Telomerase Activity in Lesions of the Thyroid: Application to Diagnosis of Clinical Samples including Fine-Needle Aspirates

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ABSTRACT

The telomerase enzyme is capable of replacing telomeric DNA sequences that are lost at each cell division. It has been suggested that the function of this enzyme is necessary for cells to become immortal, and in concordance with this hypothesis, telomerase activity has been detected in malignant tumor cells, whereas the enzyme is inactive in normal somatic cells. The measurement of this activity in human tissue samples may have diagnostic value, and in this study, we examined whether such a measurement may be useful for the detection of malignant cells within the thyroid.

Telomerase activity was assayed using the telomeric repeat amplification protocol and related to the histological diagnosis of thyroid biopsy tissue samples and of cells obtained from the thyroid by fine-needle aspiration (FNA). Extracts from 9 of 11 (82%) carcinoma biopsy tissue samples contained telomerase activity, whereas enzyme activity was detected in only 2 of 14 (14%) benign tissue sample extracts. These two positive cases were subsequently diagnosed as Graves’ disease with severe lymphocytic infiltration. Five of six (83.3%) histologically confirmed carcinoma FNA samples were identified by using the telomeric repeat amplification protocol assay, and two samples considered to be suspicious by FNA cytology were also positive. Conversely, only 4 of 48 (8.3%) benign FNA samples had telomerase. These promising data indicate that this sensitive assay could become a useful adjunct to microscopic cytopathology in the detection of cancer cells in small tissue biopsies and in fine-needle aspirates of the thyroid.

INTRODUCTION

Patients with an enlarged thyroid gland frequently present at outpatient clinics. A number of approaches are used to investigate the condition, ranging from noninvasive techniques (including palpation and ultrasonography) through minimally invasive serological tests, biochemical tests, radioisotope scanning and FNA2 to surgical biopsy. FNA cytology is the first line of investigation for a nodule in the thyroid with sensitivities and specificities of approximately 90% for the cytological detection of malignancy (1-5). The technique depends on subjective interpretation by a skilled cytopathologist (4), and there are particular problems with the diagnosis of follicular tumors with an overlap in cytological appearances between adenomas and well-differentiated follicular carcinomas. The availability of a subsidiary technique to aid interpretation would be clinically very valuable.

The enzyme telomerase is a promising candidate marker for the detection of neoplasia (6-9). Attention has been recently focused on this marker, because activation of telomerase is thought to be necessary for cells to become immortal or at least capable of extended proliferation (10, 11). The termini of eukaryotic chromosomes are specialized structures known as telomeres, which contain 2-30 kb of DNA composed of a simple hexanucleotide repeat, TTAGGG (12). In conjunction with specific DNA-binding proteins, these sequences play an important role in chromosome structural integrity and function and protect the ends of chromosomes against degradation and untimely recombination events (12-14). The noncoding sequences composing the telomeres also act as a buffer zone that prevents any compromise of function resulting from the obligatory loss of terminal DNA that occurs at each cell division. This loss occurs as a result of the inability of DNA replication processes to complete lagging strand synthesis to the chromosomal terminus (15, 16). The reduction in length of telomeric DNA beyond a critical point has been proposed to be a signal for cells to exit from the cell cycle and enter a senescent state (17, 18). This suggests that maintenance of telomere length is necessary for continued cell division and immortalization (10, 17-19). One mechanism for the restoration of telomeric repeats is the activation of the enzyme telomerase, which is a RNA-dependent DNA polymerase ribonucleoprotein (20, 21).

