicated. For fine-needle-aspirated samples, aliquots corresponding to an extract derived from approximately 10^3 cells were used for the TRAP assay. For ribonuclease (RNase) treatment, 5 μL of extract was incubated with 1 μg RNase (Boehringer Mannheim Corp., Indianapolis, IN) for 20 minutes at 37 °C. Assay tubes were prepared by sequestering 0.1 μg of CX primer (5′-CCCTTACCCTTACCCTTACCCTAA-3′) under a wax barrier (HotStart 50 PCR tube; Molecular Bio-Products, San Diego, CA). Each extract was assayed in 50 μL of reaction mixture containing 20 m*M* Tris–HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 68 m*M* KCl, 0.05% Tween 20, 1 m*M* ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 50 μ*M* deoxynucleoside triphosphates, 150 kilobecquerels [^{32}P]deoxycytidine triphosphate, 0.1 μg of TS oligonucleotide (5′-AATCCGTCGAGCAGAGTT-3′), 0.5 μ*M* T4 gene 32 protein (United States Biochemical Corp., Cleveland, OH), and 2 U of *Taq* DNA polymerase (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD, or Wako Chemicals, Osaka, Japan). Each reaction mixture contained 5×10^{-18} g (5 attograms) of an Internal Telomerase Assay Standard (ITAS) for quantitative estimation of the levels of telomerase activity and the identification of false-negative tumor samples that contain *Taq* polymerase inhibitors (*34*). ITAS is a 150-base-pair DNA standard, which is coamplified with telomerase-elongated products and is sufficiently long that it does not interfere with the visualization of the telomerase ladder. After 30 minutes of incubation at room temperature for telomerase-mediated extension from annealed TS oligonucleotides, the reaction mixture was heated at 90 °C for 90 seconds and then subjected to 31 PCR cycles at 94 °C for 40 seconds, 50 °C for 40 seconds, and 72 °C for 50 seconds. The PCR product was electrophoresed on a 10% polyacrylamide gel. To estimate telomerase activity in tissue samples, we compared the intensity of the TRAP assay-generated DNA ladder with that of the ITAS signal using the Bioimage Analyzer (BAS 2000; Fuji, Tokyo, Japan) or the Phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Southern Blot Analysis

Genomic DNA was isolated from 22 adjacent noncancerous tissues and 60 tumor tissues of untreated breast cancer patients and 33 specimens of benign breast disease tissue (one gynecomastia, 15 fibrocystic disease tissues, 14 fibroadenomas, and three phyllodes tumors) surgically obtained in Japan as previously described (*35-37*). For the analysis of length of terminal restriction fragments, 2 μg of DNA was digested to completion with 10 U of *Hin*fI, electrophoresed on 0.8% agarose gels, and then blotted onto nitrocellulose filters. The filters were hybridized to a ^{32}P-end-labeled $(TTAGGG)_4$ probe, washed, and then autoradiographed as previously reported (*35-37*). We estimated the mean length of terminal restriction fragments at the peak position of hybridization signal. To confirm complete *Hin*fI digestion, we rehybridized the same filters with a ^{32}P-labeled β-globin or a K-ras probe. To exclude the possible effect of DNA degradation, we analyzed the integrity of undigested DNA by gel electrophoresis.

Statistical Analysis

For statistical analysis, we divided tumor samples into one of two groups: tumors with undetectable telomerase activity and tumors with telomerase activity. To compare these groups, we analyzed clinical data by univariate analysis. All *P* values refer to either a chi-squared test with Yates correction or Fisher's exact test for tables and the Mann–Whitney test for nonparametric data, where appropriate. All *P* values resulted from two-sided statistical tests. In addition, multivariate analysis using the multiple logistic regression model was used to identify which, if any, other status of cancer indicators was associated with telomerase expression. This analysis was performed using the CARE Software system (Hiroshima University, Japan).

