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Breast cancer detection and diagnosis has been limited by the lack of early and accurate markers of disease. Efforts to date have largely relied on insensitive measures such as mammography or breast examination for detection, with conventional histopathology for diagnosis of breast cancer. The goal of this project is to utilize new molecular diagnostic techniques to improve breast cancer diagnosis. Towards this goal we evaluated the frequency of genetic alterations in variable short tandem repeats (microsatellites) in microdissected breast tissue including normal, in situ carcinoma, invasive, and metastatic carcinoma from the same breast lesion. On the same tissues, in parallel, we are testing the utility of a novel marker, the enzyme telomerase. In this way the prognostic and genetic changes that occur within a single breast lesion can be determined. The long range goal of this project is to develop molecular diagnostic techniques which may be used in the detection of breast cancer and to utilize these techniques in defining the genetic alterations associated with neoplastic progression in breast.

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INTRODUCTION

Subject, purpose and scope of research:

Breast cancer screening and diagnosis have relied on conventional histopathology and clinical parameters, which are often insensitive. Newly developed molecular diagnostic techniques such as detection of genetic alterations in microsatellite markers have been used to determine clonal mutations in a variety of tumors and promise to be more sensitive measures of disease extent. We hypothesized that these techniques will allow more precise definition of the genetic progression of breast neoplasia and improve the diagnosis of breast cancer.

To test the central hypothesis that molecular diagnostic techniques can be developed to aid in the detection of breast cancer, the following specific aims were proposed:

Specific Aim 1. To characterize the frequency of microsatellite alterations in breast cancer and to develop informative panels of microsatellite markers in breast cancer.

We will examine, in microdissected normal and neoplastic breast tissue, a series of microsatellites with documented genetic alterations in human neoplasia. We hypothesize that a majority of breast cancers can be detected by using a panel of markers.

Specific Aim 2. To apply such a panel of microsatellite markers to microdissected breast tissue including normal, hyperplasia, *in situ* carcinoma, invasive, and metastatic carcinoma from the same breast.

In view of our recent findings on the infrequency of microsatellite expansion in many markers in sporadic breast cancer, we have added another task to the 1st and 2nd specific aims, using a marker that was developed after this grant was approved. We examined and ascertained that telomerase is a good marker for breast cancer. We have evaluated the utility of telomerase as an early marker and as a marker of outcome of breast cancer in a large panel of tumors.

Background of previous work:

Recent molecular techniques which are being studied in the detection of malignancy include the use of tumor-specific probes. Unfortunately, at this time, there are no known pathognomonic mutations in breast cancer. For detection in the absence of a known specific mutation, other techniques are needed. One approach is based on short tandem-repeat DNA

sequences (microsatellites)[1-5]. Microsatellites are highly variable sequences commonly found throughout the human genome. Microsatellite alterations include imbalance, in which the allele is of the same size, but of greater or lesser intensity than the corresponding nomal allele, or instability, in which an allele of novel size is found in the neoplastic tissue[1-5].

Microsatellite imbalance is generally thought to result from deletion or amplification of the repeat sequence, and is commonly used as a marker of potential tumor suppressor genes. Such imbalances have been identified in numerous chromosomal sites in breast cancer, including the areas known to contain genes implicated in breast cancer such as erb B-2, BRCA-1, and nm23. Studies employing various microsatellite markers within a specific chromosomal area are proving to be a valuable tool in identifying areas of potential tumor suppressor gene loss. Several of these recent studies have identified microsatellites which are not only heterozygous in the majority of individuals studied, but evidence allelic imbalance in the majority of tumors studied.

Another important milestone in breast cancer research is the realization that the variability in results obtained in different laboratories on oncogene and tumor suppressor gene as well as loss of heterozygosity (LOH) analysis are due in large part to the inherent heterogeneity of breast tumors. Their composition varies widely with respect to the stromal/ epithelial compartment as well as to the presence of both benign and malignant lesions in close apposition. The ability to analyze genetic changes in each of the cell compartments has been made possible, firstly by sensitive PCR techniques and secondly, by the ease with which microdissection of sectioned breast tissue slices can now be performed. In fact, using these techniques, investigators have shown the loss of heterozygosity of markers on 3p, 11q, 13q, 16q, 17 p, and 17q in a significant proportion of breast tumors[4,6,7]. In addition, they showed that the same loss had taken place in DCIS in the same section of the tissue, thereby providing evidence that the LOH had taken place very early in malignant progression[4,6,7]. Such markers show great promise and may prove efficacious in identifying the hyperplasias and/or DCIS that will progress to malignancies versus those that will not.

One of the most exciting recent findings with special importance to breast cancer is the specific and frequent presence of the enzyme telomerase in cancer cells. Telomerase is a ribonucleoprotein enzyme active in germ cells, immortal cell lines, and the majority of

malignancies examined, but inactive in most normal somatic tissues. 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, while 5-25% are telomerase-negative[9-13]. Obviously, telomerase activity as a marker of invasive breast cancer is more useful if it detects 95% of the tumors rather than 75%, so the determination of the real telomerase-negative proportion of invasive breast cancers becomes crucial. In an exhaustive study of 105 breast tumors, we have determined that close to 98% of breast tumors are positive for telomerase, while normal epithelium from the same breast is not (Appendix 1). We have also found that some DCIS adjacent to the carcinoma are positive for telomerase while others are not (our unpublished observations, Appendix 3). These findings point to the potential of telomerase, along with LOH studies, to provide crucial information regarding the biology and genetics of early lesions that may or may not progress to full malignancies. Further, we completed a study of close to 300 nonmetastatic breast cancers to determine the utility of telomerase as a marker of disease outcome (Appendix 4).

BODY

In the last two years of this project, we have accomplished the goals of Specific Aim 1, which was to select microsatellite markers to apply to a panel of breast cancers in Specific Aim 2. We have also established telomerase as a promising new marker of breast cancer, and have applied this marker in parallel. During 1997-98, we have addressed Specific Aim 2 which applies this selected panel of markers to microdissected breast tissue to determine the progression of genetic alterations in normal, premalignant, and malignant tissue. In the long term, the method will hopefully provide a targeted, noninvasive, and more accurate alternative to punch biopsies.

Progress Report:

1. *Specific Aim 1A.* To characterize the frequency of genetic alterations in microsatellites in breast cancer and to develop a panel of informative microsatellite markers.

Rationale: Microsatellite alterations represent a promising technique in diagnosis and primary prevention of breast cancer, since they do not require knowledge of the specific mutation, only knowledge of areas frequently altered in breast cancer and their corresponding microsatellites.

Design: Predictive value analysis for new diagnostic method and selection of panel of markers.

Tissue. Approximately 40 breast cancer cases were identified and selected from banked, frozen tissue in the Breast Cancer Program laboratory. These specimens are from mastectomy or excision of a primary breast cancer. Normal and neoplastic tissue will be microdissected, and DNA from normal and neoplastic areas will be amplified via PCR.

Genetic markers: Genetic imbalance in microsatellites was defined as a change of greater than 50% intensity between the normal and neoplastic tissue. Genetic instability in microsatellites is characterized by an increase or decrease in the number of repeated elements and is detected by the presence of novel electrophoretic bands in neoplastic compared to the normal tissue. Any novel band will be considered abnormal and indicative of instability.

Work completed: A prescreen of DNA from 40 breast tumors has been performed as those providing the greatest likelihood of identifying microsatellite alteration.

The following markers were screened based upon the relatively high frequency (more than 50%)

of LOH seen at these loci:

Chromosome

3p: D3S2397, D3S1597, EABMD, D3S1244

6q: D6S292, D6S311, D6S310, D6S473, and D6S255,

9q21: D9S171, D9S1748, D9S1749, D9S1751, and D9S1752

11q13-23: Int2, D11S29, D11S35, and D11S528

13q12-14: D13S260, D13S263, and D13S155

16q12-24: D16S541, D16S415, D16S265, and D16S402

17p: D17S513, CHRNB1, TP53, D17S786, and D17S122

17q12-24: THRA1, D17S579, and D17S588

PCR reactions typically contained 1 ul lysate prepared from microdissected cells (20 cell equivalent)[4]. PCR products were then separated using denaturing gel electrophoresis, and allelic loss was determined by at least a 75% reduction in the relative intensity of one allele in the tumor compared to normal after autoradiography. Allelic loss was usually confirmed by observation of LOH at multiple informative markers mapped to the same chromosomal region. When only one informative marker was recognized for a chromosomal region in a particular tissue sample, LOH was confirmed by repeating the PCR amplification of that marker.

Data analysis and conclusion. The group of markers with the greatest likelihood of being abnormal are two at 3p24, two at 11p15.5 and two at 17p and 17q. We believe that these will provide a reliable and sensitive means to identify tumors, study their clonality, and to study tumor progression in Specific Aim 2. Considering the small amount of DNA obtained from each sample, limiting the number of markers will, we believe, provide optimal chances of success. We observed microsatellite instability very infrequently and not reproducibly at the same locus. These results lead us to conclude that instability in dinucleotide repeat sequences are not common in breast cancer, and will not prove to be a reliable marker for sporadic breast cancer.

2. Specific aim 1B: To validate telomerase activity as a potential marker for breast cancer:

Rationale: The reverse transcriptase telomerase is reactivated in breast tumor cells. Therefore,

telomerase could prove to be a marker that can distinguish between normal, preneoplastic and malignant breast cells.

Design: Predictive value analysis for new diagnostic and prognostic method.

Tissue. Approximately 400 breast cancer cases, and 20 cases of ductal carcinoma in situ were identified and selected from banked, frozen tissue in the Breast Cancer Program laboratory and from Duke University. These specimens are from mastectomy or excision of a primary breast cancer. Normal and neoplastic tissue were used before and after microdissection.

Work Completed: In the first funding year, we examined 105 breast tumors for telomerase activity on lysates prepared from: whole tissue, 1 frozen section after histopath examination, or microdissected tumor material. We ascertained that close to 97% of breast tumors are positive for telomerase, while 17 microdissected normal lobules were negative for telomerase. Histopathological examination was found to be crucial prior to assaying (see appendix 1). Recent studies have revealed that very weak telomerase activity is detected in some fibroadenomas, and in normal breast tissue during the luteal stage. Activity in tumors is significantly higher than in these tissues. We have also established that 70% of DCIs are positive for telomerase activity while 30% DCIS are not. The same proportion of telomerase positive DCIS are observed when the DCIS lesions appear by themselves or adjacent to carcinomas. This data suggests that telomerase negative DCIS progress to telomerase positive DCIS, or alternatively, identifies a set of DCIS that will never progress to malignancy (Appendix 3). We have, in the second year of funding, also completed a study examining telomerase activity to disease outcome in 300 patients with five or more years of followup (Carey et al, submitted to JNCI). Using cryopreserved tissues for quantitative telomerase analysis, there was no correlation between telomerase activity and disease free or overall survival (see Appendix 4). It appears that telomerase activity shows the sensitivity and specificity necessary for a tumor detection marker, but its utility as a prognostic marker is currently limited by the need for cyropreserved tissues and must await the development of more sensitive quantitation techniques.

**Methods:**

Telomerase studies:

Selective sampling of small cell populations by microdissection: Biochemical assays attempting to find differences between normal and cancerous tissue, and between lesions at different stages of the disease in clinical material have often been hampered by contamination. This can be minimized by microdissecting tissue samples under the light microscope. Briefly, unstained, formalin-fixed 5-10 micron tissue sections are deparaffinized, rinsed in ethanol and 5% glycerol in water, and immediately processed under 100X magnification. Alternatively, 10-20 μm unstained cryostat sections are placed on glass slides, and thawed rapidly to 4°C. Areas of interest are selected on adjacent sections stained with hematoxylin and eosin. Specific cells of interest are identified and scraped away using a disposable 30-gauge needle, placing the sampled nests of cells directly into 10-20ml of the appropriate lysis buffer for nucleic acid or protein assays. Particular attention will be given to the sampling of histologically distinct tumor areas whenever feasible. In addition, a large repository of breast cancer cases are available in a frozen tissue bank in the department of pathology and in our laboratory.

Telomerase assay: The standard in vitro telomerase assay is based on a primer extension reaction in which telomerase synthesizes telomeric repeats onto oligonucleotide primers. Two modifications in the protocol have decreased the number of cells necessary for detection from 10^7 to less than 10^2: a detergent lysis extraction which allows a more uniform recovery of telomerase activity at low cell numbers, and the PCR-based Telomeric Repeat Amplification Protocol (TRAP) in which the telomerase synthesized xtension products serve as templates for PCR amplification in the same reaction tube [14]. The commercially available HotStart 50™ reaction tubes (Life Technologies Inc.) facilitate the two step assay by incorporating a wax barrier which remains intact during the telomerase phase of the assay, and melts during PCR.

Statement of Work:

Specific Aims 1 - 3: To develop molecular diagnostic techniques which may be used in the detection of breast cancer and to utilize these techniques in defining the genetic alterations associated with neoplastic progression in breast.

Task 1: Months 1-12: To develop a panel of informative microsatellite markers by examining a series of potential markers in DNA obtained from microdissected normal and breast cancer tissue.

Additional Task 1 undertaken: To determine the frequency of telomerase activation in breast cancer.

Status: Completed

Task 2: Months 12-24: To characterize the frequency of these genetic alterations in breast cancer by applying the microsatellite panel to a series of microdissected normal and breast cancer tissue.

Additional Task 2 undertaken: In the microdissected specimens in which loss of heterozygosity markers were studied, we performed telomerase assays on the same lysate, or on an adjacent section. The evaluation of both types of markers-microsatellite as well as telomerase on the same tissue provided valuable information, and a direct comparison of the utility of these two markers.

Status: Microsatellite alterations were not reproducibly found in sporadic breast cancer specimens to enable the development of a reliable panel of markers. An examination of the value of telomerase for distinguishing between ductal carcinoma in situ that will progress versus those that may not, was completed (MS appended). The value of telomerase as a prognostic indicator was tested on a panel of nearly 295 breast carcinomas with 5 or more years of followup. We concluded that telomerase activity was associated with rRNA level, tumor content, and hTR, as well as proliferative index, but does not predict survival in non metastatic breast cancer patients.

CONCLUSIONS

1) A panel of microsatellite and LOH markers were selected for studying tumor progression in situ, in frozen sections of breast cancer. While LOH was frequently observed in breast specimens, microsatellite instability was infrequent. Thus instability, unlike in other types of tumors, is not commonly observed in the panel we utilized.

2) Activation of telomerase occurs in the vast majority of breast cancers. Telomerase activation occurs early in DCIS, raising the possibility that it is an early marker of breast cancer. Further, in non-metastatic breast cancer patients with five or more years of followup, telomerase activity is associated with rRNA level, tumor content, and hTR, as well as proliferative index, but does not predict long term survival.

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APPENDICES.

Publication 1: Clin Cancer Res. 4, 435, 1997

Publication 2: Cancer Res. 57: 5605, 1997

Manuscript 1: Telomerase activity in ductal carcinoma in situ... (submitted to Oncogene)

Manuscript 2: Telomerase activity and prognosis in primary breast cancer (submitted to JNCI)

Careful Histological Confirmation and Microdissection Reveal Telomerase Activity in Otherwise Telomerase-negative Breast Cancers[1]

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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[3] 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. Breast cancer tissue specimens that tested negative for telomerase activity in the original screening subsequently underwent further evaluation using two methods: (a) histological confirmation; and (b) microdissection. We found that although 22% of invasive breast cancers were telomerase negative in standard screening, only 5% were telomerase negative if the sample tested for telomerase activity was carefully histologically confirmed and microdissected, suggesting that telomerase activity is in fact nearly ubiquitous in invasive breast cancers.

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

[3] The abbreviations used are: TRAP, telomeric repeat amplification protocol; TLB, TRAP lysis buffer; ITAS, internal TRAP assay standard.

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-[(3-cholamidopropyl-dimethyl-ammonio]-1-propane sulfonate), 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.1 mM (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochlorine, 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
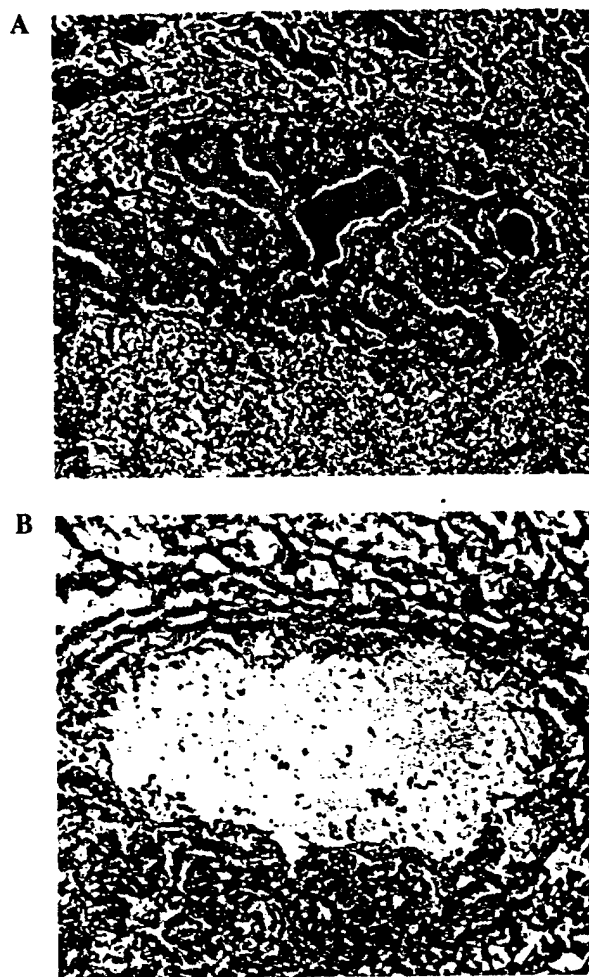

Fig. 1 Photomicrographs demonstrating the technique of microdissection. A, invasive tumor before microdissection; B, an adjacent cryosection of the same tumor after microdissection, demonstrating the removal of a pure population of tumor cells.

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 $MgCl_2$, 1 mM EGTA, 0.005% Tween-20, 50 μM deoxynucleotide triphosphate, 0.2 μg of TS oligonucleotide (5′-AATCCGTCGAGCAGAGTT-3′; Operon) end-labeled with γ^{32}P-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

Table 1 Comparison of the presence or absence of telomerase activity with tumor size and lymph node metastasis in the screened breast cancers
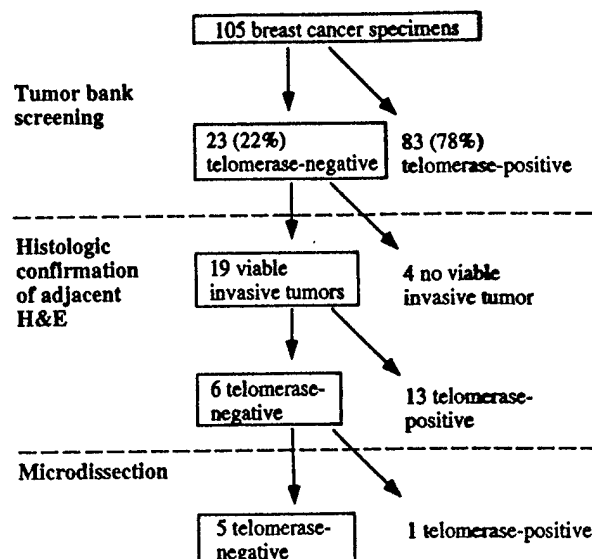
	N	Telomerase positive n (%)	Telomerase negative n (% ± SE)
Tumor size, cm			
≤2	22	20 (91)	2 (9 ± 12)
>2	80	60 (75)	20 (25 ± 10)
Unknown	3	2 (67)	1 (33)
Lymph node metastases			
Absent	31	24 (77)	7 (23 ± 15)
Present	51	37 (72)	14 (27 ± 12)
Unknown	23	21 (91)	2 (9)

(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 and 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.[4] 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).

Statistical Analysis. The proportion of tumors exhibiting telomerase activity was compared to tumor size (greater than versus less than or equal to 2 cm) and lymph node status (metastases present or absent) using two-tailed Fisher's exact test with a significance level of $P \leq 0.05$.