A highly sensitive PCR-based assay named TRAP has been

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2The abbreviations used are: FNA, fine-needle aspiration; TRAP, telomeric repeat amplification protocol; ITAS, internal telomerase assay standard.
developed for the detection of telomerase activity (22). The use of this assay has shown that telomerase activity is detectable in all but a few human cancer cell lines and in the majority of human tumors tested (9, 23–26), but activity is absent in normal tissues (6, 22). There have been studies that have assessed the utility of the TRAP assay in the detection of carcinoma cells in thyroid tissue samples (27–30), but only a few thyroid fine-needle aspirate samples have been analyzed (28). In this study, we assessed whether the detection of telomerase activity could be used as a diagnostic marker for the presence of tumor cells in thyroid samples. The enzyme activity was assayed in surgically obtained tissues from thyroid carcinoma and benign lesions. Furthermore, to elucidate whether it would be possible to detect cancer cells in material obtained by minimally invasive nonsurgical procedures, telomerase activity was assayed in samples obtained from the thyroid by FNA. This study demonstrates the feasibility of detecting malignant cells in the thyroid by applying the TRAP assay to such samples.

MATERIALS AND METHODS

Tissue Specimens. Twenty-five fresh thyroid tissue samples were obtained from surgical specimens resected at the John Radcliffe Hospital (Oxford, United Kingdom) and at the Karolinska Hospital (Stockholm, Sweden). Eleven samples were from patients with thyroid carcinoma, including five papillary carcinomas, three follicular carcinomas, two anaplastic carcinomas, and one Hürthle cell carcinoma. The other 14 samples were obtained from benign thyroid lesions, including 9 adenomas, 2 cases of Graves’ disease, 2 hyperplasias, and 1 colloid goiter. All tissues were snap-frozen immediately after resection and stored in liquid nitrogen until processed. Cryostat sections were examined by expert pathologists to confirm the histological identity of all tissue samples before analysis. FNA samples were obtained from 56 patients with thyroid lesions. Cells from FNA samples were obtained by flushing the needles and syringes with the same material used for cytological evaluation. Cell suspensions were assayed in precisely the same manner as described below. All samples were obtained in random order with the identity of all tissue samples before analysis.

Protein Extraction. Frozen tissue specimens were sectioned on a cryostat, and twenty-μm-thick sections from each specimen were mixed with 50 μl of ice-cold lysis buffer [10 mm Tris-HCl (pH 7.5), 4-(2-aminoethyl)benzenesulfonyl fluoride (0.4 mg/ml), EDTA-Na₂ (0.5 mg/ml), leupeptin (0.5 μg/ml), and pepstatin (0.5 μg/ml); ICN Pharmaceuticals]. After centrifugation at 1500 × g for 5 min at 4°C, cell pellets were used for protein extraction as described below. All samples were obtained in random order from the residue of tissue or cells used for pathological diagnosis that would otherwise have been discarded.

Telomerase Assays. Telomerase activity was assayed by the TRAP method described by Kim et al. (22). Briefly, 2 μl (6 μg of protein) of the cell extract were incubated in 50 μl of reaction mixture [20 mm Tris-HCl (pH 8.3), 1.5 mm MgCl₂, 63 mm KCl, 0.005% Tween 20, 1 mm EGTA, 50 μm deoxyribonucleotide triphosphates including [32P]dCTP (Amersham Corp., Arlington Heights, IL), 0.1 μg of TS oligonucleotide (5’-AATCTCGTGAGCAGAGTT-3’), 1 μg of T4 gene-32 protein (Boehringer Mannheim, Indianapolis, IN), and BSA (0.1 μg/ml; Sigma Chemical Co.)] and 2 units of Taq DNA polymerase (Boehringer Mannheim) at 20°C for 30 min for telomerase-mediated extension of TS primers. After heating the mixture at 90°C for 3 min to inactivate telomerase, 1 μg of CX primer (5’-(CCCTTA)₅(CCCCTAA)₃) and 10 μg of an ITAS (31) were added, and the mixture was subjected to 31 cycles of PCR with the following cycle conditions: (a) 94°C for 45 s; (b) 50°C for 45 s; and (c) 72°C for 90 s. Each PCR reaction (16 μl) was analyzed by electrophoresis on 12% polyacrylamide nondenaturing gels. The gel was dried and processed for autoradiographic exposure. To estimate the relative intensity of telomerase activity in a sample, serial dilutions of the protein extract from each case were assayed (6 μg and 0.6 μg of protein for each case). To test the specificity of telomerase-positive assays, 1 μg of DNase-free RNase (Promega, Madison, WI) was added to 5 μl of each sample, and after incubation for 20 min at 37°C, 2 μl of the treated sample were applied to the TRAP assay. The inclusion of an ITAS, a cDNA that is amplified with the TS and CX primers to generate a 150-bp product, aids the detection of false negatives that can result from the presence of PCR inhibitors in the extracts (31). The criterion for a positive TRAP assay was a hexanucleotide ladder of three or more bands that were absent from the negative controls of: (a) no sample protein; and (b) the RNase-digested sample.

