The enzyme telomerase contributes to the maintenance of telomere stability (1). Although telomerase reactivation in somatic cells is not sufficient per se for the cells to proliferate indefinitely, telomerase expression and telomere stabilization appear to occur concomitantly with the attainment of immortality in cancer cells (1–3) and may contribute to tumorigenesis and neoplastic progression. It is not yet known at what stage of cancer development telomerase is reactivated, but scientific interest has recently been focused on defining the utility of assaying for telomerase as a diagnostic and prognostic tool (4). In particular, investigators have focused on breast cancer for its high incidence and relevance. These investigations have been favored by the existence of ongoing screening programs and the possibility of obtaining samples for pathologic and biologic characterization through the use of the relatively noninvasive method of fine-needle aspiration. Only two studies (5,6) of which we are aware have provided results on the diagnostic potential of measuring telomerase activity in fine-needle aspirates (FNAs) of breast lesions. However, the actual diagnostic predictivity of results obtained with the telomeric repeat amplification protocol (TRAP) assay has not been conclusively defined, owing to the lack of histologic confirmation for some of the cytologically diagnosed lesions and the small number of cases investigated. In this study, we prospectively assessed the diagnostic accuracy of findings of telomerase activity in a consecutive series of FNAs from 116 solid breast lesions that were subsequently submitted for histologic examination—i.e., in a series that is much larger than those previously examined (5,6). Telomerase activity was measured by use of the polymerase chain reaction-based TRAP assay described by Kim et al. (2). The protein content of each sample was quantified, and the TRAP assay was performed at two protein concentrations (0.6 and 6.0 μg) per sample, as suggested by Sugino et al. (6). The reliability of the TRAP assay results was supported by the concordance of data obtained from the FNAs and their corresponding surgical specimens (Fig. 1, A) and by the demonstration of a lack of TRAP assay inhibitors in the telomerase-negative samples (Fig. 1, B). The TRAP assay results were independently scored by two of the investigators (R. Villa and M. Folini) without knowledge of the histologic diagnosis.

Women entering the study had a median age of 42 years (range, 18–86 years), had palpable solid breast lumps (median size, 2.2 cm; range, 0.5–4.5 cm), and were examined at the Division of Diagnostic Oncology and the Outpatient Clinic of the Istituto Nazionale per lo Studio e la Cura dei Tumori, Italy. Patients with telomerase activity were from women who were more than 65 years of age; these tumors were small and of a pure or mixed-lobular histologic type. No association was observed between the number of morphologically identified putative tumor cells and the TRAP assay results.