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

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.

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 resection 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
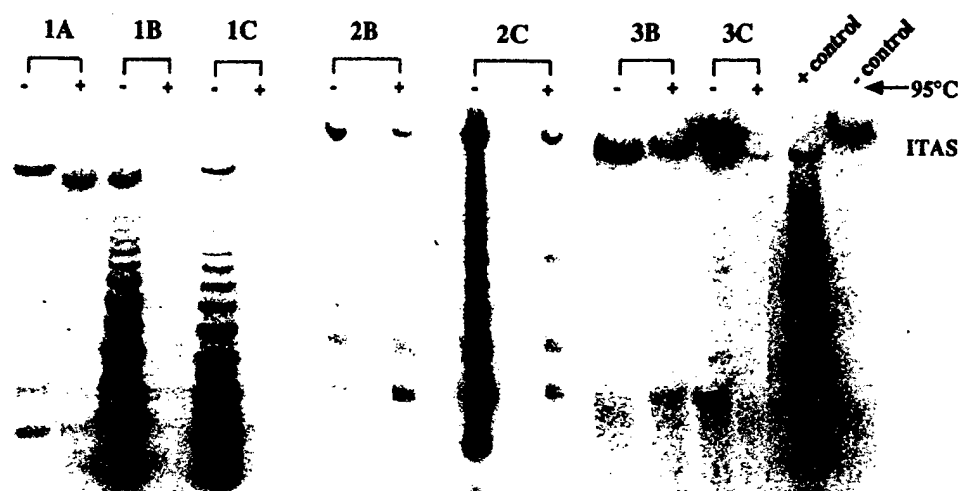
Fig. 3 Left panel: telomerase activity in one tumor that was telomerase negative in the original screening (*1A*) but was telomerase positive in the histologically confirmed whole section (*1B*). In this as in all telomerase-positive sections, the microdissected pure tumor sample also remained telomerase 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.[4] 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.*

(2), who found a correlation between breast cancer stage and likelihood of exhibiting telomerase activity, with higher stage tumors more likely to be telomerase positive. Conversely, if the true telomerase-negative rate of invasive breast cancers is very low, this tumor marker gains greatly in its potential clinical applications, particularly because in this as well as in other studies, the specificity of the TRAP assay is very high. According to recent studies, virtually no normal breast tissue or benign breast lesion exhibits telomerase activity with the possible exception of a proportion of fibroadenomas (2–6).

Previous studies of invasive breast cancers have found a telomerase-positive rate of between 73% (5) and 95% (6). Our screening efforts detected telomerase activity in 78%, which is consistent with these and other studies. Unlike Hiyama et al. (2) and Sugino et al. (5), but in concordance with findings of Nawaz et al. (4), we did not detect a correlation between telomerase activity and clinical stage at the time of screening. In fact, the five tumors that remained telomerase negative at the end of the study were of mixed size and lymph node status. Telomerase activity was examined as a continuous variable in Dr. Sugino's study (5), which was hampered by the use of an older, less quantitative assay technique (3) than those that are presently available (9, 11). In addition, these investigators did not use an internal control sequence to standardize for the efficiency of the PCR reaction (9), which may also have confounded their results. Dr. Hiyama's study (2), however, examined telomerase activity in much the same manner as our own study. Their study was slightly larger (140 invasive breast cancers) than ours (105 invasive cancers) and included a greater proportion of smaller, lymph node-negative tumors. However, this study was sufficiently powered to reliably detect an association of tumor size or lymph node status and telomerase activity. The discrepant result may reflect the potential for an excess of normal tissue in the banked sample from smaller tumors, which are more likely to be from patients who are lymph node negative.

After careful histological confirmation by H&E staining of the immediately adjacent section, the proportion of telomerase-negative breast cancers fell to 6%. Thus, by this simple maneuver, the sensitivity of the TRAP assay in detecting telomerase activity rose markedly, making telomerase activity a considerably more useful tumor marker. This finding also raises justifiable concern about the use of the official pathology reports to define banked tumor samples. Breast cancers in particular are a heterogeneous group both macroscopically and microscopically. Macroscopically, the palpated mass can include fibrosis, inflammation, and postbiopsy changes as well as cancer. Microscopically, the tumor typically contains varying amounts of inflammatory cells, stroma, normal ductal tissue, preneoplastic tissue, and in situ carcinoma as well as invasive cancer.

Further evaluation of the six persistently telomerase-negative 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 heterogeneous 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.

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Telomerase Activity in the Normal and Neoplastic Rat Mammary Gland[1]

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ABSTRACT

The 1-methyl-1-nitrosourea-induced rat mammary tumor model system is well studied, reproducible, and widely used. We have investigated whether these tumors possess higher telomerase activity than normal mammary tissue. Using the telomeric repeat amplification protocol assay, we found significantly higher telomerase activity in 36 mammary carcinomas than in 72 mammary glands of virgin rats. The level of telomerase activity in virgin rats was unaffected by strain, age, stage of the estrous cycle, or ovariectomy. However, mammary glands obtained from pregnant rats exhibited telomerase activity comparable to that found in the tumors, possibly reflecting the high epithelial content of these tissues. Indeed, isolated epithelial cells from virgin and pregnant mammary glands and from carcinomas had similar telomerase activities. Thus, telomerase activity is constitutive in the rat mammary epithelium and is not a unique characteristic of malignant transformation in this tissue. These results underscore the importance of attributing biochemical properties to specific cell types in a tissue, a situation not paralleled in the interpretation of data from *in vitro* models.

INTRODUCTION

The ribonucleoprotein telomerase is responsible for the synthesis and maintenance of the specialized structures at the ends of chromosomes called telomeres, which display many important structural and functional roles during interphase, mitosis, and meiosis (1, 2). Telomeres consist of tandemly repeated DNA sequences (TTAGGG) ranging from 5–20 kb in length in humans, 20–100 kb in length in the rat, and 100–150 kb in mice (3). They protect genomic DNA from degradation and deleterious recombination events (1, 2). Although telomerase is not usually detectable in normal somatic differentiated tissues, it appears to be reactivated in a large variety of human tumor types, resulting in the reinitiation of synthesis of telomere repeat units, stabilizing telomere length (4, 5). Recent studies have shown that, in contrast to humans, almost all somatic tissues of mice and rats (6–13) have detectable telomerase activity. The presence of telomerase activity in normal rat tissues was attributed to the presence of somatic stem cells in the tissues with regeneration potential, an idea similar to that reported in human epithelium (14), skin (15), and hematopoetic cells (16). The level of telomerase activity was, however, substantially higher in the rodent tumors compared to the corresponding normal tissues (6–12). In this study, we have evaluated telomerase activity as a tumor biomarker in rat mammary gland and MNU[4]-induced mammary carcinomas (17, 18), a model system widely used in experimental carcinogenesis research (19, 20). Based on the work cited above, we hypothesized that higher telomerase activity would be observed in

mammary carcinomas than the normal mammary gland, and that the degree of increase would be a consistent phenotype that would distinguish carcinomatous mammary epithelium from nontransformed mammary epithelium. As expected, we found high telomerase activity in mammary carcinomas. However, further investigation revealed that enzyme activity in mammary carcinomas may be a reflection of the high epithelial cell content of the tissue and that telomerase activation is not an obligatory event of malignant transformation in this system.

MATERIALS AND METHODS

Tissues. For determining telomerase activity in normal mammary glands, six female Wistar-Furth rats were used. This group consisted of three 13–15-week-old parous rats, and 3 28-day-old virgin rats. Female Sprague Dawley rats were used for studying the influence of age, parity, stage of the estrous cycle, hormonal modulation, pregnancy, and lactation on telomerase activity in breast tissue. The impact of age on telomerase activity was tested in 30-, 60-and 90-day-old virgin rats. To test telomerase activity caused by hormonal changes in the breast, mammary glands from six 10-week-old parous rats, four 35-day-old virgin rats at estrus and diestrus (2-day timed cycle), eight 10-week-old rats at early, mid, and late pregnancy, and six estrogen-reconstituted ovariectomized rats were examined. Ovariectomy was performed on six 50-day-old female rats. Four days after surgery, three rats were treated with one dose of 17β-estradiol (5 μg/0.1 ml corn oil/rat) s.c., whereas the remaining three received the vehicle. Rats were sacrificed 76 h after initiation of hormone treatment. All 12 mammary glands from each rat were snap frozen in liquid nitrogen and stored at −80°C. For comparison of telomerase in mammary glands of various strains, mammary glands were collected from 30-day-old female virgin rats of strains Sprague Dawley, Wistar Furth, Fisher 344, Copenhagen, Lewis, and Buf/N. Cell suspensions were prepared from the six mammary glands on the right side by digesting the minced tissue with collagenase (800 units) and hyaluronidase (200 units) and enriching for epithelial cells as described (21). The digest was filtered; the organoids were collected off the filter and stored frozen at −80°C. The remaining six mammary glands were snap frozen and stored at −80°C. Identical digests were obtained from three pregnant (20-day) rat mammary glands.

Mammary Tumors. Female Sprague Dawley or Wistar-Furth rats (45 days old) received a single i.p. administration of MNU (50 mg/kg body weight; Ash-Stevens). Tumors arose in MNU-treated rats 2–4 months later (17, 18). Tissues were excised, trimmed, and frozen at −80°C. Mammary glands containing small tumors (<10 mm³) from 13 rats were embedded in OCT and cryosectioned; islands of tumor cells and normal ductal epithelium from tumor-free glands were microdissected from three to four consecutive sections under ×10, using an 18-gauge hypodermic needle, and placed in lysis buffer (see below).

TRAP Assay on Tissue and Cell Lysates. The PCR-based TRAP assay for telomerase activity was used as described previously (22, 23). Ten-μm frozen sections were obtained of tissues embedded in OCT compound. One section from each tissue was stained with H&E for histological confirmation. Telomerase activity was determined in duplicate on lysates of 5–10 adjacent sections by the TRAP assay (22), and a negative control was provided for each extract by heat inactivation at 95°C for 10 min. To enable quantitation of telomerase activity levels, telomerase reactions were repeated using the TRA-Peze assay kit as described (Ref. 23; Oncor, Gaithersburg, MD); each reaction product was amplified in the presence of a 36-bp internal TRAP assay standard. The level of telomerase in each extract was determined by measuring the combined intensities of the radioactive signal for each sample and comparing it with that obtained by using a fixed amount of protein from the quantitation standard (23). In this study, instead of the quantitation controls

provided in the kit, we used one to three reactions of 0.06 μg of protein from lysates of a mouse mammary tumor cell line, MOD, per set of reactions. Tissue lysates were tested using between 0.06 and 2.0 μg of protein per reaction, depending upon the strength of the telomerase reaction following a 12–24-h exposure of the autoradiograph at −70°C. Each set of reactions included tubes without any extract, and extracts were heated at 95°C for 10 min or treated with RNase A (200 μg/ml). The average absorbance of the first eight TRAP bands above the primer band was calculated as a ratio to the ITAS. Quantitation was performed using the IP Lab Gel software. We used an arbitrary unit for quantitation of telomerase activity in our samples, which was derived for each sample as follows:

$$\text{Relative telomerase activity} = \frac{TE/I/TE^*/I}{ME/I/ME^*/I}$$

where TE, ME, and I is the intensity of the signal in the tissue extract (2.0 μg of protein), in the MOD cell extract (0.6 μg protein), and in the ITAS standard, respectively. The * denotes extracts where telomerase has been heat inactivated prior to initiation of the reaction. None of the extracts with negative and low level telomerase activity inhibited the telomerase activity of MOD, excluding the presence of an inhibitor to telomerase in these extracts.

Statistical Analysis. All Ps were derived using Wilcoxon's Rank Sum test for nonparametric data; $P < 0.05$ was considered statistically significant. The statistical analysis was performed using the JMP statistical software package (SAS Institute, Inc.) on a Macintosh microcomputer.

RESULTS

Telomerase Activity Is Detectable in Mammary Glands from Different Strains of Rat. Several rat strains are commonly used in the investigation of chemically induced mammary carcinogenesis. Thus, we first sought to determine if constitutive levels of telomerase could be detected in the mammary glands of the most commonly used rat strains. We compared the telomerase activity in two whole mammary gland lysates from two rats each of six different strains: Sprague Dawley, Wistar/Furth, Lewis, Buf/N, Fisher 344, and Copenhagen. A low level of telomerase activity was observed in the lysates prepared from pooled sections (from six mammary glands each) of the six rat strains (data not shown). In view of this observation, we proceeded to evaluate constitutive levels of telomerase more extensively in both parous and virgin Wistar-Furth rats because this a commonly used inbred rat strain in experimental mammary carcinogenesis. As shown in Fig. 1, detectable activity was readily observed in several among the 72 mammary glands derived from six rats, irrespective of their anatomical location. However, telomerase activity in the parous rats was significantly lower than in the 28-day-old virgin rats ($P < 0.01$). Although activity was variable among glands, no consistent pattern to these differences was observed. The majority of the mammary glands from both sets of rats, however, expressed telomerase activity at low or undetectable levels (Fig. 1).

Telomerase Activity Is High But Variable in MNU-induced Mammary Carcinomas in Rats. Levels of telomerase activity were evaluated in 36 mammary carcinomas induced by MNU. All carcinomas were positive for telomerase activity, although the level of activity varied greatly among individual carcinomas (Fig. 2 and data not shown). Quantitative analysis of the telomerase activity present in the tumors was performed to determine the telomerase activity in the tumor extract relative to the normal mammary gland. Telomerase levels in the mammary carcinomas were significantly higher than in the normal mammary gland ($P < 0.0001$), suggesting the potential utility of telomerase as a tumor marker.

Is Elevated Telomerase Activity a Tumor-specific Phenotype? Based on the observation that telomerase activity is low in normal mammary glands but high in mammary carcinomas, we asked whether the increased levels were associated with tumorigenesis or whether
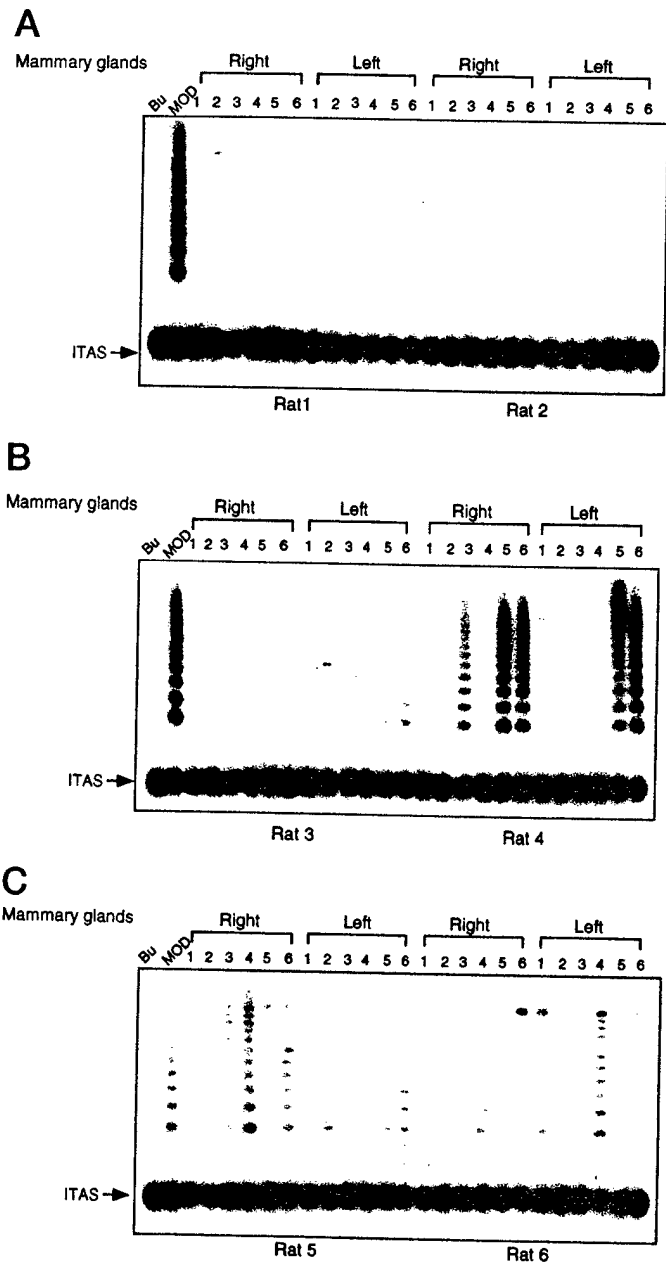

Fig. 1. TRAP assay of telomerase activity in normal rat mammary gland. Whole-tissue extracts (2.0 μg of protein) prepared from each of 12 mammary glands from rats 1–6 were tested using a commercial assay kit (TRAPeze; Oncor). *Rat 1–3*, Rat 13–15-week-old parous rats; *Rat 4–6*, Rat 28-day-old virgin rats. *Bu*, lysis buffer, negative control; *MOD*, 0.06 μg of protein from mouse mammary cell line; *ITAS*, 36-bp ITAS.

they reflected changes in growth and architecture of the mammary gland in response to hormonal stimuli. To test the effects of hormonal modulations of mammary growth, tissue lysates from two mammary glands each of rats: in diestrus and estrus; ovariectomized; ovariectomized followed by exogenous estrogen; and at early, mid, mid/late stages of pregnancy were examined for telomerase activity. Mammary glands of rats in estrus showed a slightly higher telomerase activity compared to rats in diestrus (Fig. 3A). A similar increase was observed in the mammary glands of ovariectomized rats 48 h after estrogen administration (Fig. 3A). However, telomerase activity in mammary glands of rats at early, mid, or late stages of pregnancy (Fig. 3B) was significantly higher than in virgin rats ($P < 0.0001$). In fact, the range and distribution of activities in the pregnant gland was similar to that observed for mammary tumors (Fig. 2B).
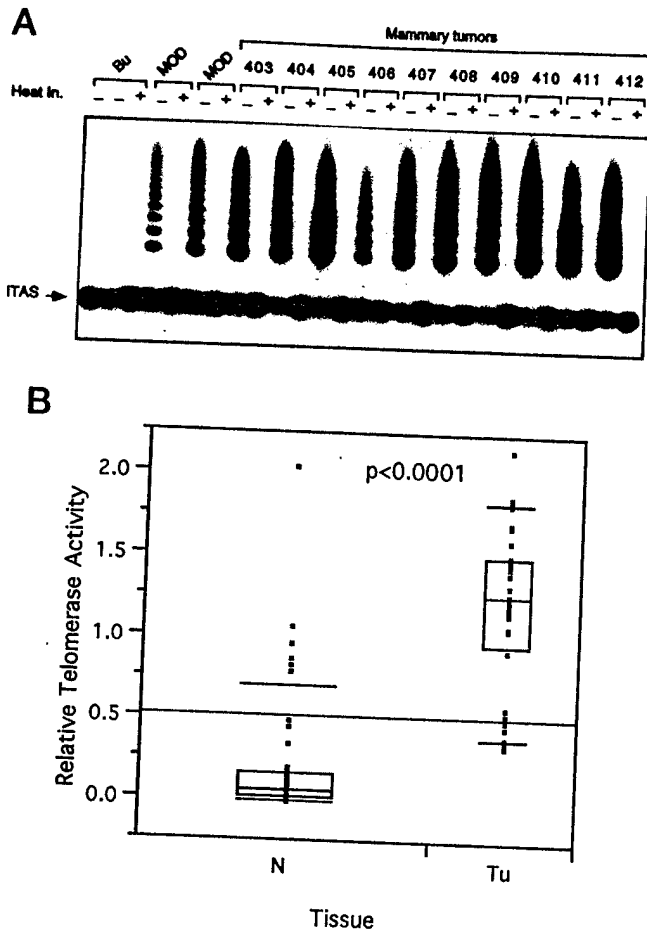
A

B

Fig. 2. *A*, telomerase activity in MNU-induced rat mammary carcinomas. *Bu*, lysis buffer; *MOD*, mouse mammary tumor cells (0.06 μg of protein). Lysates (2.0 μg) prepared from pooled cryosections of mammary tumors were subjected to TRAP (TRAPeze) assay. Samples were subjected to the TRAP assay with (+) or without (−) heat inactivation of the tissue extract. *B*, nonparametric Wilcoxon Tests (Rank Sums) were performed comparing *Relative Telomerase Activity* present in 76 normal mammary glands (*N*) to that present in 36 MNU-induced mammary tumors (*T*) using a JMP Statistics program (SAS Institute, Inc.). Quantile box plots for each group are shown, summarizing the distribution of data points for both groups; the *top* and *bottom* of the *box* represent the 75th and 25th quantile levels, respectively, whereas the upper line and lower lines represent the 90th and 10th quantile levels, respectively.