Results

Telomerase Activity in Breast Cancers Compared With Clinical Parameters

Telomerase activity was detected in 130 (93%) of 140 breast cancer tissues analyzed (Table 1), whereas telomerase activity was detected in only two (4%) of 55 adjacent noncancerous breast tissues and even then at low levels (data not shown). Histologically, there were no obvious differences in the ratio of tumor cells to stromal cells in the tumors with and without detectable telomerase activity, and the reproducibility of the TRAP assay was confirmed by sampling multiple, different sites of some of the tumors (data not shown). The frequency of tumors detected with telomerase activity differed markedly between stage I tumors (68%; 15 of 22) and other advanced stage tumors (>95%) (*P*<.0001, chi-squared test). Among 88 primary tumors resected from patients with lymph node metastasis, 85 tumors (97%) showed telomerase activity, whereas 30 (81%) of 37 of the tumors without lymph node metastasis had detectable telomerase activity (*P* = .01, Fisher's exact test) (Fig. 1). Tumor sizes also revealed an association with telomerase activity. Tumors without detectable telomerase activity were significantly smaller than those with observed activity (*P* = .003, Mann–Whitney test). These findings demonstrated that, whereas almost all advanced breast cancers (>95%) had telomerase activity, a statistically significant fraction of early breast cancers lacked detectable telomerase activity. In addition, 34 (100%) of 34 tumors from premenopausal patients had detectable telomerase activity, whereas 96 (91%) of 106 tumors diagnosed in perimenopausal or postmenopausal patients had detectable telomerase activity (*P* = .045, Fisher's exact test). Three (3%) of the 102 advanced tumors (stage II, III, or IV) examined were considered noninformative because the internal standard (ITAS) (*34*) was not amplified during the PCR reaction. Multivariate analysis of 125 specimens from patients for whom the age at surgery, stage of disease, tumor sizes, lymph node status, histology, and menopausal status were known revealed that stage classification exhibited the strongest association with the expression of telomerase activity (stage I versus stage II-IV odds ratio = 1.0 versus 73.4; 95% confidence interval = 2.0-959.0; *P* = .02)

The presence of telomerase activity was also examined in eight metastatic lesions obtained from seven patients with breast cancer: five lymph node metastases, two lung metastases, and one liver metastasis. All metastatic lesions showed high levels of telomerase activity. From these cases, four primary tumors

Table 1. Comparison of tumor stage and size and lymph node metastasis with telomerase activity in breast cancer

	No. of specimens	Telomerase activity		
		Negative	Positive	% positive
Stage				
I	22	7	15	68
II	52	1	51	98
III	46	2	44	96
IV	7	0	7	100
Unknown	13	0	13	100
Tumor size, mm				
<20	30	8	22	73
≥20	104	2	102	98
Unknown	6	0	6	100
Lymph node metastasis				
Negative	37	7	30	81
Positive	88	3	85	97
Unknown	15	0	15	100
Total	140	10	130	93