To use the detection of telomerase activity prospectively for a differential diagnosis of breast lesions, we evaluated the specificity, the sensitivity, and the predictive value of the TRAP assay results on our case series. Overall, the area under the receiver-operating-characteristic curve calculated by considering the two threshold levels of protein concentration was 0.683. The specificity (defined as the frequency of samples correctly identified as benign by the TRAP assay among the total number of histo-
Fig. 1. A) Comparison of telomeric repeat amplification protocol (TRAP) assay results from extracts of fine-needle aspirates (FNAs) of breast tumors and extracts from the corresponding surgical specimens. The comparison was performed for 15 tumors; results from five representative cases are shown. Cells in FNAs were washed once in phosphate-buffered saline, pelleted at 1000 g for 1 minute at 4 °C, resuspended in ice-cold buffer (10 mM Hepes–KOH [pH 7.5], 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, and 1 mM dithiothreitol), pelleted again, and resuspended in 10 or 20 µL ice-cold lysis buffer (10 mM Tris–HCl [pH 7.5], 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.1 mM phenylmethylsulfonil fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS, i.e., 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate, and 10% glycerol). The final suspensions were incubated for 30 minutes on ice and then centrifuged for 30 minutes at 25 000 g at 4 °C. The supernatants were recovered, and their protein concentrations were determined spectrophotometrically. The supernatants were quick-frozen in liquid nitrogen and stored at −80 °C or assayed immediately for telomerase activity. The assays (2) were performed in two steps: 1) telomerase-mediated extension of an oligonucleotide primer, i.e., TS, which serves as a substrate for telomerase, and 2) hot-start polymerase chain reaction (PCR) amplification of the telomerase product (an incremental 6-nucleotide, single-stranded DNA ladder), using the oligonucleotide primer pair, TS (forward) and CX (reverse). Reaction tubes for the TRAP assays were prepared by lyophilizing 0.1 µg CX primer onto the bottom of individual microfuge tubes and sealing it in place with 7–10 µL molten wax (Ampliwax; The Perkin-Elmer Corp., Foster City, CA). The wax was allowed to solidify at room temperature, and the tubes were stored temporarily at 4 °C. Next, a 50 µL TRAP reaction mixture, which contained 20 mM Tris–HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 50 µM each of the four standard deoxynucleoside triphosphates, 0.1 µg TS oligonucleotide, 1 µg T4g32 protein (Boehringer Mannheim, Indianapolis, IN), 0.1 mg/mL bovine serum albumin, 2 U Taq DNA polymerase, and 1–4 µL cell extract (see above), was placed above the wax barrier. For radiolabeling of products, 0.2 µL [α-<sup>32</sup>P]dCTP (deoxyctydine triphosphate; 10 µCi/µL, 3000 Ci/mmol, Amersham International, Buckinghamshire, U.K.) was added to the reaction mixtures. After 20 minutes at 23 °C for extension of the TS oligonucleotide by telomerase, the reaction tubes were transferred to a thermal cycler for 31 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 1.5 minutes. The CX primer was liberated when the wax barrier melted at approximately 70 °C. The total reaction product was resolved by electrophoresis in 0.5 × Tris–borate EDTA buffer (45 mM Tris–borate [pH 8.3] and 1 mM EDTA) in a 10% polyacrylamide nondenaturing gel, and autoradiography (with intensifying screens) of the 6-nucleotide repeat ladder was performed by overnight exposure of x-ray film at −80 °C. For samples exhibiting no telomerase signal after 18 hours, the autoradiographic exposure was extended to 72 hours; when no DNA ladder was evident after this period of time, the sample was classified as negative. For each cell extract, aliquots containing 6.0 or 0.6 µg protein, with or without ribonuclease A (RNase) pretreatment (20 µg/mL for 20 minutes at 37 °C), were used in the TRAP assay. Dilution of the cell extract was used to minimize the possibility of false negatives by reducing the contribution of tissue factors that could inhibit telomerase or the PCR step of the assay. Extracts of a human breast cancer cell line (MCF7) exhibiting telomerase activity were used as a positive control. Negative control was achieved by omitting cell extracts from the TRAP assay reaction mixtures (see ‘‘Lysis buffer’’ lane). Reproducibility of the TRAP assay was confirmed by preliminary repeated experiments. B) Example of negative samples showing positivity (lanes 1–5) after 0.1 µg protein from a telomerase-positive MCF7 cell extract (lane 6) was added to the TRAP assay reaction mixtures to demonstrate the lack of assay inhibitors.
In conclusion, our results on FNAs from solid breast lumps confirm that differences exist in telomerase activity between neoplastic and benign lesions (6–8). However, the low specificity of the TRAP assay suggests caution with respect to the application of measuring telomerase activity, at least as a single prospective diagnostic tool. In fact, the high incidence of telomerase-positive samples among benign lesions and the poor predictive values for both malignant and benign lesions are far from acceptable for routine diagnostic application and indicate the need for assay improvements to increase predictivity. Moreover, false-positive cases should be followed to monitor the evolution of the lesions, although the occurrence of a positive TRAP signal in fibroadenomas and dysplasias is in agreement with previous observations of a substantial fraction of S-phase cells in benign breast lesions (9) and may reflect the presence of a proliferative disease, mainly in young patients. Such positive signals could also be due to the presence of normal breast stem cells, which can differentiate into either epithelial cells or myoepithelial cells (6), or of normal breast tissue under hormonal stimulation (10). These findings should be taken into account, since immortality or an active proliferative state may be acquired independently of malignant transformation (11).

References


Notes

Supported by grants from the Associazione Italiana per la Ricerca sul Cancro and the Italian National Research Council (grant no. 97.00490. CT04).

We thank B. Canova and B. Johnston for editorial assistance.

Table 1. Telomerase activity in fine-needle aspirates (FNAs) of breast lesions as a function of histologic diagnosis

<table>
<thead>
<tr>
<th>Diagnosis based on surgical specimens</th>
<th>No. of specimens with TRAP* assay results</th>
<th>Telomerase activity in FNAs: No. of positive samples†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assessable</td>
<td>Unassessable</td>
</tr>
<tr>
<td>Benign lesion</td>
<td>75</td>
<td>5</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>56</td>
<td>3</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>36</td>
<td>—</td>
</tr>
</tbody>
</table>

*TRAP = telomeric repeat amplification protocol.
†A = telomerase activity detected at least at one of the two protein concentrations tested (0.6 and 6.0 μg); B = telomerase activity detected at the lowest protein concentration tested; C = telomerase activity detected at the highest protein concentration tested; and D = telomerase activity detected at both protein concentrations tested.
‡Numbers in parentheses show the frequency of positive cases among the total number of informative cases.
§Fraction of positive samples for telomerase activity, detected at least at one of the two protein concentrations tested, was significantly higher for carcinomas than for benign lesions (continuity adjusted chi-squared value = 4.975; two-sided P = .026 for one degree of freedom).