The major structural difference between virgin and pregnant mammary gland is the ratio of stroma to the epithelium. Whereas epithelial ducts are infrequently distributed in an abundance of stroma in the virgin mammary gland, the reverse is true in the pregnant mammary gland (24). We, therefore, asked whether the differences in telomerase activity could be attributed to the cellular composition of the tissue. We determined telomerase activity in lysates from epithelial organoids obtained by collagenase digestion of six mammary glands each from six pubertal and three pregnant rats. Lysates of organoids from virgin and pregnant mammary glands (Fig. 4A) showed comparably high telomerase activity.

The analysis of telomerase activity in the mammary organoids established that the epithelial cell compartment in the mammary gland was, in all probability, the source of telomerase activity. Therefore, variations in the ratio of epithelium to stroma in tissue samples can lead to large variations in measured telomerase activity in tissue samples. This result, however, did not explain the wide range of telomerase activity observed in the tumors that are predominantly composed of epithelial cells (Fig. 2B). To determine whether this variation in telomerase activity is a reflection of the heterogeneity of epithelial cell populations in the mammary gland, we tested 24 mi-

crodissected carcinoma loci and 24 histologically normal ductolobular units from an uninvolved gland from the same rat. Telomerase activity in the carcinomas and normal ductal samples varied widely from undetectable activity to very high activity (Fig. 4B). These results indicate that different ductolobular units in the breast (as well as carcinomas) express different levels of telomerase activity, a fact that was not readily apparent when pooled organoids were tested (Fig. 4A).

DISCUSSION

In this study, we provide evidence that telomerase activity is constitutive in rat mammary epithelial cells. Telomerase activity of whole tissue mammary gland homogenates from normal virgin rats was very low compared to activity in MNU-induced mammary carcinomas. However, we showed that this difference could be attributed to the vast difference in epithelial:stromal ratio between the two, because epithelial cells isolated from the mammary gland expressed the same level of telomerase activity as the mammary carcinoma.

The objective of this study was to test the hypothesis that elevated telomerase activity would be a distinguishing characteristic of mammary cancer. To provide a basis for hypothesis testing, the constitutive levels of telomerase in virgin mammary glands from animals of various ages and different rats strains were assessed. In all cases, telomerase activity was detected. This finding is consistent with reports of detectable levels of telomerase in other tissues of the rat (10–13) and with data from mice (6–9, 12). We then proceeded to assess telomerase activity in a randomly selected subset of MNU-induced rat mammary carcinomas (17, 18). A comparison of telomerase activity in whole-tissue lysates of virgin mammary glands (Fig. 1) to telomerase activity in MNU-induced mammary tumors (Fig. 2) provided strong support for the hypothesis that telomerase activity is
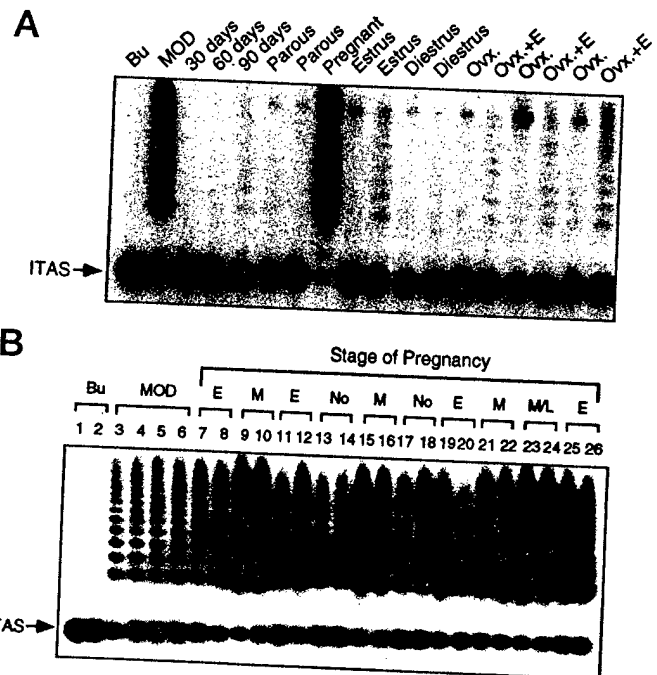
A

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Fig. 3. *A*, influence of age, parity, estrus cycle, pregnancy, and ovariectomy (*Ovx.*) on telomerase activity. *Bu*, lysis buffer; *MOD*, mouse mammary tumor cells (0.06 μg of protein); *E*, estrogen. Protein (2.0 μg) of the indicated tissue lysate was used for the TRAP (TRAPeze; Oncor) assay. *B*, influence of pregnancy on telomerase activity. *Bu*, lysis buffer; *MOD*, mouse mammary tumor cells (0.06 μg of protein). Protein (2.0 μg) of the indicated tissue lysate was used for the TRAP (TRAPeze, Oncor) assay. *No*, normal virgin mammary gland from 30-day-old rat; *E*, early pregnancy, 5–7 days postcoitum; *M*, mid-pregnancy, 10–12 days postcoitum; *M/L*, mid/late, 13–18 days postcoitum; *L*, late pregnancy, 19–21 days postcoitum. ITAS, 36-bp ITAS.
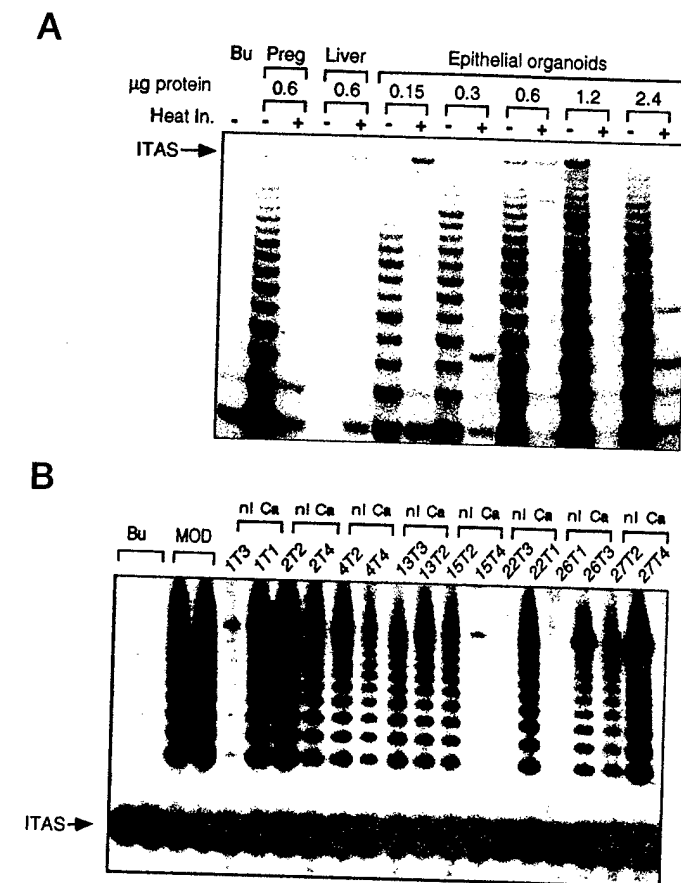
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Fig. 4. *A*, telomerase activity in epithelial organoids isolated from rat mammary glands. *Bu*, lysis buffer; *Preg*, organoids isolated from 20-day pregnant rat mammary glands; *Liver*, liver from 30-day-old female rat. Epithelial organoids were obtained by digestion of mammary glands from 30-day-old Sprague Dawley rats. Samples were subjected to the TRAP assay (22, 23) with (+) or without (−) heat inactivation (*Heat In.*) of the tissue extract. *ITAS*, 150-bp ITAS. *B*, telomerase activity in microdissected normal lobules and tumor foci in the mammary glands of MNU-treated rats. *Bu*, lysis buffer; *MOD*, mouse mammary tumor cells (0.06 μg of protein). Extracts (0.12 μg of protein) of microdissected cells from normal ducts (*nl*) or carcinoma (*Ca*) were subjected to TRAP (TRAPeze; Oncor) assays. *ITAS*, 36-bp ITAS.

higher in mammary carcinomas than in virgin mammary gland. This observation set the stage for further rigorous testing of telomerase as an intermediate tumor marker.

One of the fundamental questions in telomerase biology is whether telomerase activation occurs during rapid growth in response to mitogenic signals issued as a result of normal physiological processes or whether it occurs in response to signals specific to neoplastic transformation. Telomerase activity was not significantly affected by stage of the estrous cycle or the administration of exogenous estrogen (Fig. 3A), consistent with the notion that telomerase activation is specific to neoplastic transformation. However, the high levels of telomerase observed in the pregnant mammary gland (Fig. 3B) strongly argued against this hypothesis. In fact, the telomerase activity levels in pregnant mammary glands were as high as those seen in many mammary carcinomas. We reasoned that elevated telomerase activity might be a reflection of a change in the epithelial cell compartment of the tissues. Whereas the virgin mammary gland is mainly composed of adipose tissue and stroma interspersed with a few epithelial ductal lobular units, in the pregnant mammary gland there is an expansion in the number of ductal lobular units, resulting in a drastic increase (up to 70%) in the proportion of epithelial cells relative to stroma and fat cells. The mammary carcinomas, even more so than the mammary glands of pregnant rats, are predominantly epithelial in content but can

contain varying numbers of interlacing stromal cells (24). Mammary organoids isolated by enzymatic digestion of pregnant mammary glands showed telomerase activity comparable to that present in the virgin mammary gland from all six rat strains: Sprague Dawley, Wistar Furth, Fisher 344, Copenhagen, Lewis, and Buf/N (Fig. 4A and data not shown). These findings argue strongly that telomerase activity is constitutive in mammary epithelial cells. Constitutive activity has also been observed in rat colon (10), liver (10, 11, 13), brain (12), vagina, mammary gland, and uterus[5] and in myometrium, as well as in leimyomas, tumors arising from the myometrium of the uterus.[6]

Although the analysis on organoids represents the activity present in a particular cell type compartment in the mammary gland, it is nevertheless a pooled population of epithelial elements. We, therefore, subjected the question to more rigorous testing by measuring telomerase activity in microdissected specimens of both tumor and normal epithelial islands. We found a great variability in telomerase levels among the samples (Fig. 4B). This was not a characteristic unique to tumors, because similar variations were observed in microdissected islands of epithelial cells from normal glands as well (Fig. 4B). Thus, we can conclude that the constitutive telomerase activity present in the rat mammary epithelium is highly variable. Although the most obvious explanation for this variability is differences in the proportion of stem cells in the population, our poor understanding of mammary cell biology prevents us from speculating any further.

Reactivation of telomerase appears to be essential for maintenance of telomere length in human tumor cells (1–5). By contrast, as noted above, telomerase activity is constitutively high in many normal rodent tissues. For this reason, it seems unlikely that reactivation of telomerase is necessary for malignant transformation in rodents. However, several groups have reported a quantitative difference between telomerase activity in rodent tumors of the skin, pancreas, mammary gland, and liver when compared to normal somatic tissues (7–9, 11, 12). It appears that these investigators have not taken into account the difference in the dense epithelial cellular composition of the tumor compared to the relatively infrequent presence of epithelial cells in the normal tissue. In view of our observations, a similar detailed study is warranted in these tumor model systems as well as in human breast cancer (25, 26).

In summary, telomerase activity was significantly elevated in mammary tumors and mammary gland from pregnant animals, effects related to the increased ratio of epithelial to stromal cells in these two tissues. Although telomerase is not a marker of transformation *per se*, it remains to be determined whether the variability in the telomerase activity of morphologically similar carcinomas will be an informative tumor endpoint biomarker of prognostic value. These results underscore the importance, in interpreting telomerase activity data derived from tissues, of taking into account differences in the epithelial: stromal ratio of cells in the biopsied tissue, a situation not paralleled in the interpretation of data from *in vitro* models.

ACKNOWLEDGMENTS

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Telomerase Activity in Ductal Carcinoma *in situ* and Invasive ~~Breast~~ Cancer of the Breast[1].

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Running Title: Telomerase activity in DCIS and Invasive Breast Cancer

Key Words: Telomerase. Breast cancer. Ductal carcinoma in situ. Microdissection. Cancer progression.

Abbreviations: DCIS, Ductal carcinoma in situ; TRAP, telomere repeat amplification protocol; TLB, Telomerase lysis buffer; AEBSF, 4(-2-aminoethyl)-benzenesulphonyl fluoride hydrochlorine; CHAPS, 3-{(3-cholamidopropyl)-dimethyl-ammonio}-1-propanesulfonate; PCR, polymerase chain reaction; ITAS, internal telomerase assay standard; RT-PCR, reverse transcriptase-PCR.

Abstract.

The increasing number of breast carcinoma in-situ detected by screening procedures makes it imperative to develop improved markers to stratify the risk of invasive cancer. Telomerase has been found in most invasive cancers, and is undetectable in most normal tissues. We have investigated whether ductal carcinoma in-situ (DCIS) is the stage in which telomerase activation occurs. We have microdissected frozen tissue blocks containing both DCIS and invasive cancer to assay the telomerase activity of these two lesions. The 46 available cases of concurrent DCIS and invasive breast cancer resulted in 43 DCIS samples and 38 invasive cancer samples adequate for analysis. 70% of the DCIS samples and all invasive cancer samples had detectable telomerase activity. These results were validated by analyzing 10 additional DCIS patients without invasive cancer, 3 of which showed no telomerase activity. Mixing experiments excluded the presence of telomerase inhibitors. Furthermore, periductal inflammatory infiltrates were shown to be a potential confounding source of telomerase activity. Since 30% of the DCIS samples lack detectable telomerase, telomerase activity may be a useful adjunct in stratifying the risk of developing invasive breast cancer in preinvasive breast lesions.

Introduction.

The success of screening procedures for breast cancer is creating an increasing number of patients with the diagnosis of carcinoma in-situ. The appropriate management is problematic, since recurrence rates after local excision may reach 20%, and adjuvant radiotherapy only appears to decrease recurrence rates by 50% (Fisher *et al.*, 1998; Morrow *et al.*, 1996). Mastectomy is curative, but is an unacceptable choice for a preinvasive lesion. Therefore, the development of new markers to improve the stratification of the risk of invasive cancer is imperative.

Telomerase is a ribonucleoprotein enzyme which is responsible for maintaining the length of chromosomal telomeres. It is detectable in germ cells, tissues with rapid cellular turnover, immortal cell lines, and cancer cells, but undetectable in most normal differentiated somatic tissues (Bacchetti & Counter, 1995; Greider & Blackburn, 1996; Shay, 1995; Wright & Shay, 1995). Telomerase activity has been proposed as a potentially useful tumor marker in breast cancer, since it has been detected in most invasive breast cancers, but not in benign breast lesions, with the possible exception of some fibroadenomas (Kim *et al.*, 1994; Meeker & Coffey, 1997; Nelson, 1996; Shay & Bacchetti, 1997). In several studies of primary invasive breast cancers, approximately 75-95% of tumors demonstrate telomerase activity, while 5-25% are telomerase-negative (Bednarek *et al.*, 1997; Hiyama *et al.*, 1996; Landberg *et al.*, 1997; Nawaz *et al.*, 1997; Sugino *et al.*, 1996b). Initial results suggested that smaller, lymph node-negative tumors were more likely to be telomerase-negative than their larger, lymph node-positive counterparts (Hiyama *et al.*, 1996; Kim *et al.*, 1994), and it was postulated that telomerase activity is acquired during tumor progression to metastasis (Hiyama *et al.*, 1996). More recently, some studies have found correlations between telomerase levels and stage, traditional prognostic indicators, and clinical outcome in invasive breast cancer (Clark *et al.*, 1997; Hoos *et al.*, 1998). Other studies, however, failed to show a correlation between telomerase activity and prognostic factors or clinical outcome in breast cancer (Bednarek *et al.*, 1997; Carey *et al.*, 1998; Nawaz *et al.*, 1997; Sugino *et al.*, 1996a; Tsao *et al.*, 1997) and (Carey et al., in preparation 1998).

Telomerase activity is likely to be in some way important to cell survival during neoplastic progression, possibly by delaying cellular senescence sufficiently to allow the accumulation of the multiple genetic alterations required for the malignant phenotype (Chiu & Harley, 1997; Klingelhutz, 1997; Oshimura & Barrett, 1997; Smith & Pereira-Smith, 1996). It is not known, however, when in neoplastic progression this activation occurs. Knowledge of the circumstances under which telomerase activation occurs could help define the role, if any, telomerase plays in malignant transformation and/or tumor progression. Since a large proportion of invasive breast cancers are telomerase positive (Carey *et al.*, 1998), indicating telomerase activation may occur in early stages of the disease, we have focused our attention on ductal carcinoma in situ (DCIS), a

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preinvasive lesion of the breast, as a possible stage in carcinogenesis in which telomerase activation occurs. Previous reports have detected telomerase activity in anywhere from 60-100% of ductal carcinoma in situ (DCIS) of the breast (Bednarek *et al.*, 1997; Poremba *et al.*, 1998; Tsao *et al.*, 1997; Yashima *et al.*, 1998), but the studies have been limited by small sample sizes (n = 5-17). If a substantial subset of DCIS is indeed telomerase negative, telomerase activity may provide a very useful marker to stratify the risk of developing invasive cancer in the emerging population of women in whom screening proceedures have detected this early lesion.

Detection of telomerase activity in clinical specimens currently depends on an enzymatic assay, requiring fresh or frozen tissue samples (Kim & Wu, 1997). Since frozen specimens of DCIS lesions are not readily available due to the diagnostic requirement of excluding invasive cancer, we have analyzed samples of invasive breast cancers in which a significant proportion of the tumor consists of preinvasive lesions. We have microdissected these specimens in order to analyze distinct areas of *in situ* and invasive ductal breast cancer separately, using adjacent H&E stained sections for orientation. We report here on the telomerase activity of very small microdissected tissue samples of concurrent DCIS and invasive breast cancers. We then corroborated these results by assaying telomerase activity in a small number of samples that contained no detectable invasive cancer, and could therefore be evaluated using larger samples of tumor tissue.

Materials & Methods.

Tissue samples

215 cases of concurrent DCIS and invasive breast cancer were identified in the Johns Hopkins tumor bank records from a ten year period. Tissue blocks were obtained by a staff pathologist at the time of surgery and stored at -80°C with a coded identifier. When available, the information on patient age at surgery, tumor size, DNA index, histological grade of DCIS component was recorded. The frozen tissue blocks were embedded in O.C.T. compound (Miles, Inc. Elkhart, IN) and consecutive 8 μm cryostat sections were obtained and immediately frozen in liquid N_2 and stored at -80°C. One section was stained with H&E and reviewed with the study pathologist in conjunction with the paraffin embedded sections for the presence of >10% DCIS component. The presence of both DCIS and invasive breast cancer in the retrieved tissue blocks was histologically confirmed in a total of 46 samples. The adjacent unstained section was briefly thawed and microdissected with a 22g needle at low power magnification (100x) (Zhuang *et al.*,

1995a). At least one area of DCIS and invasive cancer tissue was sampled in all cases. Sufficient material was present to allow independent sampling of two separate areas each for DCIS and invasive cancer tissue in 17 cases. Samples of 50-100 cells were collected and lysed in 15 µl telomerase lysis buffer (TLB: 10mM TrisCl$_{7.5}$, 1mM MgCl$_2$, 1mM EGTA$_{8.2}$, 0.5% CHAPS, 0.1mM AEBSF, 5mM 2-mercaptoethanol, 10% glycerol). After incubation for 30 minutes on ice, the lysates were centrifuged at 16000g for 10 minutes at 4°C, 10 µl lysate was decanted, frozen in liquid N$_2$ and stored at -80°C. The remaining 5 µl were digested x 24h @ 37°C with 1 mg/ml Proteinase K (Boehringer) followed by heat inactivation @ 95°C x10' and stored at -80°C.

