Comparison of Telomerase and CD44 Expression as Diagnostic Tumor Markers in Lesions of the Thyroid

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ABSTRACT

The analysis of the tissue expression patterns of both the telomerase enzyme and the adhesion molecule CD44 has highlighted these molecules as potential tumor markers. In this study, the expression of these markers was analyzed in frozen tissue samples of the same human thyroid lesions, and the data were compared to evaluate their application to tumor diagnosis. The study analyzed 12 malignant specimens, including 5 papillary, 3 follicular, 2 anaplastic, 1 medullary, and 1 low-grade Hurthle cell carcinoma and 17 specimens from benign lesions, including cases of adenoma, hyperplasia, and Graves’ disease. Telomerase expression was analyzed by assay of enzyme activity using the telomeric repeat amplification protocol and by reverse transcription-PCR detection of human telomerase reverse transcriptase (hTERT) mRNA. Nine of 12 (75%) malignant samples and the two Graves’ disease samples were evaluated as positive for telomerase activity by the telomeric repeat amplification protocol assay. The presence of hTERT mRNA was detected in 8 (67%) of 12 malignant tissues and in 5 (29%) of 17 benign thyroid tissue samples. The expression of CD44 transcripts containing variant exons 7, 8, and 11 was evaluated by reverse transcription-PCR/Southern blot analysis. Of the 12 malignant samples, 9 (75%) included transcripts containing exon 7, 10 (83%) included transcripts containing exon 11, and 11 (92%) included transcripts containing exon 8. However, these CD44 exons were also present in transcripts in a high proportion of benign samples. Five (28%), 10 (59%), and 6 (35%) benign samples contained CD44 transcripts, including variant exons 7, 8, and 11, respectively. The measurement of telomerase activity proved to be the most specific for the detection of thyroid carcinoma in frozen tissue samples as a single analyte, but diagnostic accuracy was increased by the combination of telomerase and CD44 analyses.

INTRODUCTION

A close association between the activation of the telomerase enzyme and cellular immortality has been established. Telomeres are the noncoding termini of eukaryotic chromosomes and function to stabilize and maintain chromosomal structure. However, telomeric DNA is lost at each cell division as a result of the inability of DNA polymerases to replicate the 5’ end of linear DNA (1, 2), and erosion of these sequences beyond a critical point is thought to signal cell cycle arrest and entry into cellular senescence (3). The presence of functional telomerase may be necessary for cells to be capable of extended proliferation or to become immortal, and in concordance with this hypothesis, telomerase activity has been detected in the great majority of malignant tumor specimens tested (4, 5). The enzyme is undetectable in normal somatic cells; therefore, the detection of telomerase activity in human tissue samples could potentially have value for the recognition of malignant cells in clinical specimens (6–9). The development of the highly sensitive TRAP2 assay (10, 11) led to the routine detection of telomerase activity in minimal amounts of fresh and frozen tissue samples. However, the components of the telomerase holoenzyme complex have been characterized only recently, and the correlation of the presence of these subunits and telomerase activity is presently being investigated in clinical samples (12, 13). The cDNA of the catalytic reverse-transcriptase component has been described (14), and the regulation of the expression of this essential telomerase component and its role in neoplasia is of particular interest.

Another molecule whose expression patterns can be indicative of neoplasia is the cell surface glycoprotein CD44. The human CD44 gene contains at least 20 exons (15), 10 of which (exons 1–5 and 16–20) are spliced together to be translated as the standard (CD44s) isoform (Fig. 1). The remaining exons can be alternatively spliced and assembled between exons 5 and 16 to generate a number of variant protein isoforms (CD44v). These can be further modified by posttranslational glycosylation to produce a large family of transmembrane glycoproteins with a diverse array of functions (16). The standard isoform (CD44s) is ubiquitously expressed, whereas the distribution of the more complex variant isoforms occurs in a tissue-specific manner. Numerous studies have provided evidence that the expression of the CD44 gene is extensively altered in many types of tumors, including those of the colon (17), breast (18), and bladder (19). Both increased overall levels of CD44 transcripts and the ab-

