Telomerase activity in fine needle aspiration biopsy samples: Application to diagnosis of human thyroid carcinoma

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Abstract

Background: The diagnosis of thyroid follicular carcinoma by fine needle aspiration biopsy is a well known problem in thyroid pathology.

Methods: We evaluated telomerase activity (TA) in 85 fine needle aspiration biopsy (FNAB) samples from patients with thyroid nodules. Surgery samples from patients with tumor or follicular adenomas were also analyzed.

Results: Twenty of the FNAB samples corresponded to carcinomas and were positive to telomerase assay (TA > 10 Units). Among them, 4 follicular carcinomas and 1 papillary carcinoma were labeled as indeterminate by FNAB cytological examination. Four percent false positive cases and no false negative cases for TA in FNABs were reported. FNAB samples from follicular adenomas were diagnosed as indeterminate by cytological examination, but they showed no detectable TA. Tumor tissues from patients with follicular or papillary thyroid carcinomas presented TA > 10 Units, whereas follicular adenoma tissues (benign nodules) showed no TA.

Conclusion: Our results showed a good correlation between TA in FNAB samples and tumor/nodule thyroid tissue. This suggested that use of TA as a biological marker of malignancy might be a useful tool in the diagnosis of follicular thyroid carcinomas or follicular thyroid adenomas using FNAB samples.

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1. Introduction

Thyroid nodules affect almost 10% of the population [1]. Differentiated thyroid cancer is generally non-aggressive and grows slowly, but it accounts for about 1% of all cancer deaths [2]. The introduction of fine needle aspiration biopsy (FNAB) > 50 years ago has eclipsed all other techniques applied to the diagnosis of thyroid cancer due to its high degree of sensitivity (65–98%) and specificity (> 70%) [3,4]. However, 3 to 6 aspirations are necessary to obtain an accurate diagnosis [5]; cells are classified by their cytological characteristics into benign, indeterminate, and malignant. The accuracy of this classification depends on obtaining enough cytological specimens, but also on the physician’s experience; up to 30% FNABs are labeled as indeterminate [6]. According to our experience, the diagnosis of follicular neoplasm is a problem and, like other researchers, we believe that only in some cases FNAB can be used as a diagnostic tool in this type of cancer [7]. Moreover, some authors suggest that additional diagnostic markers of malignancy are needed [8].

P53 and thyroglobulin proteins could be considered good markers in thyroid tumors. Genetic alterations involving the suppressor gene p53 in thyroid tumors have been associated
with malignancy [9,10]; immunohistochemical p53 expression is more frequent in undifferentiated carcinomas than in well-differentiated carcinomas and benign thyroid tissues, in which this protein is usually undetected [11]. The expression of thyroglobulin (Tg), a specific protein synthesized by the thyroid gland, is a good marker of thyroid cell differentiation [12].

Previous reports on telomerase activity (TA) in benign and malignant thyroid tissues and FNAB samples suggested that its deregulation could be involved in tumor progression [13–16]. Telomerase determination by telomeric repeat amplification protocol (TRAP) assay has been extensively studied in many solid tumors such as breast, liver, bladder and lung carcinomas, showing that most malignant tumors have higher enzymatic activity than normal cells [17–20]. Furthermore, when applied to FNAB from malignant breast and prostate samples, positive TRAP values reached 80% [21,22]. In the case of thyroid carcinoma tissues, results on telomerase activity varied from 20% to 70% in papillary thyroid carcinoma and from 0% to 100% in follicular thyroid carcinoma, probably due to sample heterogeneity [6,23]. Thus, the use of telomerase as a marker is controversial, and some authors consider that the incidence of the enzyme in malignant specimens is low [24].

Studies carried out in our endocrinology unit were focused on thyroid tumors. We determined p53 protein and Tg mRNA levels in thyroid samples from patients with thyroid nodules. In order to identify biological markers of malignancy, we evaluated telomerase activity levels in FNABs and their corresponding thyroid tissues.

2. Materials and methods

2.1. Tissues

Fine needle aspiration biopsy samples obtained from 85 patients were processed for telomerase assay and cytology. Only 26 of these patients were operated on; 20 presented primary thyroid tumors, and 6 showed follicular adenomas. Tissue samples from the 20 patients with thyroid tumors included 4 follicular carcinomas and 16 papillary carcinomas (well differentiated as revealed by the histological examination).

