Human Telomerase Reverse Transcriptase (*hTERT*) Gene Expression in Thyroid Neoplasms[1]

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ABSTRACT

Ten percent of fine-needle aspirations (FNAs) of the thyroid are deemed "indeterminate" or "suspicious" for malignancy by the cytopathologist, but most of these lesions are benign. Therefore, additional markers of malignancy may prove to be a useful adjunct.

The catalytic component of telomerase, human telomerase reverse transcriptase (*hTERT*), has been found to be reactivated in immortalized cell lines. Reverse transcription-PCR of the *hTERT* gene revealed expression in 15 (79%) of 19 malignant thyroid neoplasms, including 6 of 6 follicular carcinomas and 9 of 13 papillary carcinomas. In contrast, *hTERT* gene expression was detected in only 5 (28%) of 18 benign thyroid nodules, including 2 of 7 follicular adenomas and 3 of 11 hyperplastic nodules. All five benign thyroids exhibiting *hTERT* gene expression had lymphocytic thyroiditis. No normal thyroids exhibited *hTERT* gene expression. Telomerase enzyme activity was examined in all 37 nodules and was found to correlate with *hTERT* gene expression in 35 (95%) nodules. The two cases in which telomerase activity and *hTERT* expression results were discrepant were in two papillary carcinomas that were telomerase activity negative and *hTERT* positive. Finally, we have demonstrated that *hTERT* gene expression can be measured in *in vivo* FNA samples. These results suggest that *hTERT* expression may be more accurate than telomerase activity in distinguishing benign from malignant and may be measured in FNA samples from suspicious thyroid lesions.

INTRODUCTION

The majority of thyroid malignancies are PCs[3] and, according to most reports, are not difficult to diagnose on FNA (1). The specificity of FNA diagnosis for these neoplasms, however, is only 56% (2). We recently examined our experience with FNA diagnosis of PCs and reported that of 24 patients who had FNAs that were suspicious for but not diagnostic of PC, 11 (48%) had benign lesions on permanent histology (3). FNA diagnosis is even more problematic in the differential diagnosis of follicular neoplasms, including Hürthle cell neoplasms, because their cytology always falls into the suspicious or indeterminate category. The difficulty in diagnosis of these neoplasms results from the fact that carcinomas differ from adenomas only in that they exhibit either capsular or vascular invasion, a feature impossible to assess on FNA.

Chromosomal ends or telomeres are specialized structures composed of a repeated sequence, TTAGGG (4, 5). Because DNA polymerases cannot replicate these ends completely, normal cells experience a progressive loss of telomeric repeats with each cell division (6). It has been hypothesized that this progressive telomere shortening down to a critical length then leads to cell senescence and death (6). Germline cells and, to some extent, stem cells and lymphocytes overcome this end-replication problem and maintain cellular proliferation by expressing telomerase, which is a holoenzyme comprised of protein and an RNA component. Furthermore, recent work suggests that the maintenance of telomeric ends is a necessary event for the sustained growth of immortalized or transformed cells and that reactivation occurs in almost all malignant neoplasms (7, 8). Relevant to this, we examined telomerase enzyme activity in thyroid neoplasms and found that 67% of PCs and 100% of FCs tested positive for telomerase activity (9, 10).

Three human cDNAs encoding the telomerase protein complex have been identified, cloned, and characterized: (*a*) *hTR*(11); (*b*) the catalytic component, *hTERT* (12, 13); and (*c*) human telomerase-associated protein 1 (14, 15). Although *hTR* has been found to be up-regulated in *in situ* as well as invasive breast carcinomas (16), both *hTR* and human telomerase-associated protein 1 genes are also expressed in most normal cells (13, 17). Initially, the *hTERT* gene was believed to be expressed in immortalized cell lines and human carcinomas, but not in normal cells (12, 13, 17–19). Recent studies, however, suggest that it is also expressed in cells that have long-term proliferative capacity and in premalignant cell types (20, 21). *hTERT* gene expression still holds promise, however, in the diagnosis of

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[3] The abbreviations used are: PC, papillary carcinoma; FNA, fine-needle aspiration; FA, follicular adenoma; FC, follicular carcinoma; hTR, RNA component of human telomerase; hTERT, human telomerase reverse transcriptase; TRAP, telomeric repeat amplification protocol; RT-PCR, reverse transcription-PCR.

carcinoma because the expression of it is much stronger in immortalized cell lines and human carcinomas than in normal or premalignant cells (12, 13, 17–19).