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2 The abbreviations used are: TRAP, telomeric repeat amplification protocol; ITAS, internal telomerase assay standard; RT-PCR, reverse transcription-PCR; hTERT, human telomerase reverse transcriptase.
The availability of reliable molecular markers to aid diagnosis and for monitoring patients during treatment would be very valuable. Both CD44 and telomerase have been reported to be altered during the malignant transformation of a wide range of tissues, including thyroid, and are therefore good candidates for use in combinatorial or multiplex assays. The present study used the TRAP assay and RT-PCR to assess the expression of telomerase and RT-PCR and isoform-specific Southern blot hybridization to analyze CD44 expression in the same tissue samples to investigate whether the two biomarkers are jointly elevated in thyroid neoplasia. Each analyte, singly and in combination, was evaluated for accuracy in identifying normal assembly of CD44 variable exons have been linked to tumor growth and to metastatic potential (20, 21). These changes appear to be present in the early stages of neoplasia and can be identified in small samples using amplification techniques, implicating CD44 as a useful marker for early diagnosis and for monitoring patients during treatment.

Patients with enlargement of the thyroid are seen frequently in outpatient clinics, and although a number of approaches are used to investigate the condition, ambiguity in diagnosis of benign conditions can lead to unnecessary surgery. The availability of reliable molecular markers to aid diagnosis would be very valuable. Both CD44 and telomerase have been reported to be altered during the malignant transformation of a wide range of tissues, including thyroid, and are therefore good candidates for use in combinatorial or multiplex assays. The present study used the TRAP assay and RT-PCR to assess the expression of telomerase and RT-PCR and isoform-specific Southern blot hybridization to analyze CD44 expression in the same tissue samples to investigate whether the two biomarkers are jointly elevated in thyroid neoplasia. Each analyte, singly and in combination, was evaluated for accuracy in identifying histopathologically diagnosed benign and malignant lesions.

**MATERIALS AND METHODS**

**Tissue Procurement.** Fresh thyroid tissue samples were collected from surgical specimens resected in the John Radcliffe Hospital, Oxford, United Kingdom. Samples were obtained in random order from the residue of tissue used for pathological diagnosis that would otherwise have been discarded. Tissues were snap-frozen immediately after resection and stored in liquid nitrogen until analysis. Histological diagnosis of all samples was made using the consensus opinion of three independent pathologists of H&E-stained cryostat sections. The 30 samples comprised 12 malignant and 17 benign thyroid lesions. The benign specimens included 2 Grave’s disease, 11 adenomas, and 2 hyperplasias. The 12 malignant thyroid samples included 5 papillary carcinomas, 3 follicular carcinomas, 1 medullary carcinoma, 2 anaplastic carcinomas, and 1 low-grade Hurthle cell carcinoma.

**Protein Extraction.** Twenty-μm cryostat sections were mixed with 50 μl of ice-cold lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate, and 10% glycerol] and incubated for 30 min on ice. The lysate was centrifuged at 10,000 × g for 20 min at 4°C, and the supernatant was immediately frozen at −70°C. The protein content in each lysate was measured and adjusted to 3 μg/μl.