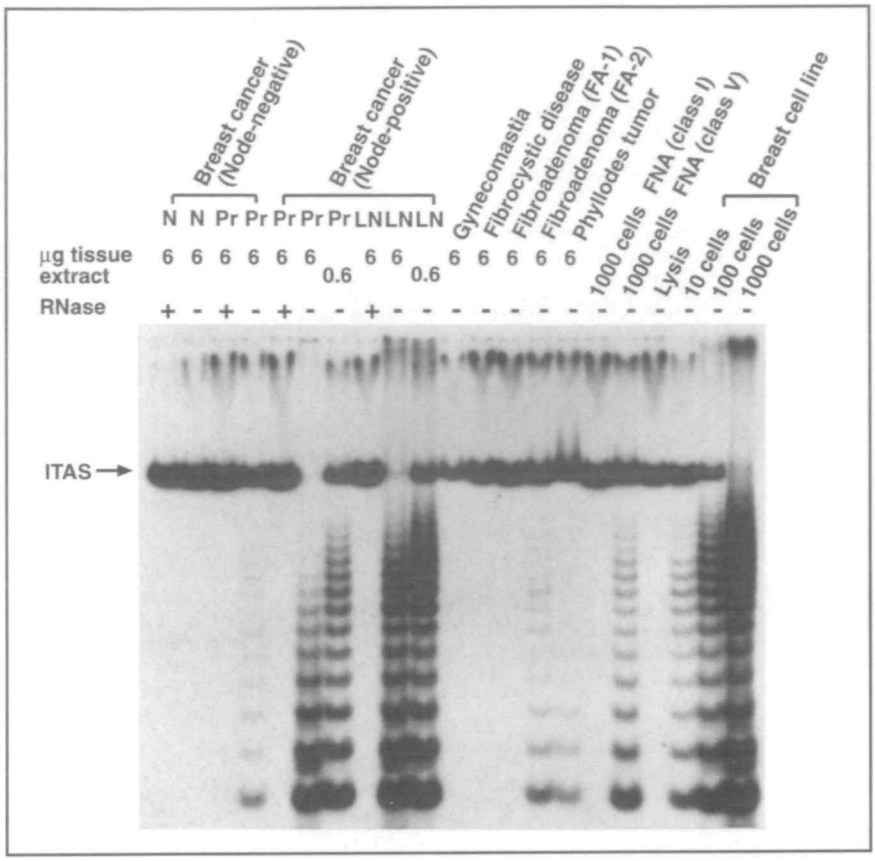

Fig. 1. Telomerase activity in breast cancer, benign breast diseases, and fine-needle aspiration samples. From each tissue sample, an aliquot of the extract containing 6 or 0.6 µg of protein with (+) or without (–) ribonuclease (RNase) pretreatment was used in each TRAP (Telomeric Repeat Amplification Protocol) assay. In samples taken by fine-needle aspiration (FNA), 10^3 cell equivalents were used for the TRAP assay. Extracts of a human breast cell line having telomerase activity were used as a positive control. Telomerase activity was detected after electrophoresis of the enzyme reaction products and autoradiography as a 6 nucleotide repeat ladder. An Internal Telomerase Assay Standard (ITAS) was used to identify noninformative specimens due to inhibitors of *Taq* polymerase affecting the TRAP assay. Primary breast cancer (Pr), lymph node metastasis (LN), fibroadenoma (FA-2), and phyllodes tumor (borderline) showed telomerase activity, whereas adjacent noncancerous breast tissue (N), gynecomastia, and fibroadenoma (FA-1) did not have detectable telomerase activity. The telomerase activity detected in the lymph node metastasis was stronger than that detected in the primary tumor. Samples (cytology: class I) obtained by FNA did not have detectable activity, whereas telomerase activity was detected in cytology class V samples.

were available; all were telomerase positive. The levels of telomerase activity observed in metastatic lesions were equivalent to or higher than those observed in analyzed primary lesions.

Telomerase Activity in Other Breast Diseases

We examined 20 fibroadenomas, 17 fibrocystic disease specimens, and one gynecomastia specimen (Table 2). In addition, we examined four phyllodes tumors (two benign, one borderline, and one malignant). Telomerase activity was undetectable in all specimens diagnosed as fibrocystic disease or in the gynecomastia sample. However, nine (45%) of 20 fibroadenomas had detectable telomerase activity. In the fibroadenoma specimens, the intensities of the telomerase signals were relatively weak (Fig. 1). There were no obvious differences in the age at surgery, tumor sizes, and histologic findings between telomerase-negative and telomerase-positive fibroadenomas (data not shown). In the phyllodes tumors, two benign tumors did not have detectable telomerase activity, whereas the one borderline tumor exhibited a low level of activity (Fig. 1) and the malignant phyllodes tumor exhibited a high level of telomerase activity.