Additionally, 17 cases with tumor blocks containing DCIS without invasive breast cancer were identified in the Duke University Hospital tumor bank records. 5 cryostat sections were prepared as described above. DCIS was histologically confirmed on H&E sections in the retrieved tissue blocks in 10 of 17 samples. The other blocks showed invasive cancer (1), atypical hyperplasia (3), benign tumor (2), and 1 normal breast tissue. The median DCIS size was 2.9 cm (range, 1-10 cm). 3 of the DCIS cases had a primary invasive breast cancer in a noncontiguous area of the breast. 2 cryosections were used for extract preparation by overlaying the frozen tissue with 50 µl telomerase lysis buffer and aspirating the resulting lysates. The 2 other sections were used for microdissections as described above.

Quantitation of tissue sample by PCR Assay of DNA content

While the whole section extracts were standardized by protein content as measured by Biorad® assay (Bradford, 1976), protein was undetectable in the microdissected samples. In order to measure the amount of tissue present in the microdissected tissue extracts, PCR amplification of genomic DNA was performed in the tissue extracts and in serial dilutions of MCF7 cell extracts (Hay *et al.*, 1994) assayed in parallel. A 175 bp fragment of the human acidic ribosomal phosphoprotein PO gene (36B4, Genbank accession number M17885) was amplified using the oligonucleotide primers 5'-GAT TGG CTA CCC AAC TGT TGCA (36B4FW) and 5'-CAGGGGCAGCAGCCACAAAGGC (36B4RV). The 36B4FW primer was end-labeled using T4 kinase (Boehringer) and ^{32}P-γ-ATP (Kim & Wu, 1997). 25 µl PCR reactions were performed with Taq DNA polymerase (Perkin Elmer) on 2 µl of proteinase K treated tissue extract using assay conditions recommended by the manufacturer. The samples underwent the following thermal cycling protocol: 94°C x5 min, (55°C x45 sec, 72°C x45 sec, 94°C x30 sec) X30 cycles. PCR products were separated in a 10% nondenaturing polyacrylamide gel in 0.5x TBE buffer (Sambrook *et al.*, 1989) and imaged by phosphorimager screen (Molecular Dynamics). PCR product bands were measured by densitometry using the IPLabGel® program (Signal Analytics, Vienna, VA 22180) on a Macintosh microcomputer. Quantitation was performed after subtracting

lane backgrounds from the genomic 36B4 signal (see figure 1). The densitometric values of the bands were divided by an average per-cell value derived from the serial dilutions of MCF7 cell extracts run in parallel, and expressed in genome equivalents.

Assessment of tissue sample preservation by RT-PCR of RNA content

RNA content in the tissue extracts was quantitated by RT-PCR amplification of a 28S rRNA fragment of 91 nucleotides (Genbank accession number M11167, bases 322-413) using the oligonucleotide primers 5'-GCT AAA TAC CGG CAC GAG ACC GAT AG (28F1) and 5'-GGT TTC ACG CCC TCT TGA ACT CTC TC (28R1). 2 µl of proteinase K treated tissue extract was used in a 25 µl reaction containing 2.5 mM manganese acetate, 50 mM bicine pH8.2, 115 mM potassium acetate, 8% glycerol, 300 µM dNTP, 1.5 µM of both primers, 2.5 U of Tth DNA polymerase (Perkin Elmer), and 0.2 µg of an internal control RNA template, rRNA-IC. rRNA-IC is an in vitro RNA transcript of 72bp in length that is amplified by the 28F1/28R1 primer set. The 28S rRNA reaction mixture was placed in a thermal cycler block and underwent the following thermal cycling protocol: 94°C x6 min, 65°C x30 min, 94°C x90 sec, (65°C x1 min, 94°C x30 sec) X12 cycles, 60°C x7 min. The reaction products were separated on a 12.5% nondenaturing polyacrylamide gel in 1x TBE buffer (Sambrook et al., 1989) and stained with SYBR™-Green I (Molecular Probes Inc., Eugene, OR) following the manufacturer's instructions, and the results analyzed by fluorescent densitometry using a Gel Print 2000I™ (BioPhotonics).

To correct for variations in RT-PCR efficiency, quantitation of 28S rRNA was performed by dividing the fluorescent signal from the sample lane by the signal from the competitive internal control (28S-IC). This quantity (28S rRNA / 28S-IC) was then compared to the fluorescent signal from dilutions of extracts of HeLa cells (Hay et al., 1994) or MCF7 cells ranging from 10 to 10,000 cells which were used to create a standard curve. Fluorescent signals were expressed in cell-equivalents (see figure 2).

Telomerase assay

A 2 µl aliquot of extract was used for the telomerase assays. Standard telomere repeat amplification protocol (TRAP) assays using TS and CX primers were performed as described (Piatyszek et al., 1995). All assays contained 10 ag of the internal telomerase assay standard (ITAS) for identification of Taq polymerase inhibitors. A paired sample was inactivated by preincubation with RNAse A (Boehringer Mannheim, Indianapolis, IN). MCF7 cell extracts and the TSR8 control template (Kim & Wu, 1997) served as positive control. For quantitation of telomerase activity, a modification of the assay using the TRAPEZE® kit (Oncor) (Kim & Wu,

1997) was used instead. PCR products were electrophoresed in a 10% nondenaturing polyacrylamide gel in 0.5x TBE buffer (Sambrook *et al.*, 1989). The DNA ladders were visualized using a Molecular Dynamics Phosphorimager (see figure 4). Densitometry was performed with the IPLabGel® program (Signal Analytics, Vienna, VA 22180) on a Macintosh microcomputer.

Statistical analysis

All p values refer to either Fisher's Exact test for tables or Wilcoxon's Rank Sum test for nonparametric data, where appropriate. Correlations where analyzed by Spearman's rank-order test. The analyses were performed using the JMP statistical software package (SAS Institute, Inc., Cary, NC 27513) on a Macintosh microcomputer. The cohort of 10 cases with tissue blocks containing DCIS only was not included in the statistical analyses of the microdissection study.

Results

Descriptive statistics of clinical parameters. The median age at surgery in 34 patients of known age was 50, with a range of 26 to 90 years. Median size of the invasive cancer was 3.2 cm in 29 patients, with a range of 1.1-10.0 cm. Median DNA Index of the invasive cancer tissue was 1.3 in 26 cases, with a range of 1.0-3.0. The median S-phase fraction of the invasive cancer tissue was 4.4 % in 22 cases, with a range of 1.0-28 %. The histological grades of the DCIS tissue samples were I (n=3); II (n=17), and III (n=14).

Quantitation of tissue samples & sample preservation. Telomerase assays are usually standardized by total protein concentration of sample extracts (Kim & Wu, 1997). Pilot experiments revealed that protein content of microdissected tissue extracts could not be reliably quantitated by standard protein assays due to the small sample size (data not shown). Semiquantitative PCR amplification of genomic DNA was performed to determine the number of cells present in the tissue extracts. The median number of genome equivalents (see methods) measured in 128 microdissected samples was 3/µl, with a range of 0.5/µl - 18.3/µl. Of 3 samples containing less than 1 genome equivalent/µl, 2 had undetectable RNA and telomerase levels. Of the samples containing 1 genome equivalent/µl or more, 30 of 64 DCIS samples (47%) and 38 of 64 invasive cancer samples (59%) had detectable telomerase activity (p>0.5). DNA content was comparable in telomerase positive and telomerase negative samples in both DCIS and invasive cancer (figure 3). All cases with telomerase negative cancer samples had detectable telomerase in extracts prepared from whole sections (data not shown). This suggested either a lack of telomerase

positive cells in the microdissected tissue or a loss of telomerase activity during microdissection of the cryosections in some samples.

In order to determine the quality of tissue preservation after microdissection, cellular 28S rRNA was measured by semiquantitative RT-PCR. Overall, 43 of 64 DCIS samples (67%) and 38 of 64 invasive cancer samples (59%) had detectable 28S rRNA levels. Telomerase positive samples had higher 28S rRNA levels than telomerase negative samples (p < 0.001) (see figure 3), and semiquantitative telomerase levels correlated with rRNA levels (p< 0.02, data not shown). None of the the samples lacking detectable 28S rRNA levels showed telomerase activity. None of the telomerase negative invasive cancer samples had detectable 28s rRNA levels. A subset of telomerase negative DCIS samples, however, had detectable 28s rRNA levels (see figure 3).

Assessment of telomerase status in DCIS and invasive cancer samples. Based on these data, the analysis of telomerase activity was limited to samples with detectable levels of 28S rRNA in order to minimize the effects of tissue degradation. Of 46 cases of concurrent DCIS & invasive breast cancer analyzed, 43 DCIS samples from 37 patients and 38 invasive cancer samples from 35 patients contained sufficient tissue for analysis as well as detectable levels of 28S rRNA. 30 (70%) of 43 DCIS samples and 38 (100%) of 38 invasive cancer samples had detectable telomerase activity. 29 (78%) of 37 patiens with DCIS and all 35 patients (100%) with invasive cancer had detectable telomerase in at least one microdissected tissue sample (see figure 4 and table 1).

In order to determine if telomerase levels were higher in invasive breast cancer than in DCIS lesions with detectable telomerase activity, we quantified telomerase activity in a subset of 17 patients in which sufficient tissue was available for 2 independent samples of DCIS and invasive cancer using the TRAPeze[TM] assay (Kim & Wu, 1997). The 11 cases in which both DCIS and cancer tissue showed detectable telomerase activity are shown in figure 5. For samples in which telomerase was detectable, no significant difference in telomerase levels was seen between DCIS and invasive cancer.

An additional cohort of 10 cases of DCIS which did not contain invasive breast cancer were analyzed to validate our results in the microdissection study using samples obtained from whole tissue sections. Assaying 0.1 µg and 1 µg aliquots of DCIS lysate, 8 of 10 samples were telomerase-positive (data not shown).

2 of the telomerase-positive DCIS samples showed periductal inflammatory infiltrates (see figure 6). To investigate the possible contribution of these infiltrates to the detected telomerase activity, all cases without invasive breast cancer underwent microdissection as well. A separate interstitial sample without ductal tissue was obtained from the 2 cases of DCIS with infiltrates. The 2 negative DCIS cases remained negative, while 7 of the 8 positive DCIS cases were again positive. As shown in figure 6, the interstitial infiltrates appeared to be the only source of

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telomerase activity in 1 of the 2 cases with periductal inflammation (figure 6, #5), while telomerase activity was detectable in both interstitial and ductal samples in the other (figure 6, #2).

A mixing experiment was performed in order to detect possible tissue inhibitors of telomerase activity. As shown in figure 7, adding an equal amount of tissue extract from telomerase-negative samples of DCIS and atypical hyperplasias to a positive control extract did not inhibit the telomerase reaction.

Correlation of semiquantitative telomerase levels with tumor parameters. Where the information was available, possible correlations between telomerase levels and parameters such as patient age at surgery, overall tumor size, DNA index and S-phase fraction of the breast cancer, and histological grade of the DCIS were examined by nonparametric testing using the Spearman rank correlations (see table 2). A strong positive correlation was found between telomerase levels and patient age at surgery for DCIS samples ($p = 0.01$) and, to a lesser degree, for invasive cancer tissue ($p = 0.07$). Cancer size correlated positively with telomerase levels in the cancer tissue ($p = 0.05$), but not in the DCIS samples ($p = 0.09$). DNA index of the cancer tissue correlated with telomerase levels in both DCIS and cancer tissue ($p = 0.02$). The S-phase fraction of the cancer tissue did not correlate with telomerase levels ($p > 0.1$), although it was closely linked to cancer size ($p = 0.01$) and histological grade of DCIS ($p < 0.0001$).

Discussion.

In this study, we examined telomerase activity in DCIS associated with invasive breast cancer using microdissection to allow selective tissue sampling. We found that 30 of 43 DCIS samples (70%) and all 38 invasive cancer samples had detectable telomerase activity in at least one microdissected tissue sample (figure 4 and table 1). Since our DCIS samples come from patients with invasive breast cancers, they probably represent a late stage of DCIS. Nevertheless, almost a third of our DCIS samples were telomerase-negative, whereas all invasive cancer samples were telomerase-positive. Similarly, 3 out of 10 cases of DCIS with no invasive component lacked detectable telomerase activity. Again, these DCIS cases do not represent the typical DCIS lesion detected by mammography today, since the average size of these DCIS was 2.9 cm, and smaller lesions may well be more frequently telomerase-negative. Thus, telomerase activation may indeed be occurring in preinvasive stages of breast cancer development, since invasive breast cancer is usually telomerase positive even in early stages (Carey *et al.*, 1998).

Unfortunately, the unfixed tissue currently required for the detection of telomerase activity is only rarely available from this type of lesion, since the surgical pathologist typically needs to examine most if not all available tissue in order to rule out evidence of invasive growth. So far,

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only four small series of DCIS have been investigated for telomerase activity. Bednarek et al. reported on six DCIS tumors, all of which showed telomerase activity (Bednarek *et al.*, 1997), and Tsao et al. reported that 9 out of 12 DCIS were telomerase positive (Tsao *et al.*, 1997). Yashima et al. reported that 11 out of 12 DCIS cases were telomerase positive, while Poremba et al. reported 10 out of 17 positive cases (Poremba *et al.*, 1998). None of these studies discuss the circumstances, such as unusual size, or presence of invasive cancer elsewhere, which allowed saving part of the DCIS without complete diagnostic evaluation. Such circumstances would also raise questions about the applicability of the results to DCIS lesions in general.

A somewhat more readily available source of DCIS tissue are frozen tissue blocks from invasive breast cancers that also contain foci of DCIS, keeping in mind that this may systematically select DCIS lesions that may be more advanced than DCIS lesions found in women without evidence of invasive breast cancer. These tissue blocks formed the basis of this investigation. This approach necessitated selective sampling of the DCIS foci from surrounding cancer tissue by microdissection techniques (Zhuang *et al.*, 1995a), which have been shown to allow selective analyses of different DCIS foci and genetically heterogenous invasive cancer samples from the same tumor (Fujii *et al.*, 1996; Zhuang *et al.*, 1995b). Unlike studies of various genetic markers, the tissue had to be dissected without fixation or staining to avoid inactivating telomerase activity (data not shown). Therefore, we selected cases in which the DCIS lesions were readily identifiable on unstained sections and large enough to allow for unequivocal sampling. Appropriate sampling was subsequently confirmed by reviewing the sections after H&E staining.

A number of technical issues had to be addressed for this study. False negative results due to insufficient sampling was controlled for by measuring the amount of genomic DNA present. Tissue degradation due to delays in surgery or surgical pathology can be assessed to some extent by the ability to detect telomerase activity in adjacent tissue. Tissue degradation during thawing and microdissection occurred in some samples since telomerase-negative microdissected cancer samples were telomerase-positive when assayed by standard methods using extracts from whole cryosections. Since RNA is considerably more labile than DNA, and could therefor serve as monitor of tissue preservation, the content of 28S rRNA, a housekeeping gene which is expressed at constant levels in various tissues, was assessed by RT-PCR. Since there was an excellent correlation between detectable 28S rRNA and telomerase activity (see figure 3), and invasive tumor samples lacking rRNA and telomerase activity could be shown to have detectable telomerase activity by standard methods, we excluded microdissected samples with no detectable 28S rRNA from this analysis. Other causes of false negative results include PCR inhibition, which was monitored by the internal telomerase assay standard (ITAS), as well as tissue inhibitors, which currently can only be detected by mixing experiments and for which the microdissected samples were insufficient. Mixing experiments were performed with telomerase-negative tissue extracts

from the small cohort of cases without invasive cancer, and no inhibitors were detected (see figure 7).

False positive results due to contaminating DNA products are assessed by inactivation of telomerase by RNAse A or heat in paired extracts. Contamination by telomerase activity from adjacent tissue can be minimized to some extent by careful microdissection techniques. We have previously reported that the interstital inflammation that is often present in high grade DCIS with comedonecrosis can be a confounding source of telomerase activity (Umbricht *et al.*, 1997). Indeed, one telomerase-positive DCIS whole-section tissue sample was negative by microdissection, and the periductal inflammation was telomerase-positive (see figure 6).

The previously reported correlation between telomerase levels and biological parameters such as patient age, tumor size, and DNA index (Clark *et al.*, 1997) was again seen in the samples for which a quantitative assay was available. Telomerase levels were not significantly higher in invasive cancer tissue than in DCIS if telomerase negative samples were excluded, suggesting that once telomerase activity reaches a certain level, no further selection occurs. Also, the correlation between telomerase and DNA index (a measure of aneuploidy) and the lack of correlation with the S-phase fraction (a measure of proliferation) suggests that telomerase is more than a simple proliferative index, although a relationship with proliferative indices has been detected in larger series (Clark *et al.*, 1997) (Carey et al., in preparation 1998).

No clinical outcome data were available for this study, so potential prognostic significance of telomerase levels could not be addressed. The prognostic information of telomerase levels in invasive breast cancer is under active investigation. A recent study showed a correlation between telomerase levels and clinical outcome in node-positive breast cancers (Clark *et al.*, 1997), but this was not the case in a similar study including patients in a somewhat earlier stage of the disease (Carey et al., in preparation 1998). The similar levels of telomerase activity seen in matched DCIS and cancer tissue (figure 5) and the possible lack of prognostic implications of telomerase levels in early stages of invasive breast cancer (Carey et al., in preparation 1998) suggest little benefit to the cancer cell in overexpressing telomerase beyond what is necessary for telomere maintenance, at least initially. This may not be the case in later stages of the disease, since high telomerase levels are associated with significantly poorer outcomes in node-positive breast cancer (Clark *et al.*, 1997). At least two hypotheses can be formulated to accommodate these data. First, these findings could be explained by a progressive perturbation of regulatory processes during cancer progression, such that reactivated telomerase activity would only initially be maintained at a level matching cellular needs. High telomerase levels would then be a marker of overall dysregulation late in cancer progression, and as such would be expected to carry an more ominous prognosis. Alternatively, and perhaps concurrently, very high telomerase levels in late stages may point to additional functions of telomerase beyond telomere maintenance, such as an increasing role in

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healing double-stranded DNA breaks as required by an ever-rising chromosomal instability often seen in solid tumors (Bednenko *et al.*, 1997; Flint *et al.*, 1994; Greider, 1994; Melek & Shippen, 1996).

A growing body of evidence suggests that telomerase activity plays an important role in cancer biology (Shay & Bacchetti, 1997). Since even in this series of late stage DCIS a significant subset lacked telomerase activity, DCIS may in fact be a stage in tumor progression in which telomerase activation begins to confer a selective advantage to malignant cell populations. Since natural history studies of DCIS suggest that a subset of these tumors never develop invasive potential (Page *et al.*, 1998), it is tempting to speculate that telomerase activation contributes to this divergence within the natural history of DCIS lesions. Furthermore, telomerase may prove to be a valuable prognostic marker for the development of invasive cancer in patients with DCIS.

Figure Legends.

Figure 1. Quantitation of tissue samples by PCR Assay of DNA content. PCR reaction products were separated in a 10 % nondenaturing polyacrylamide gel and imaged by phosphorimager screen, as shown in the upper panel (see methods). Densitometry of the product bands is illustrated in the lower panel. Quantitation was performed after lane backgrounds were subtracted by sampling identical segments above the product bands. These values bands were divided by an average per cell value derived from serial dilutions of MCF7 cell extracts run in parallel, and expressed in genome equivalents. p36B4 is a plasmid containing the 36B4 cDNA

Figure 2. Assessment of tissue sample preservation by RT-PCR Assay of 28S rRNA content. The RT-PCR reaction products were separated on a 12.5 % nondenaturing polyacrylamide gel, stained with SYBR™-Green I, and analyzed by fluorescent densitometry (see methods). Quantitation was performed by dividing the fluorescent signal from the sample lane (arrow, 28S rRNA) by the signal from the 28S rRNA competitive internal control (arrow, IC) to correct for variations in RT-PCR efficiency. This quantity (28S rRNA / IC) was then compared to the fluorescent signal from extracts of 10, 100, and 1000 MCF7 cells which were used to create a standard curve (3 left lanes). (B) indicates a buffer control lane without added extract. Fluorescent signals were expressed in cell-equivalents as indicated below the gel.