**TRAP Assay.** Telomerase activity was assayed by the TRAP method described by Kim et al. (10). 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 deoxynucleotide triphosphates including [³²P]dCTP (Amersham, Arlington Heights, IL), 0.1 μg of TS oligonucleotide (5'–ATATCCGTCCAGGACAGTT–3'), 1 μg of T4 gene-32 protein (Boehringer Mannheim, Indianapolis, IN), 0.1 μg/ml BSA (Sigma Chemical Co., St. Louis, MO), 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'-GATAGGCTCCGCCTAA-3'] and 10 attograms of an ITAS (11) were added, and the mixture was subjected to 31 cycles using the following conditions: 94°C for 45 s, 50°C for 45 s, and 72°C for 90 s. Sixteen μl of each PCR reaction were analyzed by electrophoresis on 12% polyacrylamide nondenaturing gels. The gel was dried and processed for autoradiographic exposure. To test the specificity of telomerase-positive assays, 1 μg of DNase-free RNase (Promega Corp., Madison, WI) was added to 5 μl of each sample, and after incubation for 20 min at 37°C, 2 μl (6 μg protein) of the treated sample were applied to the TRAP assay. The inclusion of an ITAS, a synthetic DNA construct added to all samples and which is amplified with the TS and CX primers to generate a 150-bp product, aids the detection of false-negatives, which can result from the presence of PCR inhibitors in the extracts (11). Criteria for a positive TRAP assay was deemed negative when fewer than three bands were observed in the presence of copious telomerase-extended template. An assay was deemed negative when fewer than three bands were observed in the presence of an ITAS product.

**RNA Extraction and cDNA Synthesis.** One hundred 20-μm cryostat sections from each frozen sample were used for mRNA extraction using the Mini Message Maker kit (R&D Systems, Minneapolis, MN). cDNA was synthesized from 50 to 100 ng of poly(A)+ selected mRNA in a 20μl volume at 42°C with AMV-reverse transcriptase using random primers (Invitrogen, San Diego, CA). The presence of intact input RNA and successful cDNA synthesis was checked in all samples by amplification of human β-actin cDNA.

**CD44 RT-PCR and Southern Hybridization.** cDNA (2 μl) was amplified with 2.5 units Taq DNA polymerase (Boehringer Mannheim) in the presence of 1 μM of a CD44 standard exon primer pair P1, P4 (Fig. 1). The PCR conditions were as follows:

**CD44 Standard Form (Exons 1 to 5 and 16 to 20)**

![Diagram of the CD44 gene structure](image)

![Diagram of the CD44 gene structure](image)

![Diagram of the CD44 gene structure](image)

![Diagram of the CD44 gene structure](image)

![Diagram of the CD44 gene structure](image)

![Diagram of the CD44 gene structure](image)

**VARIANT EXONS (Exons 7 to 15/2-10)**

(by inserted into standard form in various combinations)

**Fig. 1** Schematic diagram of the CD44 gene structure. Arrowheads, annealing positions for primers used in RT-PCR analysis. UTR, untranslated region.
follows: 94°C for 5 min and 85°C for 1 min, during which time Taq polymerase was added (Hot start), followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The presence of CD44 transcripts in all samples was confirmed by reference to amplification of the standard form of CD44 mRNA, which is ubiquitously expressed (Fig. 4). CD44-specific PCR primer sequences (see Fig. 1) were: P1, 5'-GACACATATT-GCTTCAATGCTTCAGC-3'; and P4, 5'-GATGCGCAAGAT-GATCGAGCATTCTGGGA-3'. One-fifth of the 50 μl of P1/P4 PCR reaction mixture was electrophoresed in a 1.2% agarose gel and transferred to a Hybond N+ nylon membrane (Amersham) with 0.4 N NaOH solution overnight. The resulting Southern blot was hybridized with exon-specific probes made by PCR using the primer combinations listed below and a CD44 genomic clone (c2311) template. The DNA probes were labeled with peroxidase, using enhanced chemiluminescence (ECL) direct nucleic acid labeling and were visualized posthybridization with the ECL detection system (Amersham). The conditions used for hybridization, washing, and detection were those recommended by the manufacturer. CD44 exon-specific primer sequences were (see Fig. 1): for standard exon probe, P2 (5'-CCTGAA-GAAATGTGATACGTCAAGCAG-3') and A15 (5'-ACTGGAGTTGAATCTTGCTTG-3'); for exon 7 probe, E1 (5'-TTGATGAGCCTAGTGTGACTACAGCA-3') and E2 (5'-CATTGTTGTCGTGTTCTGGAAGAGT-3'); for exon 8 probe, D1 (5'-TACGCTTCAAATACCACATCTGAGC-3') and AD1 (5'-GGTGCTGAGGATATAAATCTCTC-3'); and for exon 11 probe, EX11 (5'-TCCAGGGCAATCTCTA-3') and AE11 (5'-CAGCTGTCCTGTGTTG-3').