RESULTS

Telomerase Activity in Thyroid Tissue Samples. Nine of the 11 (81.8%) fresh tissue samples histologically diagnosed
as containing carcinoma were shown to be positive in the TRAP assay (Table 1). These positive samples were comprised of all five papillary carcinomas, all three follicular carcinomas, and one of two anaplastic carcinomas. Because the TRAP assay used in this study is nonquantitative, no correlation between the level of telomerase activity and the grade or stage of malignancy could be accurately assessed. One case of anaplastic carcinoma and the single case of low-grade Hürthle cell carcinoma gave negative assay results (Fig. 1; Table 1). Negative TRAP assay results can be caused by the presence of PCR inhibitors in the extract, but the successful amplification of the included ITAS showed that the two cases without detectable telomerase activity were not false negatives. However, the presence of telomerase inhibitors in these extracts, as observed in some breast carcinoma tissue samples (32), could not be ruled out. Of the 14 samples obtained from lesions diagnosed as benign, only 2 (14%) had detectable telomerase activity. Both of these positive samples were subsequently histologically diagnosed as Graves’ disease with severe lymphocytic infiltration. This result confirms that telomerase activity data need careful interpretation. False positive results can occur when assaying inflamed tissue. Peripheral blood lymphocytes do possess active telomerase (7), and lymphocytic infiltrate commonly occurs in Graves’ disease and in thyroiditis.

**Telomerase Activity of Thyroid FNA Samples.** Clear differences in detectable telomerase activity were observed between FNA samples that were histologically confirmed as containing malignant cells and those that were diagnosed as being from benign lesions. FNA samples cytologically judged to contain no malignant cells had no histological confirmation from solid tissue, because no surgery was performed. Only 4 of 48 (8.3%) FNA samples obtained from cases diagnosed as having benign or nonneoplastic lesions were found to contain telomerase activity (Table 2; Fig. 2). Two of the positive samples were cytologically diagnosed as Hashimoto’s thyroiditis, one sample was obtained from a case of de Quervain’s thyroiditis, and another sample was diagnosed as a benign Hürthle cell adenoma. In all four positive samples, telomerase activity was abolished when extracts were subjected to RNase pretreatment. Microscopic analysis revealed that all of these samples had high numbers of lymphoid cells present.

In contrast to the nonmalignant samples, the FNA samples that were evaluated as containing malignant cells or diagnosed as "suspicious of carcinoma" had subsequent tissue biopsy material to confirm the preliminary diagnosis. Five of six (83.3%) FNA samples obtained from histologically confirmed malignant lesions were positive for telomerase activity (Table 2). However, the FNA cytological preliminary diagnosis was inconclusive in three of these samples. Two TRAP-positive FNA samples identified as suspicious of carcinoma were subsequently confirmed as carcinoma on resection (Fig. 3, cases 1 and 2), and one TRAP-negative sample suspicious of carcinoma was subsequently identified as papillary carcinoma with cystic degeneration (Fig. 3, case 3). Two more FNA samples that were cytologically diagnosed as suspicious of malignancy were also found to be positive for telomerase activity but await final diagnosis. The specificity of all telomerase-positive assays was confirmed by pretreatment of protein extracts with RNase. Because the functional telomerase complex requires an RNA moiety, this digestion should negate a previously positive assay (Fig. 3).