We, therefore, examined 37 thyroid nodules, including 19 malignant and 18 benign neoplasms, and 12 corresponding normal thyroid specimens for *hTERT* gene expression by RT-PCR to determine whether *hTERT* gene expression correlated with telomerase activity and whether it could serve as a useful marker to differentiate benign from malignant thyroid lesions.

MATERIALS AND METHODS

Tissue and Cytological Collection

This project has been approved by the Joint Committee on Clinical Investigation at The Johns Hopkins University School of Medicine. Thirty-seven thyroid nodules from 37 patients and 12 corresponding normal thyroid specimens were studied. Tissue and *ex vivo* FNA samples from the center of the thyroid nodules were obtained. Samples were immediately placed in liquid nitrogen and stored at −80°C with a coded identifier. The tissue blocks were embedded in OCT compound (Sakura Finetechnical Co., Tokyo, Japan). Subsequently, 10 consecutive 5-μm cryostat sections were obtained and immediately frozen in liquid nitrogen before being stored at −80°C. One slide each from both the frozen section and FNA samples were stained with H&E and by Papanicolaou's method, respectively. They were then reviewed by a senior surgical pathologist (W. H. W.) and cytopathologist (D. P. C.).

Ten paired *in vivo* FNA samples from 10 patients were also collected. One slide was used for diagnosis, and the second slide was immediately frozen in liquid nitrogen and used for RNA extraction.

Materials

The cDNA for *hTERT* (pCI*Neo-*hEST2*-HA) was a gift from Dr. Robert A. Weinberg (Whitehead Institute of Biomedical Research, Cambridge, MA; Ref. 12). Human thyroid carcinoma cell lines ARO, WRO, and NPA were obtained from Drs. Guy J. F. Juillard (University of California–Los Angeles, Los Angeles, CA) and James A. Fagin (University of Cincinnati, Cincinnati, OH). Taq polymerase and PCR-related products were purchased from Perkin-Elmer Co. (Norwalk, CT). PCR primers were synthesized by Operon Technologies, Inc. (Alameda, CA). Unless otherwise indicated, other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Telomerase Activity Assay

Protein Extraction. Tissues and cells were suspended in 50 μl of ice-cold telomerase lysis buffer [0.5% (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate, 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.1 mM 4-(2 aminoethyl)-benzenesulfonyl fluoride hydrochloride (Boehringer Mannheim Co., Indianapolis, IN), and 10% glycerol], and protein extracts were prepared as described (9, 10). After 30 min of incubation on ice and subsequent centrifugation (12,000 × *g*, 15 min, 4°C), the supernatant was collected and stored at −80°C. Protein concentrations were determined by BCA protein assay kit (Pierce Chemical Co., Rockford, IL).

TRAP Assay. Aliquots of tissue or cell extract were used for standard TRAP assays (10, 22–24). Briefly, 2 μg of protein were incubated in a final 50-μl buffer containing 20 mM Tris-HCl (pH 8.3), 68 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, 0.05% Tween 20, 50 μM deoxynucleotide triphosphates, 100 ng TS primer (AATCCGTCGAGCAGAGTT), 4 μCi [^{32}P]dCTP (NEN Life Science Products, Boston, MA), and 2 units of Taq polymerase for 30 min at 25°C. After the addition of 100 ng CX primer (CCCTTACCCTTACCCTTACCCTAA), PCR was performed (30 cycles at 94°C for 30", 50°C for 30", and 72°C for 45"). All assays contained 10 ag of the 150-bp ITAS to detect Taq polymerase inhibitors. Extracts from each sample were treated in parallel at 85°C for 10 min., serving as negative control. PCR products were electrophoresed in a 10% nondenaturing polyacrylamide gel and visualized by autoradiography. Detectable telomerase activity was defined as a hexanucleotide ladder of three or more bands not present in the heat-inactivated controls. When the PCR amplification of both ITAS and 6-bp ladder were not detected, samples were subjected to phenol/chloroform/isoamyl-alcohol (25:24:1) extraction and ethanol precipitation before PCR amplification. Finally, the samples demonstrating a negative TRAP assay were mixed with the protein extract from ARO cells, which is TRAP assay positive, to determine whether the negative results were due to contaminating inhibitors.