**RT-PCR of hTERT cDNA.** A nested PCR approach used primers designed to amplify sequences in the specific "T-motif" of hTERT cDNA (12). cDNA was first amplified with 2.5 units of Taq DNA polymerase (Boehringer Mannheim) using the primer pair 5'-TGGGTGATAGTGATGTGTAGCTGTTCT (T1) and 5'-GGATGAAAGGCCGAGGAGCAGCA-3') and E2 (5'-CATTGTTGTCGTGTTCTGGAAGAGT-3'); for exon 8 probe, D1 (5'-TACGCTTCAAATACCACATCTGAGC-3') and AD1 (5'-GGTGCTGAGGATATAAATCTCTC-3'); and for exon 11 probe, EX11 (5'-TCCAGGGCAATCTCTA-3') and AE11 (5'-CAGCTGTCCTGTGTTG-3').

**RESULTS**

**Telomerase in Thyroid Tissue Samples**

**TRAP Assay.** A representative TRAP assay is depicted in Fig. 2. The presence of active telomerase in a sample is revealed by a characteristic ladder of products, corresponding to the 6-bp TTAGGG repeats comprising telomeric DNA. All samples were evaluated using 6 and 0.6 μg of total protein to confirm positivity and to dilute potential inhibitors of polymerases. RNase treatment of extracts prior to the reaction always negated positive assays, confirming ribonucleoprotein activity. Nine of 12 (75%) carcinoma samples contained telomerase activity when evaluated by the TRAP assay. The telomerase-positive samples consisted of five papillary, three follicular, and one anaplastic carcinoma. However, one case of each of a medullary, an anaplastic, and a low-grade Hurthle cell carcinoma samples revealed no telomerase activity (Table 1). In contrast, only 2 (12%) of the 17 benign samples were positive for telomerase activity. These telomerase-positive, nonmalignant samples were both from Grave’s disease lesions, and on microscopic examination, severe lymphocytic infiltration was observed in both specimens. The presence of lymphocytes may account for false-positive signals in the absence of tumor cells in TRAP assay analysis, as reported previously (22). The diagnostic sensitivity of the TRAP assay on samples from thyroid carcinomas was 75%, with specificity values of 88% on benign samples (Table 2), as judged by the gold standard of histological diagnosis.

**hTERT RT-PCR.** A nested PCR approach was used to amplify hTERT cDNA to both increase specificity of amplification and to detect the low copy transcripts in minimal thyroid tissue samples. Amplification within the "T-motif" gave a product of 196 bp (Fig. 3), according to the cDNA sequence as reported by Nakamura et al. (12). When present, the expected amplicon was always accompanied by another product ~30 bp...
larger, resulting in a doublet as visualized after electrophoresis. Similar related products have been detected previously in a number of tumor tissues when analyzed using a nested PCR regime and are believed to be the result of alternative splicing (23). Of the 12 malignant samples, 8 (67%) contained hTERT mRNA as evaluated by RT-PCR (Table 1). Analysis of three of the five papillary carcinomas and the one Hurthle cell carcinoma specimen did not reveal the presence of hTERT mRNA. Amplification of 5 (29%) of the 17 nonmalignant samples also gave positive results (5 of the 11 adenoma lesions). However, none of the adenoma samples were positive for activity in the TRAP assay. A sample of lymphocytes (L1, Table 1) was positive for hTERT mRNA as it was for enzyme activity by TRAP (see above). The diagnostic sensitivity of this assay (67%) was inferior to that of the TRAP assay (Table 2). The specificity values were also less favorable (71% overall for benign samples and only 55% for adenoma lesions).