Figure 3. DNA and RNA content of telomerase-positive and telomerase-negative microdissected tissue samples. The estimated 36B4 DNA and 28S rRNA contents of telomerase positive and negative microdissected tissue samples are shown for invasive cancer and DCIS. Mean values and standard error bars as well as quantile boxes are shown.

Figure 4. Standard TRAPeze® telomerase activity assay in microdissected tissue samples. 2 µl of extract was used in each assay. (1D) and (1C) indicate DCIS and cancer tissue samples from patient #1. A paired sample was inactivated by preincubation with RNAse A (indicated by + above the lanes). (B) buffer control without added extract. MCF7 cell extracts (+C) and the TSR8 control template (T) served as positive controls. All assays contained 10 attograms of an internal telomerase assay standard (arrow, IC) for identification of Taq polymerase inhibitors. Reaction products were electrophoresed in a 10 % nondenaturing polyacrylamide gel and visualized using a Molecular Dynamics Phosphorimager.

Figure 5. Comparison of telomerase levels in DCIS and invasive breast cancer samples from the same tissue block. Telomerase levels were measured by TRAPeze®

assay in a subset of cases in which sufficient tissue was available for 2 independent samples of DCIS and invasive cancer. The average telomerase values of the 11 cases in which both DCIS and cancer tissue showed detectable telomerase activity are shown in panel A. The similar distribution of the telomerase levels in these DCIS and cancer tissue samples is shown in panel B.

Figure 6. Telomerase activity of periductal infiltrates vs. DCIS. TRAPeze® assays of microdissected tissue samples of DCIS cases (1-6) without invasive cancer. 2 µl of extract was used in each assay. Lanes {2} and {5} indicate lysates of periductal intersitial infiltrates. Lanes 4* and 6* indicate DCIS samples that were telomerase-negative when whole section lysates were assayed. (Bu) buffer control without added extract. All assays contained 10 attograms of an internal telomerase assay standard (arrow, IC) for identification of Taq polymerase inhibitors. The tissue source of lanes 2 & 5 is illustrated at the top.

Figure 7. Assessment of telomerase inhibitors. Standard TRAPeze® telomerase activity assay mixing 1 µg of telomerase-positive control lysate with an equal amount of lysate from telomerase-negative samples of DCIS (CIS) and atypical hyperplasias (AH). (+RNAse) indicates inactivation by preincubation with RNAse A. (Bu) buffer control without added extract. All assays contained 10 attograms of an internal telomerase assay standard (arrow, IC) for identification of Taq polymerase inhibitors.

Table 1. Telomerase activity in microdissected DCIS and invasive breast cancer tissue samples. The top 2 rows summarize the results of the microdissection study. DCIS samples were significantly less often telomerase positive than invasive cancer samples (p < 0.0001, Fisher's exact test). 29 (78%) of 37 patients with adequate DCIS samples and all 35 patients with adequate invasive cancer samples had detectable telomerase (p < 0.001, Fisher's exact test). The lower 2 rows summarize the result of the study of tissue blocks without detectable invasive cancer component.

Table 2. Correlation of semiquantitative telomerase levels with tumor parameters. P values of Spearman rank correlations between tumor parameters are shown. (n) indicates the number of cases for which the information was available.

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Figure 1
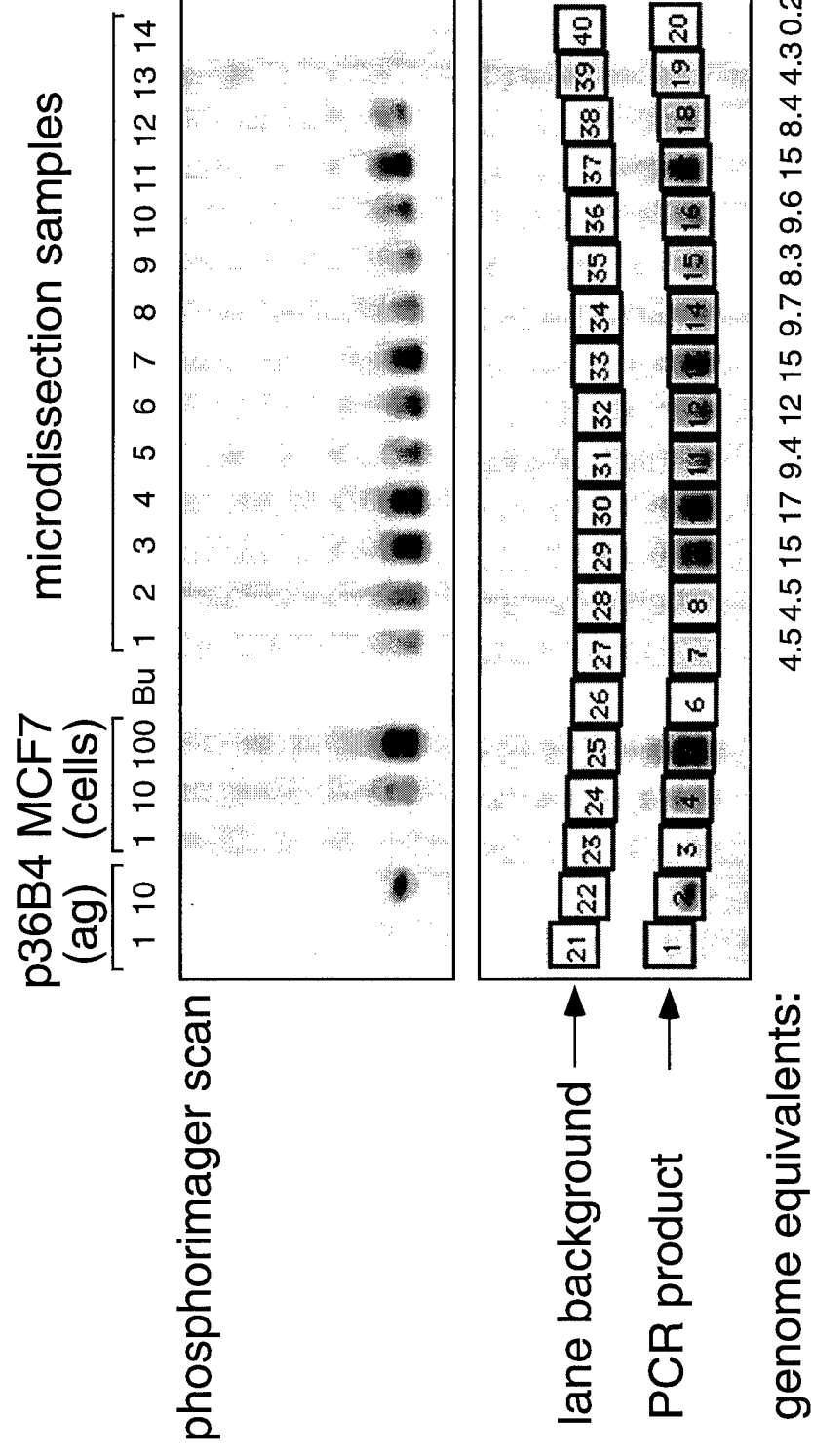
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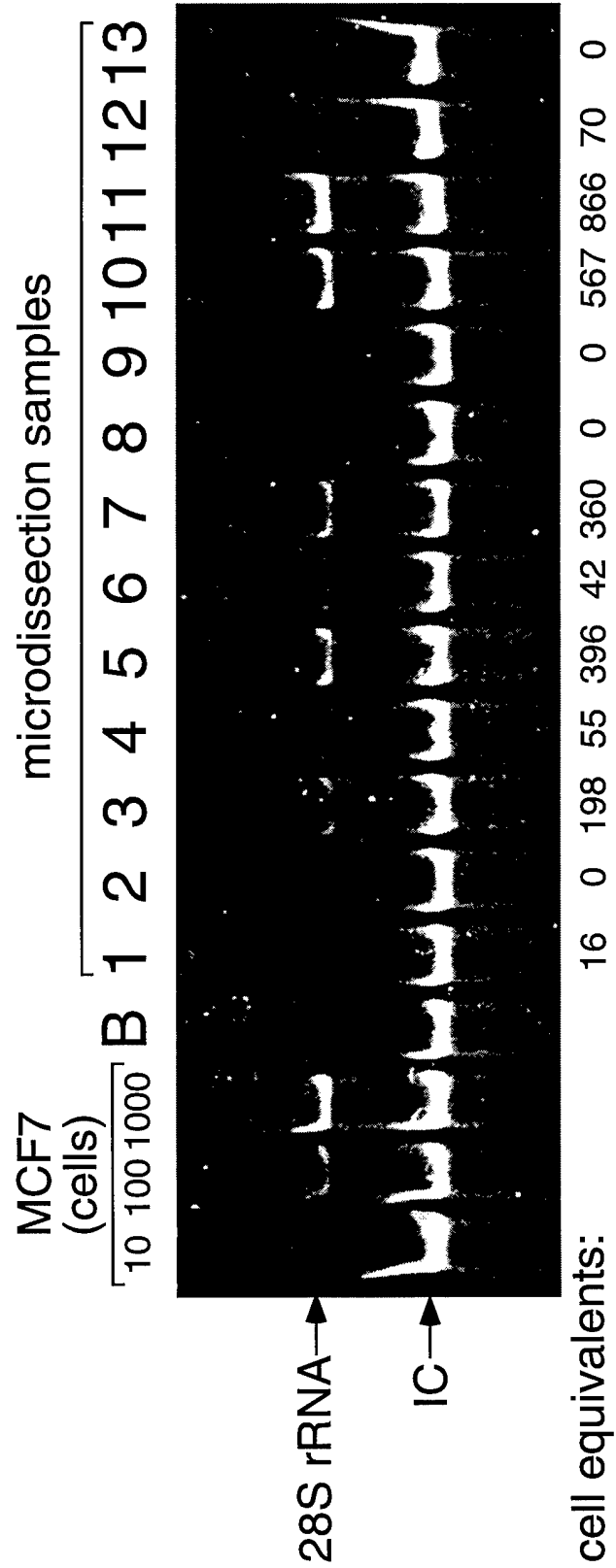
MCF7 (cells)
10 100 1000

microdissection samples
B 1 2 3 4 5 6 7 8 9 10 11 12 13

28S rRNA→

IC→

cell equivalents: 16 0 198 55 396 42 360 0 0 567 866 70 0
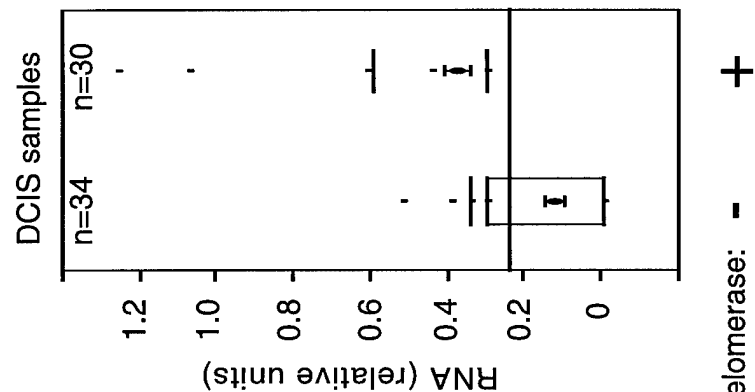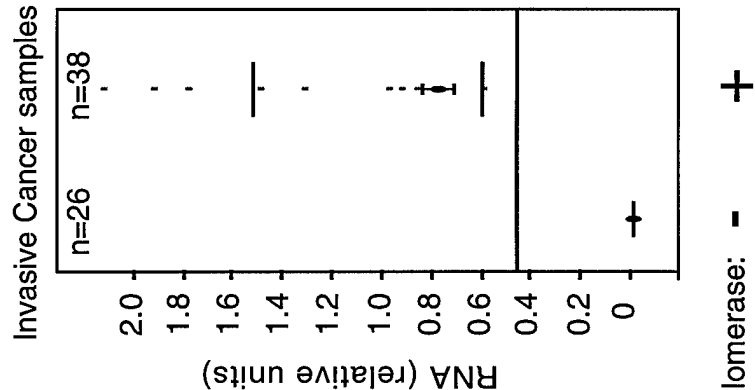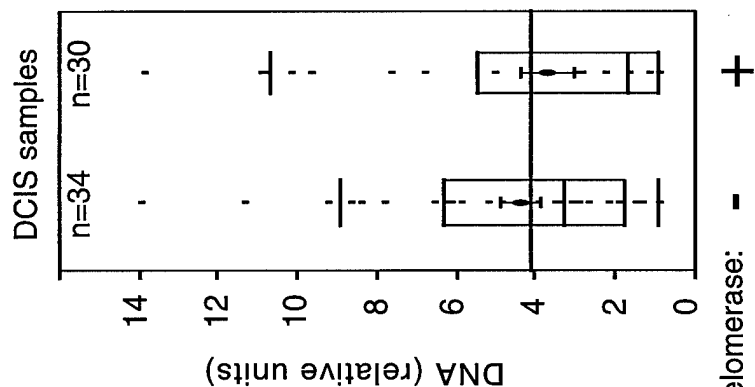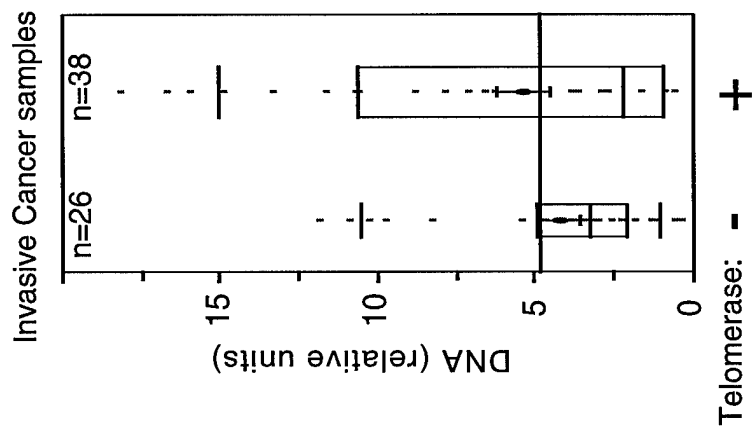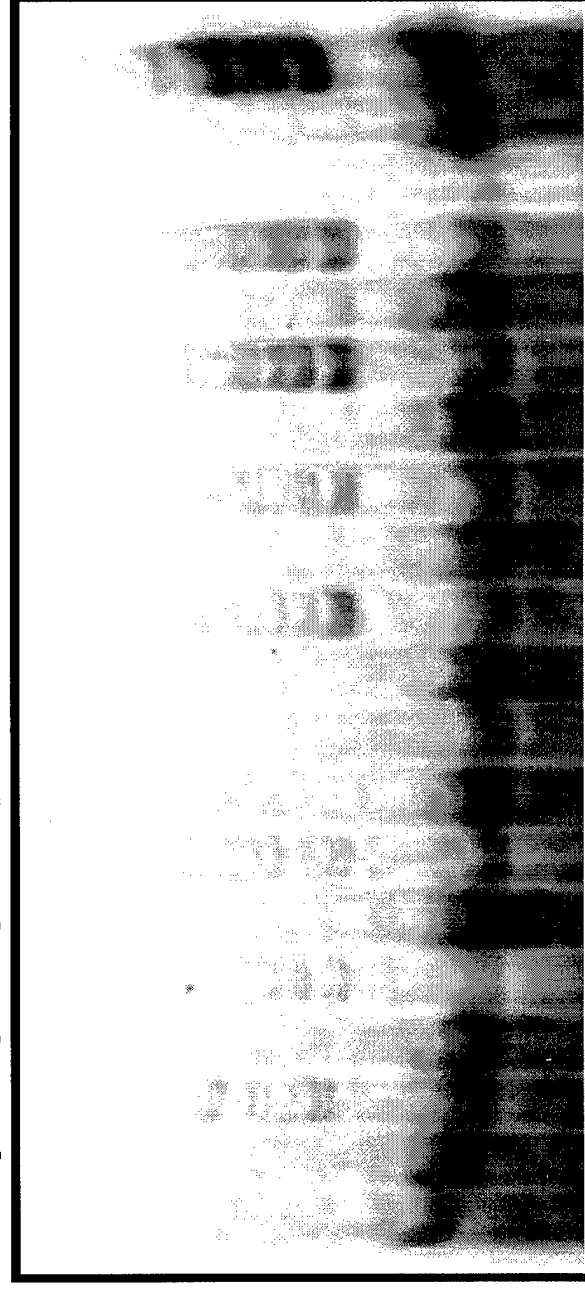
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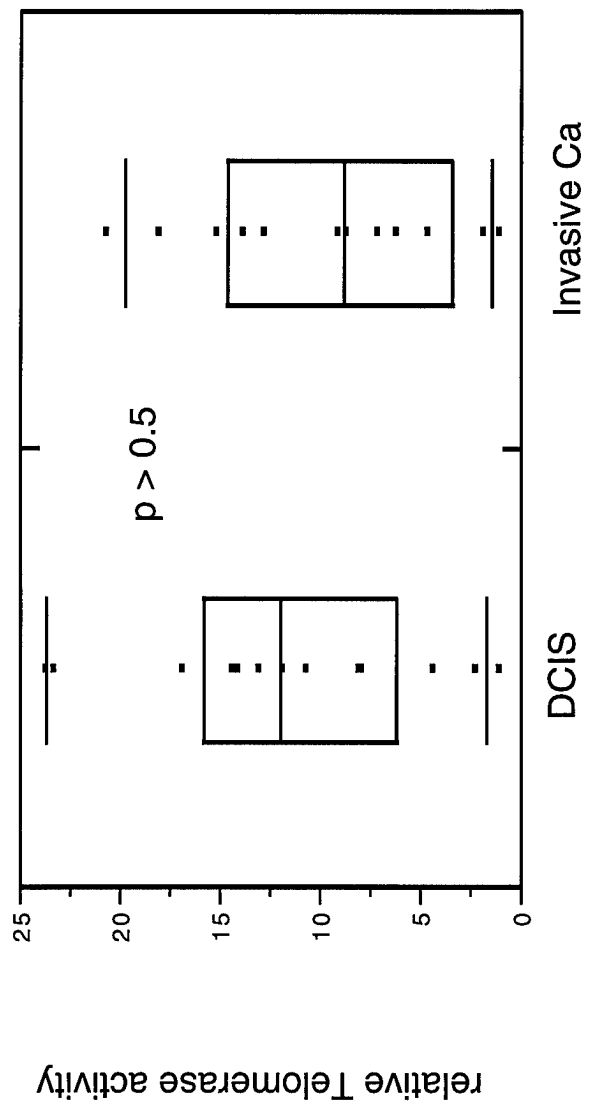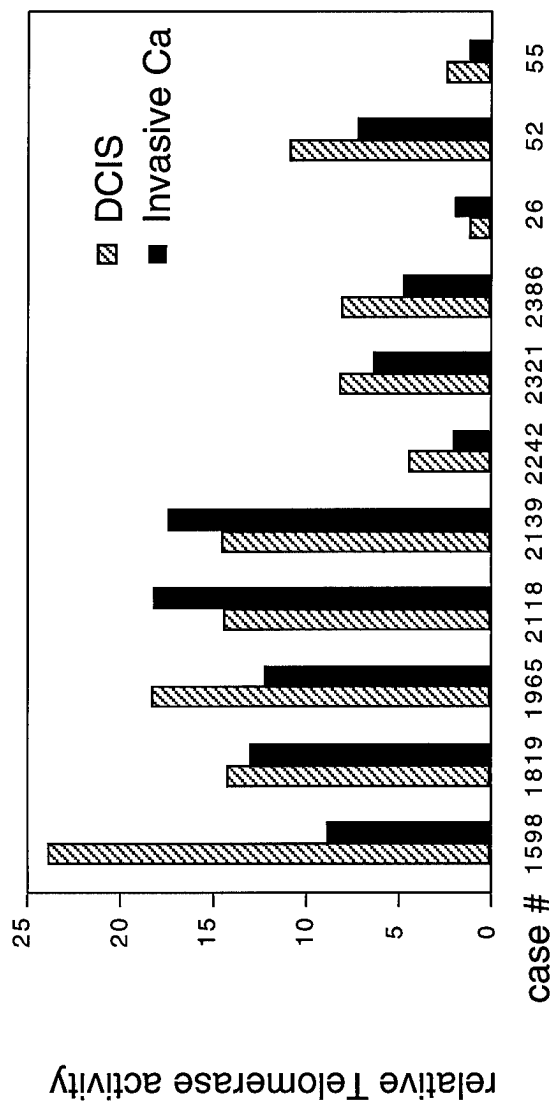
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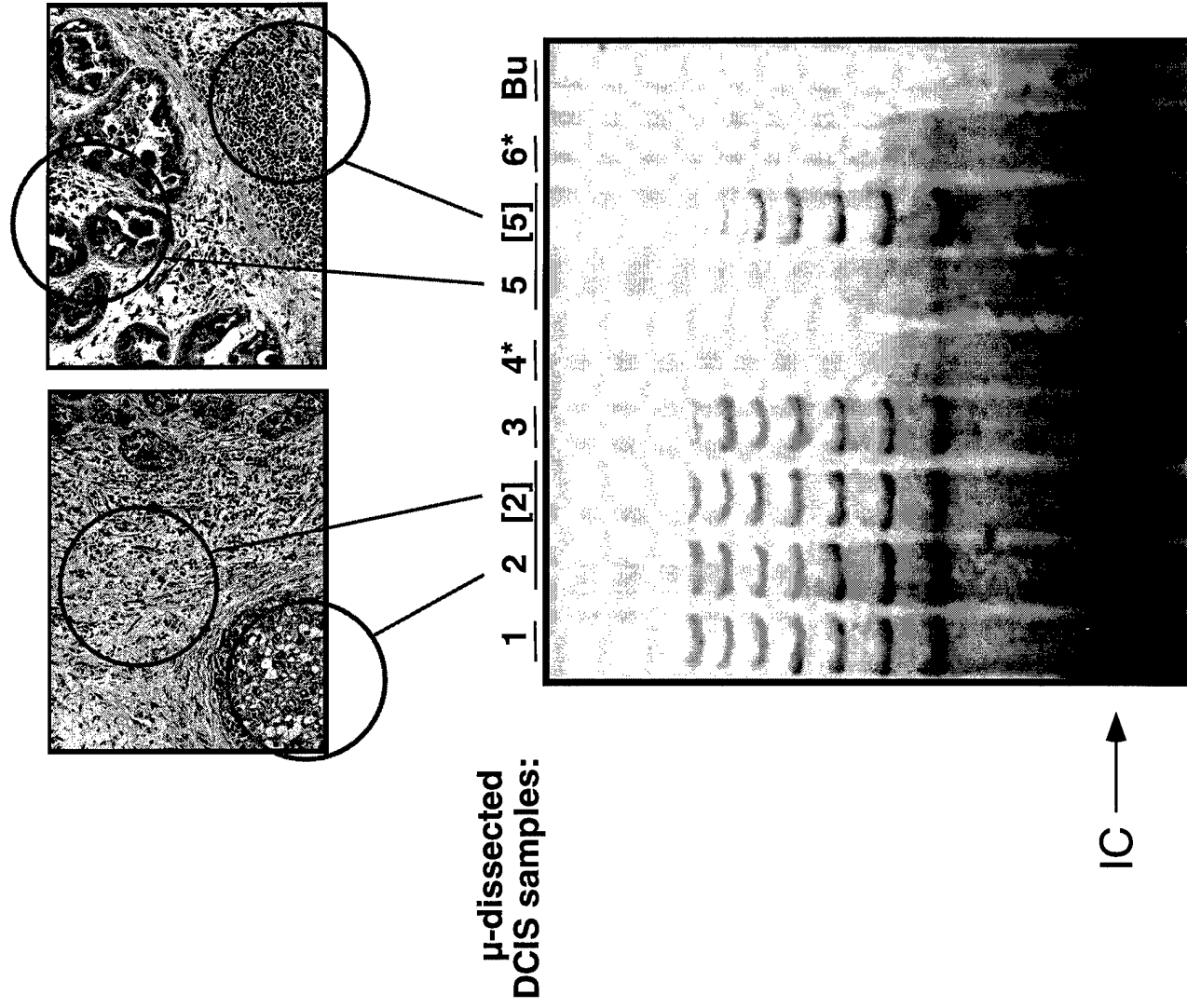
Figure 5