hTERT Gene Expression

RNA Extraction. Total RNA was extracted by TRIZOL (Life Technologies, Inc., Gaithersburg, MD). Briefly, 200 μl of TRIZOL solution were added to the slides, and the cell lysate was collected in 1.5-ml microcentrifuge tubes. After an additional 200 μl of TRIZOL were added, cell lysate was mixed by pipetting and then incubated for 5 min at room temperature. After the addition of 100 μl of chloroform, tubes were vigorously shaken, incubated for 3 min at room temperature, and centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatant was then transferred into a fresh tube, and 250 μl of isopropyl alcohol were added. After vortexing, tubes were centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatant was aspirated, and the pellets containing RNA were washed with 500 μl of 70% ethanol and dried. The concentration of RNA was measured by optical densitometer at 260 nm.

RT-PCR. Total RNA (200–400 ng) was reverse-transcribed in a 20-μl reaction containing 5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 μM random hexamers, 1 mM deoxynucleotide triphosphate, 1 unit/μl RNase Inhibitor, and 2.5 units/μl reverse transcriptase for 15 min at 42°C. After heat-inactivation of reverse transcriptase at 99°C for 5 min, 10 μl of reverse transcriptase reaction and 40 μl of PCR reaction mixture [final concentration: 2.5 mM MgCl2, 60 mM Tris-HCl (pH 8.5), 15 mM (NH4)2SO4, 1 unit of Taq polymerase, and specific primers] were mixed and PCR-amplified (35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min). PCR products were electrophoresed on a 2% agarose gel in 0.5 × Tris-Borate-EDTA buffer containing 1 μg/ml ethidium bromide. The molecular size marker used was a ϕX174-Hae digest (New England Biolabs, Beverly, MA). Primers for *hTERT* (forward: 5′-AGAGTGTCTGGAGCAAGTTGC-3′, reverse: 5′-CGTAGTCCATGTTCACAATCG-3′) were designed with the

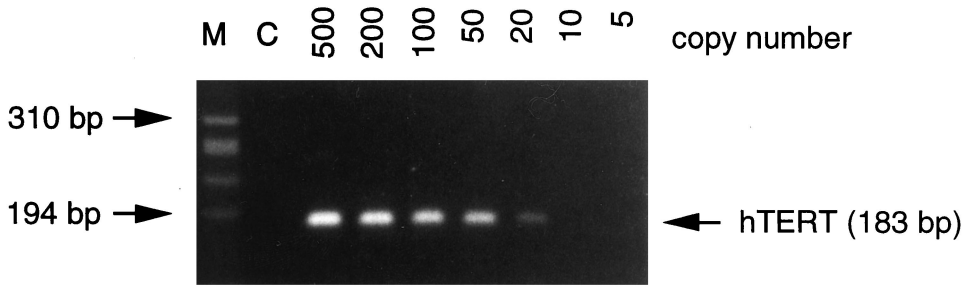

Fig. 1 PCR amplification of *hTERT* cDNA. Serial dilutions of *hTERT* cDNA were used as a template in PCR assay. Copy number of cDNA is indicated on the top, and molecular size marker (*M*) is ΦX174-*Hae*III digest. Control (*C*) contains only buffer. The molecular size of the PCR product was 183 bp.

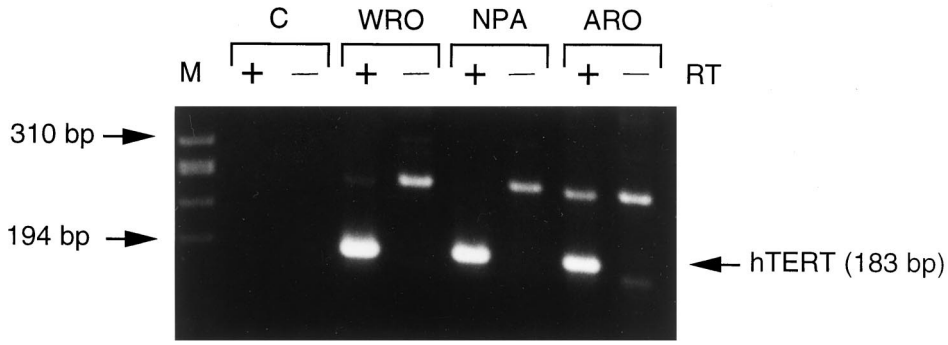

Fig. 2 *hTERT* gene expression in human thyroid carcinoma cell lines WRO, NPA, and ARO. RNA (200 ng) was used. Bands seen at 270 bp were nonspecific PCR products.