### CD44 Expression

#### CD44 Standard Isoform Expression.
RT-PCR products obtained using P1/P4 primers (see Fig. 1) were size-fractionated by electrophoresis and blotted to a nylon membrane support. Southern blot hybridization analysis was performed using exon-specific probes. The use of a probe specific for exon 5 reveals the presence of all transcripts as this exon is within the “standard” region of the gene and is present in all CD44 mRNA (Fig. 1). Accordingly, as shown in Fig. 4, all samples examined contained a wide range of RT-PCR products when hybridized with an exon 5 probe. The ubiquitous expression of the standard form of CD44 (no variant exons included between exons 5 and 16) acts as an internal control, in that a product of 482 bp must be revealed by such analysis, otherwise the assay is deemed unsuccessful. The visualization of all CD44-derived amplification products is informative. In addition to the prevalent standard product, a second intense band can be seen in both tumor and normal thyroid samples. This is termed the “epithelial” form, or CD44E (also termed CD44R1), and is prevalent in many tissues, sometimes as abundant as the CD44s isoform. This isoform contains variant exons 13 (v8), 14 (v9), and 15 (v10) in addition to the standard exons, and is revealed as an amplification product of 908 bp. Both benign and malignant thyroid tissues clearly contain this isoform in abundance (Fig. 4). The malignant samples also appeared to contain a wider range of transcripts than the benign ones, as evidenced by the presence of higher molecular weight amplification products.

#### Variant Isoform Expression.
The Southern blot analysis using a CD44s probe revealed the presence of multiple CD44 mRNA species within each of the thyroid tissue samples. To define which variant exons were included within those transcripts, Southern hybridization was performed with exon-specific probes. DNA probes to exons 7 (v2), 8 (v3), and 11 (v6) were used because they are the variant exons most reported to be expressed in correlation with neoplasia in many tissue types (17, 24). An exon 7 probe hybridized to 9 (75%) of 12 carcinoma

### Table 1 Presence of telomerase and CD44 analytes in histologically diagnosed thyroid tissue samples

<table>
<thead>
<tr>
<th>Case</th>
<th>Histological diagnosis</th>
<th>Telomerase</th>
<th>CD44</th>
<th>Score</th>
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<tr>
<td></td>
<td></td>
<td>TRAP</td>
<td>hTERT</td>
<td>Exon 7</td>
</tr>
<tr>
<td>B1</td>
<td>Adenoma</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B2</td>
<td>Adenoma</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B3</td>
<td>Adenoma</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B4</td>
<td>Adenoma</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B5</td>
<td>Adenoma</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B6</td>
<td>Adenoma</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B7</td>
<td>Adenoma</td>
<td>-</td>
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<td>+</td>
</tr>
<tr>
<td>B8</td>
<td>Follicular adenoma</td>
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<td>-</td>
</tr>
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<td>B9</td>
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<td>+</td>
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<tr>
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<tr>
<td>B17</td>
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</tr>
<tr>
<td>L1</td>
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</tr>
<tr>
<td>M1</td>
<td>Papillary carcinoma</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M2</td>
<td>Papillary carcinoma</td>
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</tr>
<tr>
<td>M3</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M4</td>
<td>Papillary carcinoma</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M5</td>
<td>Papillary carcinoma</td>
<td>+</td>
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</tr>
<tr>
<td>M6</td>
<td>Follicular carcinoma</td>
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<td>+</td>
</tr>
<tr>
<td>M7</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M8</td>
<td>Follicular carcinoma</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M9</td>
<td>Anaplastic carcinoma</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M10</td>
<td>Anaplastic carcinoma</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M11</td>
<td>Medullary carcinoma</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M12</td>
<td>Hurthle cell carcinoma</td>
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Table 2  Diagnostic validity of telomerase and CD44 assays in benign and malignant thyroid tissue samples

<table>
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<tr>
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<td>hTERT</td>
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</tr>
<tr>
<td>Exon 7</td>
<td></td>
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<td>Exon 11</td>
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<tr>
<td>Histology</td>
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</table>

Combination of the total data obtained revealed that in only four cases were all analytes present, and these four were histologically diagnosed as carcinoma (2 papillary and 2 follicular). Four additional cases of carcinoma were positive for all CD44 exons analyzed; however, this was also observed in two cases of adenoma, one of hyperplasia and the benign thyroid specimen. If the total analyte number is 5 (CD44s must be present in all useful samples), 9 of the 12 malignant samples had a positive score of 4 or higher. Conversely, of the 17 benign lesions, only 1 sample had a positive score of >3.