Figure 6

Figure 7
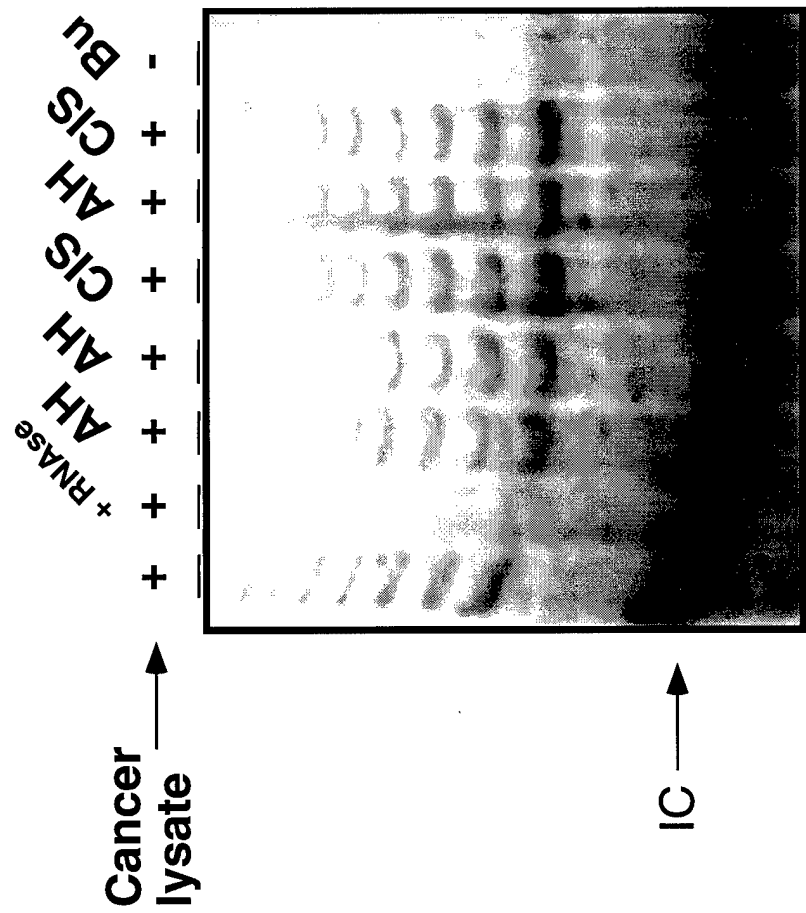
Table 1

	telomerase activity			
μ-dissected	+	-	% pos.	
DCIS (n = 43)	30	13	70	p < 0.0001
Ca (n = 38)	38	0	100	
whole section	+	-	% pos.	
DCIS (n = 10)	7	3	70	p < 0.0001
benign & nl (n = 6)	0	6	0	

Table 2

	Telo/rRNA (Ca)	Telo/rRNA (DCIS)	DCIS Grade	Ca Size	Age	Ca-DNA Index	Ca-S-Phase
Telo/rRNA (Ca, n=38)			0.09	0.05	0.07	0.02	0.11
Telo/rRNA (DCIS, n=43)			0.35	0.09	0.01	0.02	0.65
DCIS Grade (n=34)	0.09	0.35		0.13	0.27	0.14	<.0001
CaSize (n=29)	0.05	0.09	0.13		0.21	0.98	0.01
Age (n=34)	0.07	0.01	0.27	0.21		0.56	0.25
Ca DNA Index (n=26)	0.02	0.02	0.14	0.98	0.56		0.06
S-Phase (n=22)	0.11	0.65	<.0001	0.01	0.25	0.06	

Telomerase activity and prognosis in primary breast cancers

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Telomerase activity and breast cancer outcome
Key words:
telomerase activity, breast cancer, tumor marker, prognosis

Abstract

Background. Recent studies associate telomerase activity with prognostic factors and with survival. We compared quantitative telomerase activity in primary tumors with traditional prognostic factors and outcome in a group of nonmetastatic breast cancers.
Methods. Telomerase activity was measured in 295 invasive breast cancers by the quantitative telomeric repeat amplification protocol (TRAP) method. Telomerase expression was compared to 28S rRNA level, tumor content, and clinical variables including outcome. For clinical correlations, telomerase activity was standardized by two methods: 1) a correction for cellularity using 28S rRNA levels, and 2) a correction for the histologically determined invasive proportion of the specimen. In addition, telomerase template RNA (hTR) levels were measured by reverse transcriptase-polymerase chain reaction in a subset of tumors and correlated with telomerase activity, prognostic factors, and outcome.
Results. Telomerase activity was found in 67% of primary breast cancers overall, 80% of breast cancers with measurable 28S rRNA levels. hTR was measurable in 98 (34%) of 287 tumors. Telomerase activity level was significantly associated with hTR (p=0.03), rRNA levels (p=0.002), and tumor content of the sample (p=0.04). Telomerase activity was also associated with the proliferative index (p<0.01) of the tumor but not with any other prognostic variable. Neither uncorrected nor corrected telomerase activity nor hTR was associated with relapse-free or overall survival in this study.
Conclusions. Telomerase activity level was associated with rRNA level, tumor content, and hTR, as well as the proliferative index of invasive breast cancers but its measurement in samples from this group of nonmetastatic breast cancer patients did not predict survival.

Introduction

Mitosis in organisms with linear chromosomes is complicated by the ìend-replication problemî , in which the DNA termini are not fully copied. Over successive replications, more and more of the telomere is thus lost. This process is now known to be causally linked to normal cellular senescence .

Telomerase is a ribonucleoprotein enzyme which adds noncoding repeat sequences to the ends of linear chromosomes. By adding TTAGGG to chromosomal ends, this enzyme counteracts the end-replication problem. Telomerase is active in germline cells, immortalized cell lines, and the majority of malignancies. It is generally inactive in normal somatic cells. For this reason, telomerase activity is believed to play an integral role in cancer cell immortality. We and others have shown that telomerase is clearly active in the majority of invasive breast cancers. Telomerase activity has been associated with survival in neuroblastoma and gastric cancer but the relationship between the level of activity and outcome has not been widely studied. Efforts to define this relationship have been aided by recent modifications to the telomeric repeat amplification protocol (TRAP), the most commonly used assay of telomerase activity, allowing improved quantitation of telomerase activity. Using such a quantitative TRAP assay, Clark et al.. recently found a correlation between telomerase activity level and overall survival in breast cancer patients with axillary lymph node metastases. In addition, methods to detect the RNA component of human telomerase

(hTR) have been developed, including Northern analysis, and in situ hybridization . Since telomerase is a ribonucleoprotein, stability of RNA in clinical samples could be an important factor in accurate telomerase activity quantitation. Another potentially crucial factor is the natural heterogeneity of breast cancers, which often contain tumor cells admixed with various types of normal cells. Using the quantitative TRAP method for telomerase activity and RT-PCR for rRNA (index of RNA stability) and hTR levels, we examined 295 primary invasive nonmetastatic breast cancers for telomerase activity and hTR. We then compared these levels standardized to RNA integrity and tumor content to traditional breast cancer prognostic factors and outcome.

Methods

Patient population

Patients were identified by analysis of the Johns Hopkins University and Duke University Tumor Banks. Criteria for inclusion were: 1) Resection of primary invasive breast carcinoma between 1986 and 1991, 2) adequate frozen sample from the patientís primary tumor available for analysis, and 3) clinical variables and survival data pertaining to each tumor available from the Tumor Registries of each institution and the pathology reports. All patients meeting these criteria were included.

Tissue samples

All tissue samples were obtained from excess clinical specimens stored at -80(C since resection. Institutional guidelines for the acquisition and maintenance of such specimens were followed.

Each tumor was cut by 10(m cryosections onto two or three slides. One section was stained with hematoxylin and eosin (H&E) and evaluated by the study pathologist. If a section was judged to have <10% invasive tumor or to exhibit necrosis, the tumor was either recut until an adequate sample was obtained or the tumor was removed from the study. The second 10(m section underwent lysis and evaluation of telomerase activity by a modified TRAP method and evaluation of hTR level by RT-PCR (13,14).

Prognostic factors

Clinical variables measured included age at diagnosis, gross tumor size (in centimeters), axillary lymph node status, and whether the patient had received adjuvant chemotherapy or hormonal therapy. Laboratory prognostic factors examined included measurement of the estrogen receptor (ER) content, DNA index, and the proliferative index (PI) of the primary tumor. Age at breast cancer diagnosis and tumor size were coded as continuous variables. Lymph node status was coded categorically, with a ìpositiveî result if any lymph nodes microscopically contained tumor. Chemotherapy and hormonal therapy were grouped together as ìadjuvant therapyî and coded categorically. No distinction was made among different adjuvant chemotherapeutic regimens. The only adjuvant hormonal therapy used was tamoxifen, which was coded as ìadjuvant therapyî only if the patientís tumor was known to be ER-positive. Some patients with ER-negative tumors received tamoxifen. Because it is unlikely that they benefited from this therapy, these patients were coded as ìno adjuvant therapyî. Patients who received hormonal therapy but in whom the tumorís ER status was unknown, were coded as ìunknownî rather than as ìadjuvant therapyî. The ER in the primary tumor was measured with a standard biochemical assay by a commercial reference laboratory (Nichols Institute, San Juan Capistrano, CA) and coded dichotomously as ìpositiveî or ìnegativeî.

ER results were considered ìpositiveî if at least 10 femtomoles of receptor per milligram of cytosolic protein was found. DNA indices were obtained from individual pathology reports, and were grouped ordinally, with ìdiploidî defined as a DNA index 0.90 ñ 1.10, ìnear-diploid aneuploidî defined as 1.10 ñ 1.30, and ìaneuploidî as > 1.3 or < 0.90. These cutpoints are derived from Dressler et al. with the diploid range broadened due to the spectrum of reported values (Dressler LG, personal communication). The proliferative index was measured using two different methods: For the tumors from the Johns Hopkins Tumor Bank, S-phase fraction was performed by a commercial laboratory according to standard flow cytometric methods (Nichols Institute, San Juan Capistrano, CA). For the tumors from Duke, Ki-67 was measured. S-phase fraction was categorized as ìlowî or ìhighî based on the formal pathologic report from the reference laboratory, with tumors graded as low and intermediate grouped together as ìlowî PI. Ki-67 was categorized as ìlowî (less than or equal to 7% staining) or ìhighî (greater than 7% staining), with this cut-off value based on the mean of the distribution among the tumors. The two proliferative indices have been shown to correlate with each other (18,19).

Outcome data

Outcome data was obtained from the Tumor Registries of the Cancer Centers from each institution, with follow-up updated yearly according to cancer center protocol. These data were assessed as relapse-free survival (RFS), defined as the period from diagnosis to local or systemic relapse or last known follow-up if no relapse had occurred, and overall survival (OS), defined as the interval from diagnosis to death or last known follow-up.

Telomerase activity assay

Tissue extracts were prepared as previously described. Briefly, between 30-60(l cold lysis buffer (10mM Tris-HCl (pH7.5), 1mM MgCl2 , 1mM EGTA, 0.1mM AEBSF (4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochlorine), 5mM (-mercaptoethanol, 0.5% CHAPS, 10% glycerol) was added to the whole 10(m tissue section removed from the slide by scraping with a needle. This sample was homogenized with a motorized pestle, incubated on ice for 30 minutes, then pelleted by microcentrifuging at 10,000Xg for 30 minutes at 4(C. The supernatant was removed, and the protein concentration measured using a BCA protein determination kit (Pierce, Rockford, IL). The extracts were diluted with cold lysis buffer to a final concentration of 1mg/ml.

The quantitative TRAP assay was performed as described (9,21) with a few modifications. One (g of TS substrate primer (5íAATCCGTCGAGCAGAGTT-3í) was end-labeled by addition to 10 (l of reaction mixture (10(Ci (P32) (-ATP (3000Ci/mmol), 1X One Phor AllTM buffer (Pharmacia, Uppsala, Sweden), 5U T4 polynucleotide kinase) and incubation at 37(C for 20 minutes followed by 95(C for 5 minutes. The 50(l TRAP reaction mixture contained 20mM Tris-HCl (pH8.3), 1.5mM MgCl2, 63 mM KCl, 0.05% Tween-20, 1mM EGTA, 50(M dNTPs, 1(l of the TS end-labeling reaction mixture, 0.1(g of ACX return primer (5í-GCGCGG(CTTACC)3CTAACC-3í), 0.1(g NT internal control primer (5í-ATCGCTTCTCGGCCTTTT-3í), 0.01amol TSNT internal control (5í-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3í), 0.1mg/ml BSA, 2U Taq DNA polymerase (Perkin-Elmer, Foster, CA), and 0.5 - 1(l of tissue extract (0.5(g protein). The TRAP reaction mixtures were placed in a thermal cycler block preheated to

30(C for 10 minutes for 1 cycle, then 94(C for 30 seconds followed by 60(C for 30 seconds for 27 cycles. One half of the 50(l reaction mixture was run on a 0.5X TBE, 15% polyacrylamide nondenaturing gel. The gel was dried and the results analyzed by autoradiography using a PhosphorImagerTM (Molecular Dynamics, Sunnyvale, CA) (Figure 1). Each extract was tested in duplicate, with a negative control for each extract created by heat inactivation at 75(C for 10 minutes.

Quantitation of telomerase activity was determined by comparing the radioactive signal from the sample lane to the signal from 0.1 amol of the R8 quantitation standard (5í-AATCCGTCGAGCAGAGTTAG(GGTTAG)7-3í). Measurements of the signal from the TRAP product bands, the TSNT internal control, and the R8 quantitation standard were made independently. The telomerase level (TPG) was calculated as:

$$TPG = \frac{(\,(TP-TP\text{í})\,/\,TI\,)}{(\,(R8-B)\,/\,RI\,)} \times 100$$

where TPG represents total product generated, TP and TPí represent the radioactive counts from the test extract and its inactivated control, respectively, TI represents the counts from the internal control, R8 represents the counts from the quantitation standard, B is the counts from the buffer-only control lane, and RI represents the internal control of the R8 quantitation standard.

Telomerase template RNA (hTR)

hTR levels were quantitated using kinetic RT-PCR with the oligonucleotide probe labeled with fluorescent reporter/quencher dyes. The 15(l reaction mixture included 1x TitanTM buffer, 0.1(g/(l BSA, 5mM DTT, 0.3mM dNTPs, 1.5(M hTRF2 (5í-TGTCTAACCCTAACTGAGAAGG-3í) forward primer and 1.5(M hTRR2 (5í-CTCTAGAATGAACGGTGGAAGG-3í) reverse primer, 0.6(l TitanTM enzyme mix (reverse transcriptase-AMV, Taq DNA polymerase, Pwo DNA polymerase) (Boehringer-Mannheim, Indianapolis, IN) and a final concentration of 50fmol/(l of NK2 probe (5í-6FAM-TTTGCTCCCCGCGCGCTGTTTT-TAMRA-3í). A HeLa cell dilution series and blanks (CHAPS lysis buffer) were included in each run to provide a standard curve that expressed each hTR unit as a HeLa cell equivalent. The reactions were mixed by gently tapping with a fingertip, and placed at 50(C for 20 minutes, then 94(C for 1 minute, then placed on ice. Ten (l of the reaction mixture was placed in a glass capillary tube and placed in a LightCyclerTM (Idaho Technologies, Idaho Falls, ID). Standard readings were made at 60(C and 80(C to normalize for small temperature-dependent variations during fluorescence measurements. The reaction mixture was denatured by heating to 94(C for seven seconds then underwent 18 cycles as follows: three seconds at 95(C, 15 seconds at 60(C, three seconds at 72(C. Ramp speed was set at 5(C/second. Fluorescence was measured at the end of each 72(C extension phase. hTR levels were quantitated using the LightCyclerTM quantitation software.