computer program, GeneWorks (Oxford Molecular Groups, Campbell, CA) and based on the published cDNA sequence (Gene Bank Accession number AF015950 and AF018167; Refs. 12 and 13). The amplified lesion by PCR products contains one intron (25). Correct bp sequences of PCR-amplified products were confirmed by partial sequencing. β-actin (Clontech, Laboratories, Inc., Palo Alto, CA) served as positive control, and reverse transcriptase-negative tubes served as negative control.

RESULTS

Sensitivity of PCR Detection of *hTERT* Gene. To examine the specificity and sensitivity of detection of the *hTERT* gene by PCR, serial dilutions of the cDNA were PCR-amplified. The size of the PCR product was ~183 bp (Fig. 1), the expected size based on the published sequences (12, 13) and the primers selected. As few as 20 molecules of cDNA amplified by PCR were consistently detectable (Fig. 1).

Telomerase Activity and *hTERT* Gene Expression in Thyroid Carcinoma Cell Lines. We examined telomerase enzyme activity and *hTERT* gene expression in the three human thyroid carcinoma cell lines ARO, WRO, and NPA (26, 27). All three cell lines exhibited telomerase activity (data not shown) and *hTERT* gene expression (Fig. 2). Serial dilutions of protein extracts and RNA from ARO cells were further examined. Telomerase activity was detectable in 20 ng and 100 ng protein from ARO and WRO cells, respectively (data not shown). *hTERT* gene expression was detectable with only 20 pg and 4 pg RNA from ARO and WRO cells, respectively (Fig. 3). Twenty to 100 μg of protein and 10–50 μg of RNA were routinely obtained from 1×10^6 cells. Therefore, the number of cells necessary to yield a detectable telomerase enzyme activity assay and *hTERT* gene expression were ~200–1000 and 1, respectively. These results suggest that the detection of *hTERT* gene expression is more sensitive than that of telomerase activity, although actual gene copy number may affect the sensitivity from clinical specimens.

Finally, *hTERT* RT-PCR products from ARO cells were sequenced directly or after being subcloned into a pCRII vector (Invitrogen, Carlsbad, CA). The cDNA sequence was identical to the previously published *hTERT* sequence (12, 13).

***hTERT* Gene Expression in Thyroid Nodules.** *hTERT* gene expression was detected in 15 (79%) of 19 malignant thyroid nodules, including 6 of 6 FCs and 9 of 13 PCs, and in only 5 of 18 (28%) benign thyroid nodules, including 2 of 7 FAs and 3 of 11 hyperplastic nodules (Fig. 4 and Tables 1 and 2). β-actin gene control was detected in all samples. All five benign thyroid nodules that exhibited *hTERT* gene expression had a background of lymphocytic thyroiditis, suggesting that the known false positive results seen from lymphocytes with telomerase enzyme activity is also present with *hTERT* gene expression (9, 10). Neither thyroid nodules without lymphocytic thyroiditis nor normal thyroid specimens exhibited *hTERT* gene expression.

Overall, the sensitivity and specificity for the detection of malignant neoplasms was 75% and 72%, respectively (Table 3). The accuracy was 74%. After excluding the samples containing lymphocytic thyroiditis, the sensitivity, specificity, and accuracy were 71%, 100%, and 85%, respectively. Most importantly, the positive predictive value was 100% after excluding these samples.