DISCUSSION

The development of the sensitive TRAP assay (10, 11) has enabled the evaluation of telomerase activity in most types of human cancer. The reported frequencies of positive TRAP assays of solid tumor samples are ~90%, and it is now widely accepted that, except for a few specialized cell types, telomerase activity in cells of somatic origin is indicative of immortal transformation. Clinical samples obtained by noninvasive collection methods have also been analyzed for telomerase expression. Exfoliated cells in the urine (7, 25) or colonic lumen (26) or in oral rinses (27) have been analyzed, and the presence of telomerase activity has enabled the detection of small numbers of exfoliated tumor cells in such samples.

Previous studies that have analyzed telomerase activity in samples taken from the thyroid (28–30) have revealed activity in the majority of thyroid carcinomas but not in most benign lesions or normal thyroid tissue. In line with previous reports on a range of tumors, this study revealed that 75% of samples obtained from histologically confirmed malignant lesions contained active telomerase, whereas only 12% of samples obtained from benign lesions were positive. The diagnostic specificity of histologically diagnosed nonmalignant samples (88%) is also comparable with previous reports (4, 30).

The presence of the hTERT catalytic subunit of the telomerase holoenzyme has been established as essential to telomerase activity (31, 32), and the expression of hTERT mRNA has been correlated with the telomerase activity status of immortal cell lines (23, 32) and in the majority of human samples examined (33, 34). However, in some analyses of complex tissue samples, this correlation is not so apparent (14, 35, 36), and in this study, the TRAP assay was both more sensitive and more specific than hTERT RT-PCR for the detection of tumor cells in thyroid lesions. The use of hTERT RT-PCR for accurate analysis may be premature because of the incomplete understanding of the gene structure and the patterns of hTERT mRNA splicing that may be important in the expression of the functional enzyme. Kilian et al. (23) described multiple forms of hTERT...
mRNA, as revealed by RT-PCR analysis, presumably produced by alternative splicing mechanisms (23); until this date, only a single copy of the hTERT gene has been identified in the human genome. In this study, the use of a nested PCR regime resulted in the amplification of the expected product plus a second, equally intense, band ~20–30 bp larger, as observed in the previous report (23). The use of primers annealing to the human T-motif is specific for hTERT amplification, but ensuing knowledge of the exon/intron arrangement of the gene may lead to better primer design for PCR-based analysis.

The presence of inhibitors of either the telomerase enzyme or the polymerase used in PCR has been reported to be a potential problem in the analysis of a total protein cell extract (6, 11). However, the inclusion of an internal TRAP assay standard (11) and the susceptibility of the enzyme to RNase degradation of its RNA moiety have helped to validate telomerase activity assay data. RT-PCR analysis also has its difficulties. Handling and homogenization of solid tissue specimens may lead to partial degradation of RNA before stabilization in chaotropic lysis buffer is complete, and the low abundance of hTERT transcripts makes any loss a concern. Although we used the same cDNA for CD44 and hTERT PCR, there is no internal reference for hTERT to check the PCR reaction directly. When amplifying transcripts from the ubiquitously expressed CD44 gene, it is clear that if transcripts encoding the standard isoform are not amplified, then the RT-PCR analysis is flawed. Ironically, although the limited cellular expression of the telomerase catalytic subunit should increase the specificity of detection using this analyte, the lack of an internal control for PCR-based hTERT analysis results in difficulty in the identification of false-negative data. The generation of false-positive data is most likely a result of the heterogeneous nature of solid tissue specimens. Telomerase is known to be active in stem cell lineages and in lymphocytes (22, 37). Indeed, the problem of lymphocytic infiltration into normal or benign tissue is a major problem for molecular detection methods, including both telomerase and cytokeratin infiltration into normal or benign tissue is a major problem for molecular detection methods, including both telomerase and CD44 analyses, especially with the sensitivity conferred upon the assays by PCR amplification.