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**Fig. 1** Telomerase activity in lesions of the thyroid. Tissue extracts (6.0 and 0.6 μg of protein) prepared from frozen thyroid tissue samples were analyzed using the TRAP assay. *Case 1*, TRAP negative, histologically diagnosed as adenoma; *Case 2*, TRAP positive, Graves’ disease; *Case 3*, TRAP negative, low-grade Hürthle cell carcinoma; *Cases 4 and 5*, TRAP positive, follicular cancer and papillary cancer, respectively. In TRAP positive cases, telomerase activity was abolished by pretreatment with RNase A (+). An ITAS was included in each reaction for the detection of inhibitors of the PCR.

**Table 2** Telomerase activity in FNAs of the thyroid

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Cases</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant*</td>
<td>6</td>
<td>5 (83.3%)</td>
<td>1</td>
</tr>
<tr>
<td>Nonmalignant</td>
<td>48</td>
<td>4 (8.3%)</td>
<td>44</td>
</tr>
<tr>
<td>Suspicious</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
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*Diagnosis of malignant FNA samples is the histologically confirmed final diagnosis. Diagnosis of nonmalignant and suspicious FNA samples is cytological only.
DISCUSSION

The development of the sensitive TRAP assay by Kim et al. (22) has allowed the application of telomerase activity as a molecular marker in the diagnosis of numerous kinds of human cancer. It is now widely accepted that telomerase activity is only present in cells of somatic origin if they have become immortalized and are therefore neoplastic. Many studies have confirmed this by the assay of samples of solid tumors originating from numerous tissues, including lung (23), gastric and colorectal (24, 25), breast (32), and bladder (26) tissue. The reported frequencies of telomerase activity in such tumor samples are around 80%. However, to be useful as a diagnostic marker for malignancies, the TRAP assay needs to be applicable to the analysis of clinical specimens obtained before surgical intervention. To this end, a number of telomerase studies on clinical samples obtained by relatively noninvasive collection methods have been performed. Exfoliated cells naturally voided into the urine (26) or colonic lumen (33) or collected from oral rinses (34) have been analyzed for telomerase activity to detect small numbers of naturally shed tumor cells as an early diagnostic marker of carcinoma. We have also assessed the diagnostic efficacy of the TRAP assay on samples obtained from breast lesions by the relatively noninvasive technique of FNA and compared the data with results from solid breast tissue samples (32). The diagnostic sensitivity of the TRAP assay for the identification of histologically confirmed malignant tumors was 81.8% on solid tissue and 83.3% on FNA samples, with a specificity value of 86% for solid tissue. TRAP assay results on nonmalignant FNA samples had a specificity of 91.6%, but this is using cytological diagnosis only. As judged by the gold standard of microscopical diagnosis, the assay of samples obtained by noninvasive techniques from those tissues thus far studied is encouraging and warrants additional investigation in other tissues.

The thyroid is an organ that commonly contains suspicious lesions and that is accessible to preliminary biopsy without surgical excision. It is estimated that >50% of the general population will have detectable thyroid nodules if examined by ultrasonography (35). Ten percent of presented cases undergo surgery for diagnostic investigation of lesions suspicious for malignancy as assessed by FNA, but only around 40% of these are subsequently confirmed as carcinoma (28). Clearly, any molecular-based assay that could aid the diagnosis of cytologically ambiguous samples of the thyroid would be advantageous for both the patient and the health care burden. Two previous studies have analyzed telomerase activity in tissue biopsy samples taken from the thyroid. The first report of telomerase activity in thyroid tissue and nodules by Haugen et al. (27) showed activity in a large percentage of thyroid carcinomas, but not in benign adenomas or most normal thyroid tissue. They also showed that the level of telomerase activity may correlate with tumor invasiveness. Interestingly, as was demonstrated with FNA samples in this study, they found that the small number of noncarcinoma samples that tested positive for telomerase activity were those that had inflammation. Umbricht et al. (28) examined follicular tumors and found telomerase activity in all
Fig. 3 Telomerase activity in malignant FNA samples. Tissue extracts (6.0 and 0.6 μg of protein) prepared from FNA samples were analyzed by the TRAP assay. Cases 1 and 2, TRAP positive, histologically diagnosed as anaplastic cancer and follicular cancer, respectively. Telomerase activity was not detected in samples pretreated with RNase A (+). Case 3, TRAP negative, histologically diagnosed as papillary cancer with cystic degeneration; Case 4, TRAP positive, cell lysate from a breast cancer FNA sample used as a positive control. An ITAS was included in each reaction for the detection of inhibitors of the PCR.

carcinomas studied, whereas it was undetectable in normal thyroid tissues taken from sites adjacent to the tumors.