Table 2. Telomerase activity in other breast lesions

	No. of specimens	Telomerase activity		
		Negative	Positive	% positive
Fibrocystic disease	17	17	0	0
Gynecomastia	1	1	0	0
Fibroadenoma	20	11	9	45
Phyllodes tumor	4	2	2*	50

*One of these tumors was borderline, and the other was malignant.

Length of Terminal Restriction Fragments in Breast Cancer Tissues, Adjacent Breast Tissues, and Tissue Samples From Cases of Benign Breast Disease

Telomere lengths were examined for 22 adjacent noncancerous tissues and 60 primary breast cancer tissues, including two tumors without detectable telomerase activity (Fig. 2, B). The terminal restriction fragment lengths of all adjacent tissues ranged between 8 and 15 kilobase pairs (kbp), whereas those of 60 breast cancer tissues varied between 3.4 and 27 kbp. We classified samples as shortened terminal restriction fragments when the lengths were shorter than 8 kbp and as elongated when the lengths were longer than 15 kbp. Among these primary cancers, the terminal restriction fragment lengths were shorter than 8 kbp in 13 tumors (22%) and longer than 15 kbp in seven tumors (12%). There was no apparent difference between altered length of terminal restriction fragments and tumor stage, tumor size, or lymph node status (data not shown). However, all seven tumors with elongated terminal restriction fragments and 11 of 13 tumors with shortened fragments showed strong telomerase signals. Of 40 tumors without altered lengths of terminal restriction fragments, telomerase activity was not detected in two tumors, whereas 26 tumors showed strong telomerase signals. In addition, there were 22 cases in which both breast cancer tissue and adjacent noncancerous tissue were available