Finally, 10 *in vivo* FNA samples of thyroid nodules were also examined for *hTERT* gene expression. Of these, four PCs

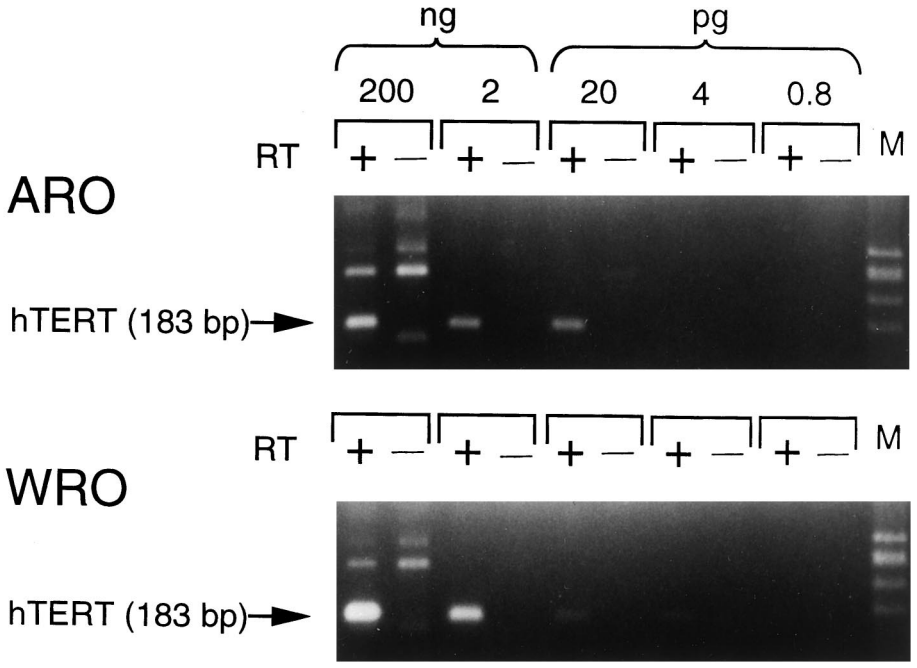

Fig. 3 *hTERT* gene expression in ARO and WRO cells. Serial dilutions of total RNA from both cells were used as a template in PCR assay. The RNA content is indicated on the top, and molecular size marker (*M*) is ΦX174-*Hae*III digest.

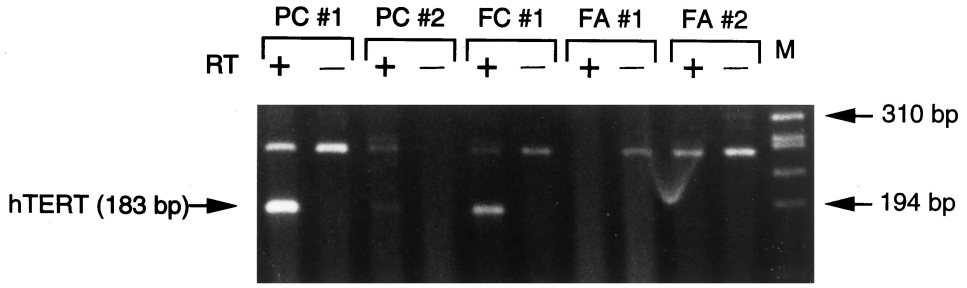

Fig. 4 RT-PCR analysis of *hTERT* gene expression in thyroid neoplasms. *RT*, reverse transcriptase; *M*, molecular size marker is ΦX174-*Hae*III digest. RNA (200 ng) from FNA samples and 20 ng RNA from ARO cells were used.

and one Hashimoto's thyroiditis demonstrated positive *hTERT* gene expression, but five hyperplastic nodules were negative (Fig. 5). In one case (PC #2), four bands instead of one were seen in the *RT(+)* lane.

Correlation between *hTERT* Gene Expression and Telomerase Activity. Telomerase activity was also examined in the 37 nodules (Table 1). There were only two PCs, in which there was a discrepancy between the two assays, namely, that were telomerase activity negative and *hTERT* gene expression positive. The correlation between the two assays was statistically significant ($P < 0.00001$) by Fisher's exact test.

DISCUSSION

Reactivation of the holoenzyme telomerase has been found in a wide variety of carcinomas (7, 8, 28). Telomerase activity has also been detected in carcinoma cells from various cytological samples [lung carcinoma cells in pleural fluid (29) and bronchial washings (30), colorectal carcinoma cells in colonic luminal washings (31), bladder carcinoma cells in urine or bladder washings (31–35), squamous carcinoma cells in oral rinses (36), and breast carcinoma cells in FNAs (37–39)]. Therefore, the detection of telomerase activity holds promise as a diagnostic marker in the detection of thyroid malignancy. Although we previously demonstrated the ability to detect telomerase activity in FNA samples from suspicious thyroid nodules (10), others have reported that the sensitivity from cytological specimens is less than that from tissue samples (34, 36). These false negative results may be due to difficulties in obtaining high-quality specimens because of inadequate cellular material and/or protein concentration. The telomerase enzyme may also be unstable with exposure to temperature fluctuations and easily degraded by proteinases and nucleases.