They also reported that telomerase was undetectable in the majority of benign follicular tumors; thus, the assay of telomerase activity may provide a diagnostic marker aiding the ability to distinguish microinvasive follicular thyroid cancer from benign follicular tumors. The ability to identify invasive follicular thyroid tumors is particularly difficult cytologically, and the uncertainty of diagnosis leads to unnecessary surgery.

This study, in line with previous reports on thyroid tissue and, indeed, those of the great majority of tumor biopsy studies (9), further confirms that telomerase activity is present in the majority of solid tumor biopsies analyzed. The detection of telomerase activity in thyroid cancers suggests that the activation of this enzyme is necessary for the malignant progression of neoplasms of the thyroid. The positive frequency observed with solid thyroid tumor samples in this study was 81.8%, which is consistent with previous reports. However, for clinical use as a diagnostic marker for malignancies of the thyroid, the telomerase assay has to be applicable to the assessment of FNA specimens.

In this study, 83% of FNA samples obtained from histologically confirmed malignant lesions were TRAP positive, compared with only 8% of samples taken from benign lesions. The diagnostic specificity of cytologically diagnosed nonmalignant samples (91.6%) is closely comparable with the accuracy reported for FNA cytology alone (5). With the borderline cases, the TRAP assay indicated carcinoma correctly in two of three suspicious samples; hence, the diagnostic use of the TRAP assay on thyroid cancer samples, including FNAs, seems feasible.

The assay of homogenized tissue extracts alone has limitations. A number of studies have reported telomerase activity in noncancerous tissues obtained from sites adjacent to tumors, and false positive results can be caused by the presence of isolated cancer cells or lymphocyte infiltrates (22, 23) in the tissue. In this study, two frozen samples diagnosed as Graves’ disease and two FNA samples diagnosed as Hashimoto’s disease gave positive results with the TRAP assay. For these reasons, the TRAP assay in its present form should not yet be used as a stand-alone diagnostic tool, but it has great potential value as an adjunct to a skilled pathologist in the evaluation of cytologically difficult diagnoses.

The elucidation of the stage at which telomerase activity is induced in the progression to carcinoma is of great interest for both clinical and biological purposes. The sensitivity of the TRAP assay may allow the analysis of microdissected samples to further elucidate the temporal details of telomerase activation in proliferative lesions. However, attempts to make meaningful correlations with tumor stage require the accumulation of more quantitative data. Although the TRAP assay has been optimized for semiquantitative analyses (31, 36), the inherent problems of a PCR-based assay, the sensitivity to degradation of a ribonucleoprotein, and the balance of as yet undefined telomerase inhibitors and cofactors present in tissue extracts make this a difficult task.

The sensitivity, specificity, and accuracy of FNA for the detection of malignancy have eclipsed the utility of other diagnostic methods, and, as a result, this relatively simple procedure has assumed a dominant role in determining the management of patients with thyroid nodules. However, the success of FNA is dependent on several important factors, of which skillful cytological interpretation is the most subjective. Furthermore, any data obtained from FNA samples needs careful interpretation, because it is possible that in cases of multiple thyroid nodules, samples may be obtained from benign nodules, whereas others may be malignant. However, in the absence of a clear cytological diagnosis, and where there is no lymphoid infiltrate, a TRAP assay could greatly aid the pathologist in evaluating borderline cases. Although the assay of telomerase activity is not 100% specific for malignant samples, the use of this test
could lead to a reduction in unnecessary surgery for patients with benign lesions and, more importantly, could aid the pathologist to make more reliable and earlier diagnoses of low-grade carcinomas. New developments regarding the detection of the expression of telomerase will be forthcoming, due to the fact that the telomerase gene has recently been identified, and these can only improve the specific application of this molecular marker of malignancy.

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