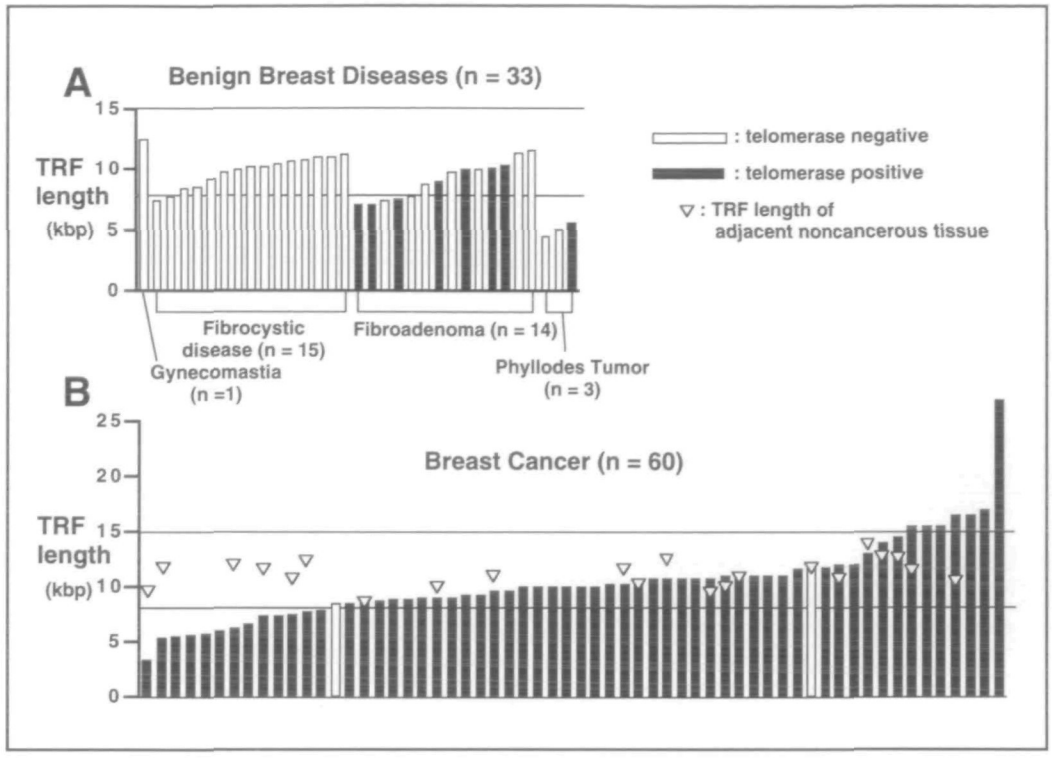

Fig. 2. Telomere lengths in benign breast diseases, breast cancer tissues, and adjacent noncancerous breast tissues. For each tissue sample, 2 μg of genomic DNA was digested to completion with *Hin*fI and was used in Southern blot hybridizations. **A)** Mean terminal restriction fragment (TRF) lengths in benign breast diseases (n = 33), including one gynecomastia, 15 fibrocystic disease tissues, 14 fibroadenomas, and three phyllodes tumors. The TRF lengths in most of these specimens were between 8 and 15 kilobase pairs (kbp), whereas all three (two benign and one borderline) phyllodes tumors showed reduced TRF lengths (4.8, 5.4, and 6.8 kbp, respectively). Seven of 14 fibroadenomas and one borderline phyllodes tumor had detectable telomerase activity. **B)** Mean TRF lengths in breast cancer tissues (n = 60) and in adjacent noncancerous tissues (n = 22). The mean TRF lengths in breast cancer ranged between 3.4 and 27 kbp, whereas those in adjacent noncancerous tissues ranged between 8 and 15 kbp. Among 60 samples examined for TRF length, 58 had telomerase activity. The two cancers without detectable telomerase activity had mean TRFs of 8.7 and 11 kbp, respectively.

for terminal restriction fragment determinations (Fig. 2, B). We also examined the telomere length of 14 fibrocystic disease tissues, 15 fibroadenomas, three phyllodes tumors, and one gynecomastia specimen (Fig. 2, A). The lengths of terminal restriction fragments in most of these tissues (except for phyllodes tumors) were in the normal range between 8 and 15 kbp. It is interesting that all three phyllodes tumors examined (two benign and one borderline) showed shortened lengths of terminal restriction fragments (<7 kbp).

Telomerase Activity in Fine-Needle-Aspirated Samples

Using extracts derived from 10^3 cells, we examined telomerase activity in 33 fine-needle-aspirated samples (Table 3). In 18 samples without detectable telomerase activity, 17 (94%) were diagnosed as class I, II, or III (suspected to be benign) by cytology. The one remaining sample without detectable telomerase activity was diagnosed as class IV; upon surgery, this tumor was determined to be an invasive breast cancer. One telomerase-negative, cytologically diagnosed class I tumor was subsequently surgically resected, and it was found to be a fibroadenoma. Among the 33 fine-needle-aspirated samples, 15 (45%) had telomerase activity (Fig. 1). Of the 15 telomerase-positive samples, 14 (93%) were diagnosed as class IV or V (suspected to be malignant) by cytology, and all were subsequently determined to be invasive breast cancer at surgery. The remaining one tumor was diagnosed as class III by cytology, and the patient has been followed without surgical resection. These findings indicate that fine-needle aspiration may be useful in the detection of telomerase activity as a diagnostic marker for breast cancer.

Table 3. Telomerase activity in breast samples obtained by fine-needle aspiration