Severe abnormalities have been observed in CD44 patterns of expression in many types of common human tumors by both protein and RNA analyses. These are manifested by markedly increased levels of unusual CD44 transcripts and proteins in many tumors compared with corresponding normal tissues. RT-PCR analysis consistently reveals an extensive array of amplimers in tumor samples, but it is clear that the expression of the variant exons is particularly affected. However, the correlation with altered CD44 expression and tumor progression is not always proven, because some studies have reported no evidence for altered CD44 expression in tumor cells (38, 39) over normal counterparts or have described a loss of CD44 in invasive tumors (19, 38). Such differences can result from the type of samples analyzed, because expression patterns can be tissue specific, but it has also been shown that CD44 expression is plastic, even in transformed cells, and can change dramatically during tumor progression and spread (19, 40). Alternatively, differences in results may stem from the sensitivity of the techniques used, for example the amplification of RNA by PCR as compared with nonamplified, immunohistochemical methods. Overall, although abnormal CD44 expression has been observed in tumors of many organs, careful analysis has not revealed any tumor-specific combination of variant exons or isoforms.

Normal thyroid tissue has been reported to express predominantly CD44s, with only trace amounts of restricted variant isoforms present (41–43). This study confirmed that the overall expression of CD44v transcripts is markedly increased in malignant tissues, as evidenced by the presence of the three variants tested (v2, v3, and v6) in all but 3 of the 12 malignant samples. Conversely, only 4 of the 17 benign samples contained transcripts containing all these variant exons. Ermak et al. (41) have reported that papillary thyroid carcinoma exhibited increases in variant 6 expression, and this was also observed in this study; however, v6 was expressed not only in the papillary carcinomas but in all types of thyroid carcinoma investigated. No reports have previously analyzed the expression of v2 transcripts in thyroid lesions, and it was observed that the presence of this exon was equally indicative of neoplasia, as was v6 expression. The summation of previous reports of CD44 analysis in a wide range of tumor tissues suggests that there is no

Fig. 4 RT-PCR/Southern blot hybridization analysis of CD44 expression in thyroid frozen tissue. cDNA was amplified across the variant region between standard exons 3 to 17. PCR products were electrophoresed and blotted to nylon filters for hybridization. Replicate blots were hybridized with exon 7 (v2), exon 8 (v3), exon 11 (v6), and standard (exon 5) probes, respectively. Lanes 1-5 contain samples from specimens diagnosed as benign. Lanes 6–9 contain samples from malignant lesions. Arbitrary case numbers are indicated above the lanes. Arrows, molecular weight markers are indicated in bp.
tumor-specific isoform that can be used alone as a diagnostically reliable marker. Rather, it is the presence of numerous variant isoforms and of misprocessed transcripts, not normally in the corresponding healthy tissue, that is more indicative of neoplasia (20, 44). This characteristic “chaos” of CD44v transcription is visually obvious on a hybridized blot, but it is not easy to infer from simplified, more routine analyses.

In this study, the detection of telomerase activity proved to be the best single diagnostic marker for malignancy in thyroid lesions, and although not 100% specific for detection of malignancy, the development of this test could lead to a reduction in unnecessary surgery by aiding the pathologist in evaluating borderline cases. However, increased diagnostic accuracy was achieved by combination of the analytes used in this study. The use of multiplex molecular assays, which simultaneously analyze a panel of markers in biopsy material, is more likely to approach reliable and acceptable values of sensitivity and specificity for routine clinical use.

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REFERENCES


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