The recently cloned *hTERT* gene seems to be the most critical gene among telomerase-related genes in the regulation of telomerase activity. Thus, *hTERT* gene expression and telomerase activity in human carcinoma cell lines should correlate (12, 17). Several studies have demonstrated this correlation in a number of human carcinomas, including breast, testis, colon, ovary, pancreas, prostate, and liver (12, 17, 19, 40). We demonstrated correlation in 35 of 37 samples tested and found the

Table 1 *hTERT* gene expression and telomerase activity in thyroid nodules

No.	Final diagnosis	Telomerase activity	*hTERT*	Sample collection[a]
1	Hyperplastic nodule	−	−	Frozen
2	Hyperplastic nodule	−	−	Frozen
3	Hyperplastic nodule	−	−	Frozen
4	Hyperplastic nodule	−	−	Frozen
5	Hyperplastic nodule	−	−	FNA
6	Hyperplastic nodule	−	−	FNA
7	Hyperplastic nodule	−	−	FNA
8	Hyperplastic nodule	−	−	FNA
9	Hyperplastic nodule by Hashimoto's thyroiditis	+	+	Frozen
10	Hyperplastic nodule by Hashimoto's thyroiditis	+	+	FNA
11	Hyperplastic nodule by Hashimoto's thyroiditis	+	+	FNA
12	FA	−	−	Frozen
13	FA	−	−	Frozen
14	FA	−	−	Frozen
15	FA	−	−	Frozen
16	FA	−	−	Frozen
17	FA with lymphocytes	+	+	FNA
18	FA with lymphocytes	+	+	Frozen
19	FC	+	+	Frozen
20	FC	+	+	Frozen
21	FC	+	+	Frozen
22	FC	+	+	Frozen
23	FC	+	+	Frozen
24	FC	+	+	FNA
25	FVPC[b]	−	−	FNA
26	FVPC	−	−	FNA
27	FVPC	−	−	FNA
28[c]	FVPC	−	+	FNA
29	PC	+	+	Frozen
30	PC	−	−	FNA
31[c]	PC	−	+	FNA
32	PC	+	+	FNA
33	PC	+	+	FNA
34	PC	+	+	FNA
35	PC	+	+	FNA
36	PC + Hashimoto's thyroiditis	+	+	Frozen
37	PC + Hashimoto's thyroiditis	+	+	FNA

[a] All FNAs were *ex vivo;* frozen represents frozen section.
[b] FVPC, follicular variant of PC.
[c] Cases in which *hTERT* gene expression and telomerase activity results are discrepant.

Table 2 Summary of *hTERT* gene expression in thyroid nodules

Diagnosis	All cases[a]	Excluding cases with lymphocyte infiltration[a]
Hyperplastic nodule	3/11 (27%)	0/8 (0%)
FA	2/7 (29%)	0/5 (0%)
FC	6/6 (100%)	6/6 (100%)
PC	9/13 (69%)	7/11 (63%)

[a] The results are summarized by final histopathological diagnosis and presented by (number of cases with *hTERT* gene expression)/(number of cases examined).

sensitivity of *hTERT* gene detection to be higher than that of telomerase activity in that 200-1000 times fewer cells were needed to obtain a positive result. Importantly, the number of false positives did not increase with the use of the *hTERT* RT-PCR assay, although recent work has demonstrated that some normal cells exhibit *hTERT* gene expression (20, 21).

Not all malignant thyroid neoplasms, however, demonstrate telomerase activity (9, 41–44). We have shown that all FCs (10), but only two-thirds of PCs demonstrate telomerase

Table 3 Acuracy, sensitivity, specificity, and positive and negative predictive values using *hTERT* gene expression in the detection of thyroid malignancy

All calculations were done as follows: (*a*) accuracy = 100 × [(number of benign without *hTERT* gene expression) + (number of malignant with *hTERT* gene expression)]/(total number of cases examined); (*b*) sensitivity = 100 × (number of malignant with *hTERT* gene expression)/(number of malignancy); (*c*) specificity = 100 × (number of benign without *hTERT* gene expression)/(number of benign); (*d*) positive predictive value = 100 × (number of malignant with *hTERT* gene expression)/(number of both benign and malignant with *hTERT* gene expression); and (*e*) negative predictive value = 100 × (number of benign without *hTERT* gene expression)/(number of both benign and malignant with *hTERT* gene expression).