Case patient No.	Age, y	Cytology	Telomerase activity*	Histology after surgery†
1	88	I	Negative	ND
2	32	I	Negative	ND
3	46	I	Negative	ND
4	44	I	Negative	ND
5	75	I	Negative	ND
6	29	I	Negative	ND
7	43	I	Negative	ND
8	43	I	Negative	Fibroadenoma
9	40	I	Negative	ND
10	55	II	Negative	ND
11	73	II	Negative	ND
12	19	II	Negative	ND
13	30	II	Negative	ND
14	71	II	Negative	ND
15	50	II	Negative	ND
16	50	III	Negative	ND
17	44	III	Negative	ND
18	78	IV	Negative	Invasive ductal carcinoma
19	48	III	Positive	ND
20	69	IV	Positive	Invasive ductal carcinoma
21	69	IV	Positive	Papillary carcinoma
22	51	V	Positive	Invasive ductal carcinoma
23	49	V	Positive	Invasive ductal carcinoma
24	53	V	Positive	Invasive ductal carcinoma
25	71	V	Positive	Invasive ductal carcinoma
26	49	V	Positive	Invasive ductal carcinoma
27	83	V	Positive	Invasive ductal carcinoma
28	77	V	Positive	Invasive lobular carcinoma
29	51	V	Positive	Invasive ductal carcinoma
30	63	V	Positive	Invasive ductal carcinoma
31	78	V	Positive	Invasive ductal carcinoma
32	77	V	Positive	Invasive ductal carcinoma
33	48	V	Positive	Invasive ductal carcinoma

*Telomerase activity estimated from extracts containing approximately 10^3 cells.

†ND = surgery was not done.

Discussion

In the present study, telomerase activity was detected in almost all malignant breast tumor samples (present in 95% of advanced stage breast cancer specimens but absent in 19%-32% of less advanced breast cancer specimens), whereas the activity was not detected in most normal breast tissues or in benign breast tumors analyzed (with the exception of fibroadenomas). Our results indicate that telomerase reactivation may be an important step in the progression of normal breast epithelial tissue to breast cancer, as previously hypothesized (*26*). The low levels of telomerase activity detected in two adjacent noncancerous breast tissues may have been caused by the presence of occult microinvasion.

In somatic cells without telomerase activity, telomeres progressively shorten and, after many cell divisions, undergo cellular senescence (*21,22*). In immortal cells, unlimited growth capacity appears to be acquired concomitantly with telomere stabilization, which is likely due to reactivation of telomerase activity (*25*). In recent studies, telomerase activity has been detected in approximately 85%-90% of tumor samples from many types of malignant tumors, including lung cancer (*28*), colorectal cancer (*29*), gastric cancer (*31*), and hepatocellular carcinoma (*30*). In the present study, 93% of the breast cancers analyzed showed telomerase activity. These results suggest that almost all human cancers consist of a population of cells that have acquired immortality.

Clinical stage, histology, tumor size, and lymph node status are known to be prognostic factors in breast cancer patients (*6,7*). The tumors without telomerase activity were at a significantly earlier clinical stage and were smaller than the tumors with telomerase activity. The incidence of lymph node metastasis in the patients with telomerase-negative primary tumors was lower than that in the patients with telomerase-positive primary tumors. The metastatic lesions of breast cancer samples were telomerase positive, and the activity in these cases appeared stronger than that in primary lesions. One explanation may be that telomerase activity is acquired during the progression of breast cancer to metastatic lesions. Alternatively, contrary to the more homogeneous population in metastatic lesions, primary breast tumors exhibit heterogeneous clusters of cells, where the telomerase-positive population is diluted by intercalating stromal and connective tissue cells (*38*).

Telomere length has been examined in many types of tumors (*35-37*). Altered lengths of telomeres occur in less than half of the breast cancers when compared with normal adjacent breast tissues of the same patients (*39*) as well as in the other types of tumors (*36*). In the present study, although almost all tumors have telomerase activity, altered telomere lengths were found in only 20 (33%) of 60 breast cancers when compared with normal breast tissues from the same respective patients. It is interesting that all tumors with elongated telomere length and most tumors with reduced telomere length exhibited high levels of telomerase activity. Contrary to the low frequency of these altered telomere lengths, the frequency of detectable telomerase activity was very high. Southern blot analysis measures the mean length of terminal restriction fragments of all cells contained in the tumor specimen—not only tumor cells but also stromal and connective tissue cells. These results indicate that altered telomere length may be difficult to detect using Southern blot analysis until the majority of the cancer cells have telomeres at altered length. On this assumption, the tumors whose telomeres were stabilized at altered lengths have high levels of telomerase activity because such tumors may consist almost exclusively of cancer cells with telomerase activity. Thus, to identify whether the cancer cells have acquired immortality, telomerase activity is likely to be a better indicator than alterations of telomere length.