Telomerase activity and hTR standardization

The usual method of TRAP assay standardization is by the addition of lysate containing a prescribed amount of protein, which fails to correct for varying cellularity, degradation, and tumor:stroma ratio. To correct for such heterogeneity, two methods were used. In the first method (cellularity corrected TA), the assay was standardized to the number of viable cells present by amplification of the 28S rRNA component (see below) in the lysate. The telomerase activity was then divided by the 28S rRNA level in

order to obtain a telomerase level standardized to the viable cellularity of the sample as determined by 28S rRNA levels. In the second method (histologically corrected TA), histopathologic evaluation of the H&E-stained tissue sample was performed by a single individual, the study pathologist, in order to determine the proportion of tumor cells within the total cells of the sample. The total telomerase activity was then divided by this tumor cell proportion to estimate the TPG per tumor cell. A final correction used both methods together to estimate the telomerase activity present standardized to the number of viable tumor cells by dividing the telomerase activity by the product of the 28S rRNA level times the proportion of invasive tumor cells.

The distribution of quantitative telomerase activity can be nonparametric, so analyses were performed both with telomerase activity treated linearly and logarithmically transformed as previously described.

The same methods were used to correct the hTR signal for sample cellularity (cellularity corrected hTR), the proportion of invasive tumor in the sample (histologically corrected hTR), and both corrections (bicorrected hTR). Logarithmic transformation of these hTR values was also performed.

28S rRNA RT-PCR

In order to standardize TPG results to sample cellularity, 28S rRNA levels were measured using primers designed to amplify positions 322-413 (EZ(RNA PCR kit, Perkin-Elmer, Foster, CA).

The 25(l 28S rRNA reaction mixture contained 1X EZ buffer, 2.5 mM Mn(OAc)2, 300nM dNTPs, 2.5U of thermostable Tth DNA polymerase, 1.5(M of 28F1 primer (5í-GCTAAATACCGGCACGAGACCGATAG-3í), 1.5(M of 28R1 primer (5í-GGTTTCACGCCCTCTTGAACTCTCTC-3í), 200ng of 28S-IC internal control sequence for monitoring PCR efficiency, and 2(l of the CHAPS tissue extract at 2 mg/ml protein concentration. The 28S rRNA reaction mixture was placed in a thermal cycler block preheated to 94(C for 6 minutes for 1 cycle, 65(C for 30 minutes for 1 cycle, 94(C for 1.5 minutes for 1 cycle, followed by 94(C for 30 seconds / 65(C for 1 minute for 12 cycles, ending with 72(C for 7 minutes for 1 cycle. One half of the 25(l reaction mixture was run on a 12.5% polyacrylamide nondenaturing gel. The gel was stained with SYBR-GreenTM I DNA dye (Molecular Probes, Eugene, OR) according to manufacturerís directions, and the results analyzed by fluorescent densitometry using a Gel Print 2000ITM (BioPhotonics Corp., Ann Arbor, MI) (Figure 1).

Quantitation of 28S rRNA was determined by dividing the fluorescent signal from the sample lane by the signal from the 28S rRNA competitive internal control (28S-IC) to correct for variations in PCR efficiency. This quantity (28S rRNA / 28S-IC) was then compared to the fluorescent signal from dilutions of CHAPS extracts of HeLa cells ranging from 30 to 10,000 cells. A standard curve was created from 3-fold dilutions of the HeLa cell extract, and 28S rRNA levels were interpolated from it. These levels were expressed in cell-equivalents.

A second measurement of 28S rRNA level was made on the same samples to standardize the hTR measurement. This 28S rRNA level was measured using kinetic PCR quantitation. The 15(l reaction mixture included 1x RT-PCR buffer, 0.1(g/(l BSA, 3mM MgCls, 0.4mM dNTPs, 1x PCR enhancer, 0.5mM MnSO4, 1.5(M 28F1 and 28R1 primers (see above), 2.5U Tth thermostable DNA polymerase with intrinsic reverse transcriptase activity (Epicentre Technologies, Madison, WI), 1/10,000 dilution of SYBR

GreenTM dye, and 2(l CHAPS extract. A set of six controls and two blanks were included in each run to provide a standard curve and the reaction was performed using the LightCyclerTM as described above under methods of hTR measurement. The two methods of 28S rRNA measurement correlated significantly with each other (p=0.008).
Data analysis

Telomerase activity was measured on a continuous scale for uncorrected TA and corrected TA (cellularity corrected TA, histologically corrected TA, both corrections together). In order to account for nonparametric distributions, all telomerase activity was examined both untransformed and following logarithmic transformation. The association between telomerase activity and continuous variables was evaluated using simple linear regression with significance testing of the regression coefficient; the association between telomerase activity or hTR and the variables measured categorically was evaluated using Wilcoxon rank sum tests for nonparametric data or ANOVA / Studentís t-test for parametric data.

There are no clear cutpoints for telomerase activity or hTR levels, so in outcome assessment this variable was treated continuously and Coxís proportional hazard modeling was used to assess the contribution of the clinical variables and telomerase activity to outcome. The statistical analysis was performed using the JMP 3.1.6 statistical program (SAS Institute, Cary, NC).

Risk of relapse-free and overall survival is presented as the adjusted relative risk associated with each prognostic variable. The relative risk associated with the continuous variables (age at diagnosis, tumor size, telomerase activity) should be interpreted as the relative risk with each incremental unit increase in the variable. The relative risk associated with nominal variables (lymph node status, ER status, adjuvant therapy) should be interpreted as the risk of possessing the variable compared with not possessing the variable.

Results

A total of 338 breast cancers were evaluated for entry into the study. Of these, 30 were removed from study because the sample was insufficient for study or contained less than 10% tumor cells in the histologically confirmed section, and 13 were removed from the study for known mishandling of the frozen tissue which could affect telomerase enzymatic activity. These exclusions left 295 breast cancers from patients with resections from 1986-1991 who: 1) had breast cancer samples frozen for research purposes and 2) were included in the clinical databases maintained at each institution. Eighty-four of the samples came from Johns Hopkins University, 211 came from Duke University. Twenty-two samples were obtained from patients found to be metastatic at diagnosis. Fifteen samples were obtained from patients with bilateral breast cancer either synchronously or metachronously within 10 years. These 37 samples were excluded from the outcome analysis. After excluding those patients who were metastatic at diagnosis or who had a history of bilateral breast cancer, there were 256 breast cancers eligible for outcome analysis.
General characteristics

The patientsí age at diagnosis ranged from 27 to 87 years old, median 56 years old. Information regarding traditional prognostic factors is given in Table 1. Only 54% of tumors were found ER-positive, which is at the low end of the reported range of 50-

85% , and clearly lower than the 88% reported by Clark et al.. One hundred forty-seven of 282 patients (52%) had received adjuvant chemotherapy, and 82 of the 116 patients (71%) known to be ER-positive had received hormonal therapy. An additional 89 patients received hormonal therapy in spite of unknown (54 patients) or known ER-negative status (35 patients). Fifty-three samples were taken from tumor masses in or near the axilla, although none resembled lymph nodes histopathologically.

Of the 256 patients eligible for the outcome portion of the study, median follow-up was 5.7 years, range 0.1 to 10.4 years. Ninety-nine (39%) are known to have relapsed, and 99 (39%) died of any cause over the duration of the study.

Telomerase activity (TA) and hTR levels

Of the 295 tumor samples, 199 (67%) had detectable telomerase activity, 250 (85%) had detectable 28S rRNA, and 98 (34%) of 287 had detectable hTR. None of the samples lacking rRNA had measurable telomerase activity or hTR. Of the samples with measurable rRNA, 80% were positive for telomerase activity and 38% were positive for hTR. Eight samples had insufficient quantities for both telomerase activity and hTR determination. Evaluation of hTR levels included only those tumors with a measurable value.

Uncorrected TA ranged from 0 to 151.0 TPG, median 1.00 TPG, interquartile range (IQR) 0 to 6.80 TPG. The distribution of telomerase activity is given in Figure 2. Ninety-six patients were telomerase-negative. Excluding the telomerase-negative samples, median TPG was 3.9. Uncorrected TA was associated with the 28S rRNA levels (p=0.002), and was also significantly associated with the proportion of tumor cells in the sample (p=0.04). Ribosomal RNA level ranged from 0 to 1974.0, with a median of 13.4.

Cellularity corrected TA using the 28S rRNA correction ranged from 0 to 27.35 TPG, median 0.07 TPG, IQR 0.01 to 0.26 TPG. This distribution excluded 45 samples which lacked measurable RNA levels. Histologically corrected TA using the proportion of tumor cells in the sample ranged from 0 to 210.43 TPG, median 1.89 TPG, IQR 0 to 11.21 TPG. Telomerase activity incorporating both corrections ranged from 0 to 30.39 TPG, median 0.12 TPG, IQR 0.01 to 0.44 TPG.

Examination of the telomerase activity in those tumors excluded from the outcome portion of the study due to metastases at diagnosis did not reveal a significant difference from the nonmetastatic tumors (p=0.37). Telomerase activity was significantly (p=0.03) higher in the masses in or near the axilla (median uncorrected TA 2.6 TPG) compared with the rest (median uncorrected TA 1.0 TPG). Addition of this variable to the outcome models did not change the results.

The median hTR signal was 8.4, IQR 2.4 to 60.0. The hTR level correlated directly and significantly with uncorrected (p=0.03) telomerase activity.

Association with other prognostic factors

Examination of the relationship between telomerase activity and other prognostic factors revealed that telomerase activity was significantly and directly associated with the proliferative index when measured as uncorrected TA(p<0.01), cellularity corrected TA (p=0.02), histologically corrected TA (p=0.01), or as both cellularity and histologically corrected TA (p=0.03). Neither uncorrected nor corrected TA was consistently associated with the patientís age at diagnosis, size of the primary tumor, lymph node status, estrogen receptor status, aneuploid or diploid status, or whether the patient had

received adjuvant chemo- or hormonal therapy. These results were unchanged by exclusion of the RNA-negative samples.

Examination of hTR association with other prognostic variables revealed a significant association with negative axillary lymph node status in uncorrected (p=0.04), cellularity corrected (p=0.03), and bicorrected hTR (p=0.04) measurements. In no model was hTR associated with proliferative index.

Outcome

Univariate Cox proportional hazard modeling revealed a significant negative association between positive lymph nodes and higher proliferative index on the one hand, and both relapse-free and overall survival (Table 2) on the other. There was also a trend towards a relationship between the tumor size and age at diagnosis and both relapse-free and overall survival. Unlike Clark et al., estrogen receptor status did not predict either relapse-free or overall survival, nor did whether the patient had received adjuvant therapy, a not surprising result given that, at that time, only poor-prognosis patients received adjuvant therapy.

As shown in Table 2, neither uncorrected nor corrected (cellularity corrected TA, histologically corrected TA, both corrections together) telomerase activity was associated with relapse-free or overall survival in univariate modeling. The presence of telomerase activity coded as a dichotomous variable (1 = present, 0 = absent) was also not associated with relapse-free (relative risk (RR) 0.99, 95% confidence interval (CI) 0.65 - 1.53) or overall survival (RR 0.95, 95% CI 0.63 - 1.48). Logarithmic transformation of uncorrected or corrected telomerase activity did not alter these results. Exclusion of the samples lacking detectable RNA levels also did not affect the results. Evaluation of only those tumors with telomerase activity > 5 TPG (n=76) did not affect the results.

Univariate Cox proportional hazard modeling did not reveal an association between uncorrected or corrected hTR level and relapse-free or overall survival. Logarithmic transformation did not alter these results. Comparison of those tumors which did not have measurable hTR with those tumors which did have measurable hTR did not alter the results. Multivariate modeling was not performed because there were too few tumors with measurable hTR to create reliable models.

Multivariate modeling was performed both as full models using all variables as well as reduced models (Table 3). The selected multivariate models presented in Table 3 include variables found to be significantly or nearly significantly associated with relapse-free and overall survival in univariate analysis. Full models (including age at diagnosis, tumor size, lymph node status, proliferative index, ER status, and if adjuvant therapy given) or other reduced models did not give notably different results. In most multivariate models, markedly shorter relapse-free and overall survival was strongly predicted by positive axillary lymph node status at resection. Shorter relapse-free and overall survival were also correlated with older age at diagnosis in most models, although the effect was smaller. A significant association between higher proliferative index and shorter RFS was seen, however this relationship was not consistent among models. No consistent association was seen between relapse-free or overall survival and tumor size, estrogen receptor status, or whether the patient had received adjuvant therapy. Neither corrected nor uncorrected telomerase activity consistently predicted relapse-free or overall survival, although in two instances an association was seen: 1) higher uncorrected TA was associated with longer relapse-free survival in the most reduced

model including only lymph node status, proliferative index, and telomerase activity (RR 0.90 for each 10-unit increment in TA, 95% confidence intervals 0.78 - 0.99, p=0.04), and 2) higher uncorrected TA, logarithmically transformed TA, and histologically corrected transformed TA were associated with longer relapse-free survival in the models including all variables (age at diagnosis, tumor size, lymph node status, with or without ploidy, proliferative index, ER status, whether the patient had received adjuvant therapy) (RR 0.80, 95% confidence intervals 0.60 - 0.95, p=0.008). In both instances, this association between telomerase activity and outcome was not robust to additions or deletions of variables to the model, and in particular was eliminated by exclusion of the RNA-negative samples. No other model demonstrated an association of uncorrected or corrected telomerase activity with survival. These models were examined using telomerase activity as a continuous variable both including all samples and excluding the telomerase-negative samples without notable changes in the results. Telomerase activity was also examined as a dichotomous variable with no independent association seen between the presence of activity and either relapse-free or overall survival.

Discussion

This study used an improved TRAP-based semiquantitative telomerase activity assay and RT-PCR for hTR in primary breast cancers and found that telomerase activity correlated with hTR in those tumors with detectable hTR. Telomerase activity was also associated with the proliferative index in the primary tumor. In 256 nonmetastatic tumors, we did not find that telomerase activity level predicted the outcome of the patient in either univariate or multivariate modeling. This study also evaluated the impact of RNA stability and tumor content on telomerase expression in primary breast tumors, but did not find that correction for either or both of these parameters significantly altered our results.

Telomerase activity was found in 67% of tumor samples. We and others have found telomerase activity present in 73-95% of frozen banked breast cancer samples (3-6,20). Forty-five tumors were 28S rRNA-negative. As expected, they were uniformly telomerase-negative, suggesting that enzymatic degradation is the cause of the absence of telomerase activity in those tumors. When these tumors are excluded, 80% of the tumors exhibit telomerase activity. Exclusion of these RNA-negative tumors did not alter the results.

Cancers vary markedly in their cellularity and the nuclear to cytoplasmic ratio. In addition, breast cancers are both microscopically and macroscopically heterogeneous, with varying proportions of stromal cells, fat, inflammatory cells, and preneoplastic and in situ malignant cells as well as true invasive malignant breast cancer cells. The majority of the nonneoplastic cells are expected to have no telomerase activity. For these reasons, the usual method of standardizing the TRAP assay by protein content may not truly reflect the enzyme activity of the invasive tumor. We examined two different methods of controlling for tissue integrity and sample cellularity that could be applied to telomerase measurements. The first method used the 28S rRNA level as a measure of the sampleís viability and cellularity by nuclear content, and is similar to the method used in the study by Clark and colleagues. This method does not distinguish between malignant and nonmalignant cells. The second method used involved the histopathologic evaluation of an adjacent section of tumor by a breast pathologist, who determined the proportion of

the entire sample tested which comprised nonnecrotic invasive tumor. Tumor specimens used in this study have a potential 10-fold difference in the invasive tumor component of the tested sample.

We found that quantitative telomerase activity, both uncorrected and corrected, was highly significantly associated with the proliferative index. Previous studies have also found an association between telomerase activity and proliferative index (10,23,24) . This association may be the result of a greater need for telomeric end maintenance in rapidly proliferating tumors in order to avoid critical shortening of the telomeres. More likely, however, the association of telomerase activity with proliferative index reflects the fact that noncycling cells are telomerase-quiescent, lowering the overall and per cell telomerase activity level. Quantitative telomerase activity in this study was not associated with the patientís age at diagnosis, the tumor size, presence or absence of lymph node involvement, ploidy, or estrogen receptor status. Although some studies in breast cancer have found such an association (5,10), several have failed to detect an association of telomerase levels with traditional prognostic factors.

Few studies to date have examined telomerase activity and survival. Using a different method of quantitation, one group found an association of telomerase activity in neuroblastoma with poorer outcome in a univariate model. The same group demonstrated that the presence of telomerase activity in gastric cancer was associated with shorter survival. Using a similar technique as employed in this study, Clark et al. examined telomerase activity and relapse-free and overall survival in a group of lymph node-positive breast cancer patients, and found that higher telomerase activity was associated with poorer overall survival. Another group of researchers used the same quantitative TRAP assay in non-small cell lung cancers, but did not demonstrate a relationship between telomerase activity and outcome.

Both Clark et al. and this study detected a strong association between telomerase activity and proliferative index, however Clark et al. also found an association between quantitative telomerase activity and overall survival, where we did not find such an association. There are several differences between the two studies. Clarkís study included only patients with axillary lymph node metastases at diagnosis, a poor-prognosis group likely representing a later stage in progression, whereas our study contained mixed stages of breast cancers. This may imply a difference in the role of telomerase in early versus late stage breast cancers.

Telomerase activity has been examined in earlier stages, including the preneoplastic lesion ductal carcinoma in situ (DCIS) (24,26,27). Many DCIS lesions were found to have telomerase activity, suggesting telomerase activation occurs at this early stage in carcinogenesis and telomerase activity could be useful in stratifying preinvasive breast lesions. However, the levels of telomerase activity in the DCIS lesions did not differ from the levels in adjacent invasive breast cancers, just as this study found no difference between the levels of telomerase activity in those patients with a good and those with a poor outcome. These data suggest that activation of telomerase occurs early, and once activated, the quantitative level of the enzyme as currently measured in cell extracts does not vary significantly in the early stage lesions. Thus, it is possible that earlier in the progression of breast cancer activation of telomerase may be important for telomere maintenance, but higher levels of activity may not confer an advantage to the cancer cells. However, in the late stage tumors examined by Clark et al., as the neoplastic cell

develops greater genomic instability, telomerase functions other than telomere maintenance (repair of DNA strand breaks (28-31), may become increasingly important for cancer cell survival and progression. This would allow more genetically unstable and theoretically more malignant clones to prosper. The chromosome healing function of telomerase has implications also in terms of sensitivity to chemotherapy. It is likely that a high proportion of the advanced stage patients in the study by Clark et al. received adjuvant chemotherapy, while fewer than 45% of the patients in this study did. If telomerase acts to heal damaged DNA, then higher levels of telomerase may counteract the cell killing induced by treatment with the DNA-damaging alkylating agents and anthracyclines commonly used in adjuvant therapy of breast cancer.

There are also important methodologic differences between this study and that of Clark et al. We used frozen sections of breast tumor whereas all previous studies used either whole or pulverized tumor. By using the frozen sections instead of whole or pulverized specimens, this study had the advantage of directly controlling the pathology and tumor contents of the specimens that were used in the TRAP assay. For example, 22 tumors were excluded from our study for having less than 10% invasive cancer in the specimens. These included tumors with a large DCIS component, and a less prominent invasive component. Such tumors may have both lower telomerase activity and a better prognosis. Using frozen sections, we were able to show that quantitative levels of the enzyme measured in a tumor sample may not be due solely to differences in cellular levels of telomerase, but are also due to differences in the number of immortal cancer cells in a tumor. This hypothesis is supported by the correlation between the total number of cells in a tumor (measured by the rRNA level) and tumor content with telomerase activity level that we have observed in this study.