	All cases	Excluding cases with lymphocyte infiltration
Accuracy	74%	85%
Sensitivity	75%	71%
Specificity	72%	100%
Negative predictive value	77%	77%
Positive predictive value	71%	100%

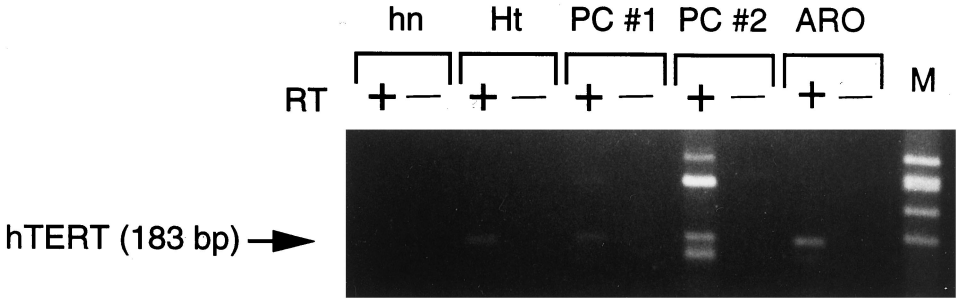

Fig. 5 RT-PCR analysis of *hTERT* gene expression in thyroid FNA. *hn*, hyperplastic nodule; *Ht*, Hashimoto's thyroiditis; *NG*, nodular goiter; *RT*, reverse transcriptase; *M*, molecular size marker is ΦX174-*Hae*III digest. Final diagnosis was confirmed by histological examination.

activity (9) and *hTERT* gene expression. One reason could be that protein extracts may contain PCR inhibitors of the telomerase assay. We, however, demonstrated that telomerase-negative samples remained negative even after samples were extracted by phenol/chloroform to eliminate these inhibitors (10). The possibility that samples may contain reverse transcriptase inhibitors is also unlikely because the mixture experiments using protein extract from TRAP-negative samples and ARO cells demonstrated no decrease in telomerase activities of the ARO cells. The results may be due to the fact that these neoplasms have another mechanism in place for telomere maintenance, as evidenced by elongated telomeres in the absence of telomerase activity (45). Another explanation is that some malignant neoplasms may not require the reactivation of telomerase even when they are clinically detectable (46, 47).

An additional complicating factor includes the fact that benign thyroid nodules that have concomitant lymphocyte infiltration are also telomerase activity positive (9, 10, 41, 44). To overcome this problem, thyroid FNA samples containing lymphocytes may require *in situ* techniques to detect telomerase activity or *hTERT* gene expression. Along these lines, Yahata *et al.* (30) and Ohyashiki *et al.* (48) have already demonstrated an *in situ* TRAP assay on cytological specimens and *in situ* hybridization for *hTERT* mRNA (21).

Two cases in our study exhibited a discrepancy between the telomerase activity assay results and *hTERT* gene expression. Additional studies using a large number of thyroid neoplasms are needed to better understand the exact mechanisms for this phenomenon. Cases exhibiting *hTERT* gene expression and no telomerase activity could be explained by the sensitivity of these two assays. Another possibility includes the fact that *hTERT* gene expression may represent an earlier event than the reactivation of telomerase enzyme in tumorigenesis. Finally, one *in vivo* FNA sample (PC #2) demonstrated additional RT-PCR products. Because these extra products were not seen in other specimens, we assume that they are nonspecific products, but cannot exclude the possibility that they represent alternative splicing products (20, 25).

Measurement of *hTERT* gene expression has the potential to discriminate benign from malignant thyroid nodules and be detected in FNA material. Although not yet a perfect marker of malignancy, its detection holds promise as an important adjunct in the differential diagnosis of suspicious thyroid lesions and, therefore, may improve the selection criteria for patients who require surgical treatment.

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