In cases of benign breast disease, telomerase activity was not detected in the fibrocystic disease and gynecomastia samples. Surprisingly, nine (45%) of 20 fibroadenomas analyzed exhibited low levels of telomerase activity. The mean terminal restriction fragment lengths in most fibroadenomas ranged between 8 and 15 kbp, which is similar to those of adjacent noncancerous breast tissues. That some fibroadenomas had telomerase activity cannot easily be explained at present because the pathogenesis of fibroadenoma is not well understood. Several reports (*40-42*) indicate that the origins of fibroadenoma are heterogeneous; our present results seem to support this theory. Fibroadenomas are benign tumors that are commonly diagnosed in young women. An analysis (*43*) demonstrated that fibroadenoma is associated with a long-term risk for breast cancer. It remains to be determined whether patients with telomerase-positive fibroadenomas are at greater risk of subsequently developing breast cancer than patients with telomerase-negative fibroadenomas. Thus, immortality may not always be acquired concomitantly with malignant transformation. In contrast, telomerase activity was not detected in either of two benign phyllodes tumors, whereas it was detected in a borderline tumor and in a malignant phyllodes tumor. Phyllodes tumors are relatively large, usually grow rapidly, and consist of an extremely hypercellular stroma accompanied by proliferation of breast ductal structures (*44*). The reduction in telomere length in benign phyllodes tumors is likely due to many cell divisions in the absence of sufficient telomerase activity. It is possible that telomerase may be reactivated in phyllodes tumors when they acquire malignant potential. However, additional studies are required to address this issue.

Among the fine-needle-aspirated breast samples, all telomerase-positive specimens were subsequently shown to be breast cancer except for one tumor that was diagnosed as class III, and this patient has been followed without surgical resection. In this study, telomerase activity was analyzed from an extract estimated to have been made from 10^3 aspirated cells. Since peripheral blood mononuclear cells are reported to exhibit low levels of telomerase activity when 10^4 or more cells are examined (*28*), we confirmed that telomerase activity could not be detected in 10^3 total blood cells, permitting us to eliminate the possible contributions of telomerase activity from positive peripheral blood cells. Clinically, aspiration cytology is being used more frequently to diagnose breast cancer before surgery. Fine-needle aspiration of the breast is a well-established technique for diagnosing breast lesions, and its specificity has been reported to be greater than 90% (*45*). Thus, detection of telomerase activity in fine-needle-aspirated samples in combination with morphologic examination may be useful in detecting

immortalized cancer cells not only in breast but also in other organs for which fine-needle aspiration or cytology is applicable.

In summary, telomerase activity in breast cancer may be useful in early diagnosis and in obtaining an accurate diagnosis. In the future, the development of telomerase inhibitors may lead to novel approaches for the treatment and management of breast cancer. However, since analysis of the relationship between telomerase activity and patient survival was not possible at present, it remains to be determined whether lack of telomerase activity predicts for favorable outcome in breast cancer patients.

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Notes

Supported by research grants from the Geron Corporation (Menlo Park, CA), United States Army Medical Research grants DAMD 17-94-J-4023 and DAMD 17-94-J-4007, the Susan Komen Breast Cancer Foundation, and a Grant-in-Aid from the Japanese Ministry of Education, Science and Culture.

We acknowledge the following colleagues for providing clinical support for this study: Professor Y. Matsuura, Professor K. Dohi, and Professor T. Toge (Hiroshima University); Dr. N. Tatsumoto (Miyoshi Central Hospital); and Dr. K. Yashima (The University of Texas Southwestern Medical Center). We also thank Dr. A. Seo (Department of Public Health, Hiroshima University) for help with the statistical analysis.

Manuscript received September 19, 1995; revised November 14, 1995; accepted November 28, 1995.