However, using frozen sections also meant that the amount of sample that was used in the TRAP assay was lower (0.5(g protein) than in the study by Clark et al. (2(g protein), and that the tissue was subjected to more manipulation. Overall, the level of telomerase activity was ten-fold lower in this study (median TPG 1.00 vs. 10.00), which may have made accurate quantitation of telomerase more difficult. The low level of telomerase observed in this study certainly reflects the lower amount of tissue specimen used in this study, but could also reflect differences in tissue handling or patient selection. Although studies suggest that telomerase is relatively robust, the potential for enzymatic degradation exists, and is the reason for the 28S rRNA correction. However correction by the RNA level did not alter our results. It is possible that the lower telomerase activity measured in our study was too low for accurate quantitation. However there was no trend towards an association between telomerase activity, corrected or uncorrected, with outcome when the analysis was limited to only those tumors with telomerase activity levels greater than 5 TPG.

It is also possible that telomerase activity level as measured by the TRAP-based assay used in this study does not adequately reflect the telomeric end maintenance in the cancer cells themselves. The TRAP assay requires that cells be lysed with viable enzyme extracted and given the appropriate substrates so that, if the enzyme is present, artificial telomeric extension will occur. In vivo, telomeric end maintenance is likely the result not only of telomerase enzymatic activity, but also other factors such as telomere-associated proteins, some of which appear to have telomerase inhibitory functions. If telomeric end maintenance in cancer cells such as those obtained from the patient population studied

here does provide a selective advantage, a less artificial assay than the TRAP assay or a different means of obtaining samples may be required to demonstrate it. Alternative methods may be needed for asssessing telomerase expression in vivo, for example, by examining the telomerase RNA or protein component levels in the tissue sections by in situ hybridization or immunohistochemistry.

In spite of detecting telomerase RNA component (hTR) levels in only 34% of tumor samples, we found that in those tumors with measurable hTR, hTR correlated significantly with telomerase activity levels. This differs from many studies which failed to detect an association between telomerase activity levels and hTR levels (33-35). This discrepancy may in part reflect differing methods of hTR acquisition. Most other studies examined hTR from purified RNA (33-35), where our study did not, likely contributing to the low sensitivity of our assay for hTR. It is possible that an association of hTR with telomerase activity is found in those tumors with higher levels of hTR, but not at lower hTR levels. Some studies have found an association between hTR levels and proliferative index. However we did not detect such an association in this study, despite the strong association of telomerase activity with proliferative index. There was a trend towards a higher hTR in those tumors with high proliferative index, although this association did not reach significance (p=0.16). In this subset of tumors, we found no association of hTR levels with outcome.

Currently accepted prognostic factors in breast cancer are used to aid in clinical decision-making regarding extent of surgery and adjuvant therapy, although these factors are only moderately able to predict outcome. Because of this uncertainty, women with breast cancer are occasionally undertreated and often overtreated. Additional markers which may aid in prediction of relapse and survival are needed. Although this study of samples from nonmetastatic breast cancer patients as described did not show correlation between telomerase level and clinical outcome, further study of the distinction between early and late stage cancers is warranted. The presence and level of telomerase between early and late stage cancers may have different implications in tumorigenesis, and futher studies will be required to delineate this aspect of the role of telomerase in breast cancer.

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Figure legends

Figure 1. A) A representative TRAP assay of three breast cancer specimens. The characteristic six base pair ladder of telomerase extension products is seen in all three samples and is absent in the heat-inactivated lanes, confirming telomerase enzyme activity in the samples. The 36 base pair internal control sequence used for standardization of polymerase chain reaction efficiency is seen as the lowest band in each lane. Also shown is the R8 quantitation standards and the buffer-only negative control assays which were run with each set of breast cancer specimens. B) A dilutional series of the 28S rRNA assay demonstrating the direct correlation between the RNA signal and

the number of HeLa cells tested. The internal control sequence (RNA IC) used to standardize for polymerase chain reaction efficiency is seen as the lower band. Results from five breast cancer samples are demonstrated in lanes S1 through S5, and Bu represents a buffer-only negative control.

Figure 2. Distribution of uncorrected telomerase activities. This excludes 96 tumors which were telomerase-negative. The distributions of corrected activities were not remarkably different from that displayed.

Figure 1A

Figure 1A

Figure 1B

Figure 2

telomerase activity (TPG)
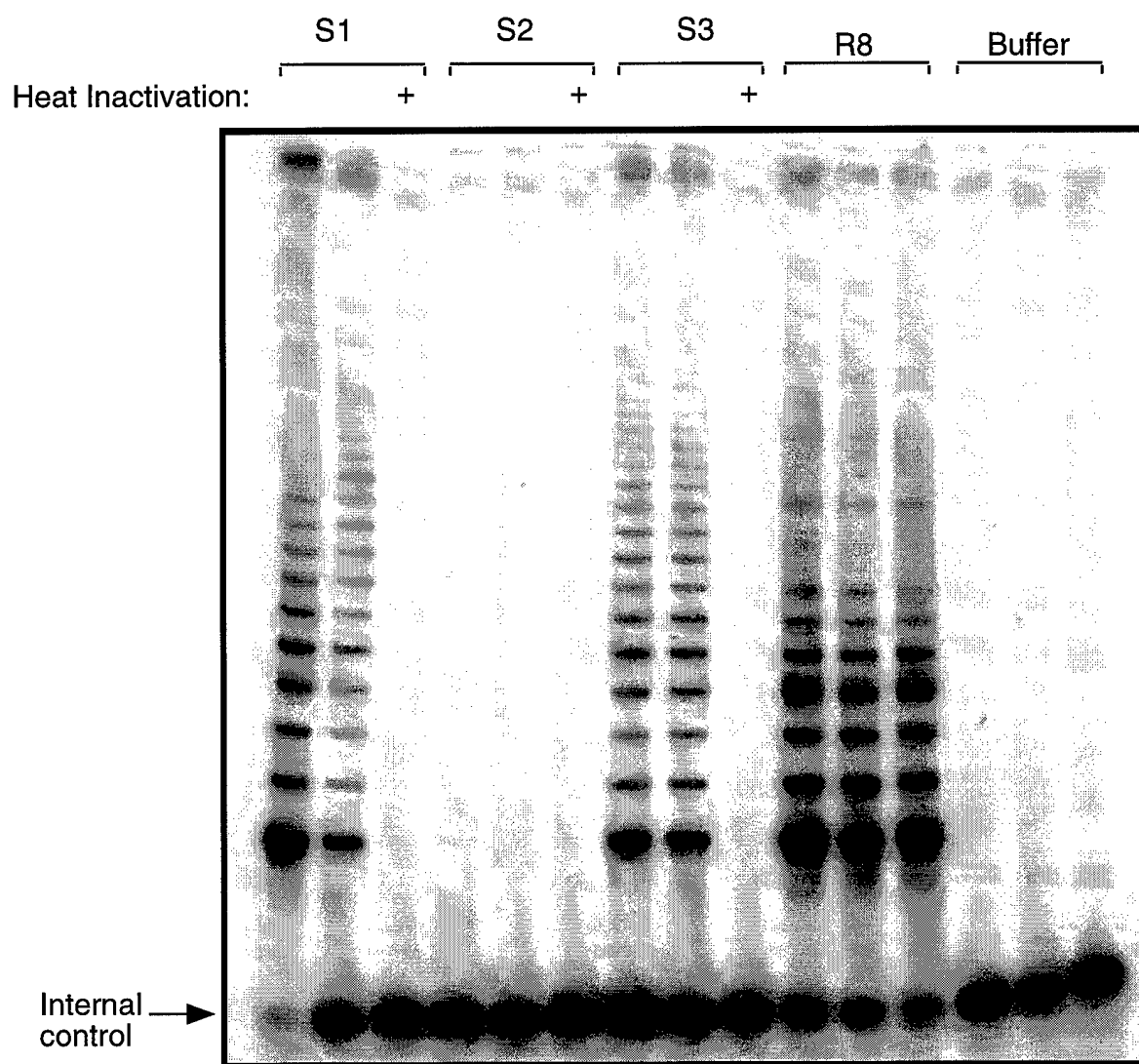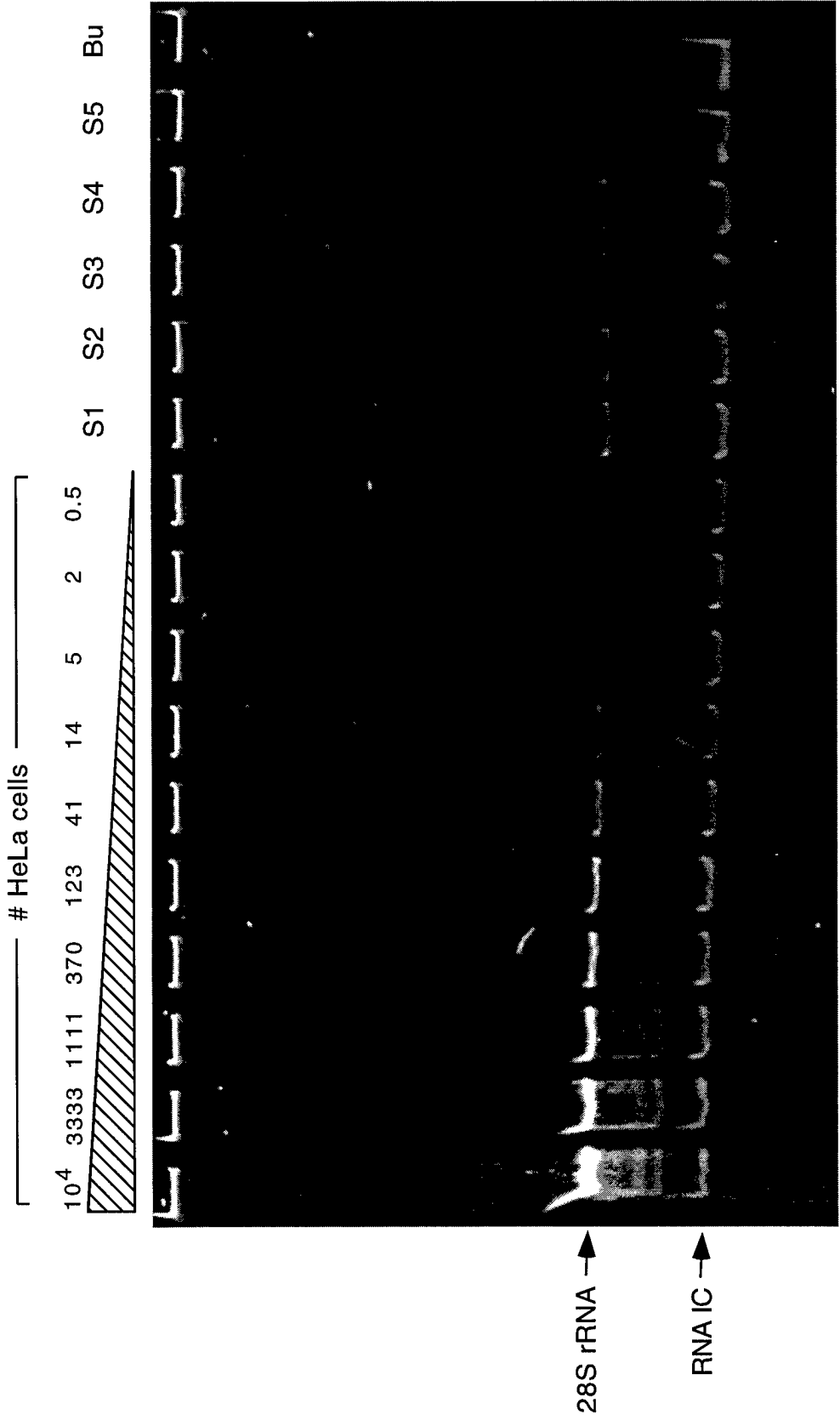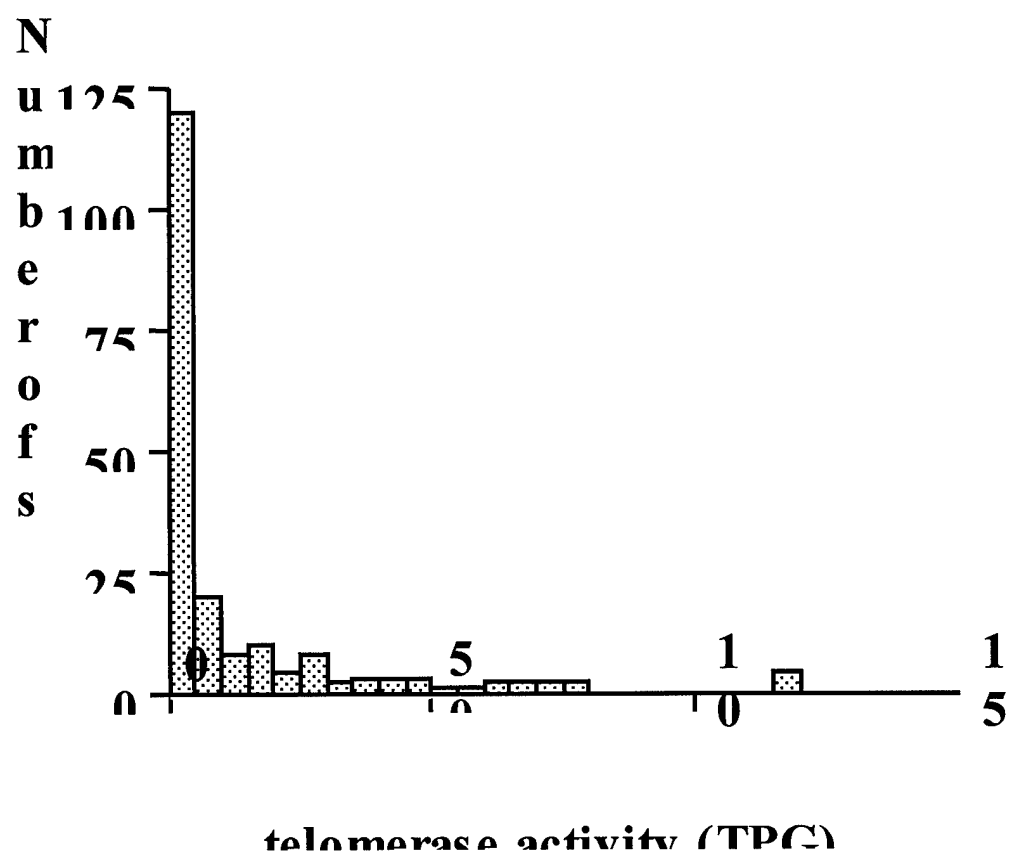
Table 1. Distribution of traditional prognostic factors.

Prognostic factor	Number (n=295)
Tumor size known	259
≤ 2 cm	66 (25%)
2 - 5 cm	147 (57%)
> 5 cm	40 (15%)
Inflammatory	6 (3%)
Lymph node status known	238
negative	83 (35%)
positive	155 (65%)
Estrogen receptor status known	216
negative	100 (46%)
positive	116 (54%)
Known if adjuvant therapy given	251
no	68 (27%)
yes	183 (73%)
DNA index	147
Diploid	43 (30%)
Near-diploid aneuploid	15 (10%)
Aneuploid	89 (60%)
Proliferative index	173
low	99 (57%)
high	74 (43%)

Table 2. Relative risks in univariate proportional hazard models for relapse-free and overall survival.

Factor	Relapse-free survival			Overall survival		
	RR[a]	CI[b]	p[c]	RR[a]	CI[b]	p[c]
age at diagnosis[d]	1.01	1.00-1.03	0.07	1.01	1.00-1.03	0.06
tumor size[e]	1.09	0.99-1.21	0.08	1.11	1.00-1.23	0.06
lymph node positive	3.73	2.17-6.90	<0.01	3.59	2.09-6.64	<0.01
aneuploidy	0.92	0.68-1.28	0.63	0.90	0.67-1.26	0.55
high proliferative index	2.00	1.21-3.33	<0.01	1.88	1.14-3.12	0.01
estrogen receptor positive	0.76	0.48-1.21	0.25	0.76	0.48-1.20	0.25
adjuvant therapy	1.44	0.89-2.47	0.14	1.40	0.86-2.40	0.18
uncorrected TA[fg]	0.98	0.89-1.05	0.60	0.98	0.89-1.06	0.71
cellularity corrected TA[h]	1.00	0.91-1.06	0.91	1.00	0.91-1.07	0.94
histologically corrected TA[g]	1.00	0.94-1.04	0.88	1.00	0.94-1.05	0.92
Telomerase activity with both corrections[h]	0.99	0.92-1.04	0.74	1.00	0.92-1.04	0.87

[a] RR=relative risk; [b] CI=95% confidence intervals; [c] p=p-value; [d] risk ratio for each additional decade; [e] risk ratio for each additional centimeter; [f] TA = telomerase activity; [g] risk ratio for each increment of 10 TPG; [h] risk ratio for each increment of 1 TPG

Table 3. Adjusted risk ratios (RR) of relapse-free (A) and overall (B) survival. Telomerase activity was incorporated into the models as uncorrected activity or standardized by one or both correction methods. In the models using uncorrected and histologically corrected telomerase activity, there were 51 relapses and 53 deaths; in those models where RNA-negative samples were automatically excluded (cellularity corrected TA and both corrections together), there were 46 relapses and 47 deaths.

A. Relapse-free survival

Factor	Uncorrected TA (n=256)			Cellularity corrected TA (n=217)			Histologically corrected TA (n=256)			Both corrections (n=217)		
	RR[a]	CI[b]	p[c]	RR[a]	CI[b]	p[c]	RR[a]	CI[b]	p[c]	RR[a]	CI[b]	p[c]
age at diagnosis[d]	1.19	0.96-1.48	0.11	1.36	1.08-1.72	0.01	1.20	0.97-1.49	0.09	1.36	1.08-1.72	<0.01
tumor size[e]	1.09	0.95-1.23	0.20	1.09	0.96-1.24	0.21	1.09	0.95-1.23	0.21	1.09	0.95-1.24	0.22
lymph node positive	3.58	1.68-8.82	<0.01	3.77	1.66-10.14	<0.01	3.54	1.66-8.72	<0.01	3.73	1.65-10.04	<0.01
high proliferative index	2.07	1.13-3.81	0.02	1.79	0.93-3.40	0.22	2.04	1.10-3.79	0.02	1.79	0.92-3.47	0.08
telomerase activity	0.91	0.79-1.07	0.11	1.03	0.93-1.11	0.27	0.96	0.88-1.02	0.25	1.02	0.93-1.09	0.59

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B. Overall survival

Factor	Uncorrected TA (n=256)			Cellularity corrected TA (n=217)			Histologically corrected TA (n=256)			Both corrections (n=217)		
	RR[a]	CI[b]	p[c]	RR[a]	CI[b]	p[c]	RR[a]	CI[b]	p[c]	RR[a]	CI[b]	p[c]
age at diagnosis[d]	1.20	0.96-1.50	0.10	1.38	1.09-1.76	0.01	1.22	0.98-1.52	0.08	1.38	1.08-1.76	<0.01
tumor size[e]	1.10	0.96-1.25	0.17	1.11	0.95-1.28	0.17	1.10	0.96-1.25	0.16	1.11	0.96-1.28	0.16
lymph node positive	3.18	1.50-7.84	<0.01	3.35	1.48-8.98	<0.01	3.14	1.48-7.74	<0.01	3.30	1.47-8.85	<0.01
high proliferative index	1.77	0.96-3.25	0.06	1.54	0.80-3.00	0.19	1.71	0.93-3.10	0.08	1.53	0.79-2.98	0.20
telomerase activity	0.94	0.81-1.04	0.25	1.05	0.95-1.13	0.28	0.98	0.91-1.05	0.62	1.04	0.95-1.11	0.34

[a] in units of 10 TPG; [b] in units of 1 TPG; [c] risk ratio with each additional decade; [d] risk ratio with each additional centimeter; [e] risk ratio with each additional unit

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