Samples from 3 patients with undifferentiated tumors were also studied. Echography showed that both differentiated and undifferentiated tumors were solid nodules between 1.5 and 6.0 cm diameter, and scintigraphy determined that they were cold. Patients were clinically and biochemically (T3, T4, free T4, TSH) euthyroid. Samples from the 6 patients with follicular adenomas were also studied.

Surgical material for telomerase assays and RNA studies was stored at −70 °C until further processing. Tumor tissues were dissected by our own pathologist to avoid contamination with necrotic and connective tissues. In the case of p53 immunohistochemistry, neutral buffered formalin fixed tissue was used. The Ethical Committee of the Hospital Israelita approved these studies.

2.2. Telomerase assays

Cells were processed according to the protocol described in the TELOTAGGG Telomerase PCR ELISA Roche SAIC kit [25]. They were added 100 μl pre-cooled lysis reagent, incubated in ice for 30 min, and centrifuged at 15,000 × g for 20 min at 4 °C. Supernatant was collected, protein concentrations were determined, and cell extracts were used in the telomerase assay following the Telomerase PCR ELISA procedure. Telomeric repeats were added to a biotin-labeled primer by telomerase contained in the sample, followed by the amplification of the elongation product by PCR. Aliquots of the product were denatured, bound to a Strept/Avidin coated 96-well plate, and hybridized to a DIG-labeled telomeric repeat-specific probe. An antibody to DIG, conjugated to peroxidase, was subsequently bound to DIG and visualized by virtue of the enzyme’s ability to metabolize tetramethyl benzidine (TMB), generating a colored reaction product. Sample absorbance was measured at 450 nm using an ELISA micro-titer plate reader within 30 min after the addition of the stop reagent. An internal standard was used to calculate relative TA (expressed in Units).

2.3. Thyroglobulin RNA studies

Thyroglobulin mRNA steady state levels were determined by dot blot hybridization. RNA purification and dot blot hybridization were performed according to procedures previously described [26]. The human probe used in this Tg study has been previously described [27]. Filters were hybridized with human Tg cDNA probe and rehybridized with an 18s human ribosomal probe in order to control RNA amount in nitrocellulose membranes. Probes were labeled with (32P) CTP by a random primer. After hybridization, filters were washed and autoradiographed, and spots were quantified by densitometric scanning using a Shimadzu Scanner. Results are expressed in arbitrary units obtained from the densitometric tracings and corrected according to 18s ribosomal RNA concentration.

2.4. P53 immunohistochemistry

Sections were prepared and mounted onto silane coated slides. Slides were then boiled in citrate buffer in a microwave oven for 10 min, and p53 protein was evaluated using monoclonal antibody to p53 protein at 1:100 dilution (clone DO7 — Novocastra, Newcastle, England) according to a technique already described [28]. Immunohistochemical p53 staining was examined and labeled as negative or positive; positive cell percentage was estimated counting 300 tumor cells in 40 high power fields. Negative controls were adjacent normal thyroid tissues seen on hematoxylin stained samples.

2.5. Statistics

Statistical analysis was carried out including all the processed samples in each study by a computer program using one way analysis of variance [29].

3. Results

Only 20 FNAB samples exhibited TA with values ranging between 10.6 and 133.4 Units (Table 1). From these, cytological
analysis showed 15 papillary carcinomas and 5 indeterminate cases. These patients were operated on and their diagnoses were confirmed by post-surgery histological examination. Samples considered indeterminate were 4 follicular carcinomas and a papillary carcinoma (Table 2). Another 3 positive samples with TA lower than 10 Units (3.4, 5.2 and 9.1 Units) were cytologically diagnosed as colloid goiters and these patients were not operated on.

From the 85 samples analyzed, 62 had no detectable TA (<0.1 Units). Cytological diagnosis included: 53 colloid goiters, 2 Hashimoto’s thyroiditis, 1 Graves’ disease, and 6 indeterminate cases. Patients with indeterminate cytology underwent surgery; they showed follicular adenomas (benign nodules) later on confirmed by histology (Table 2).

Cytological examination (Fig. 1a) showed 69% negative cases (colloid goiter, Hashimoto’s thyroiditis and Graves’ disease); TA (Fig. 1b) was not detected in 72% cases, in agreement with cytological results. On the contrary, 4% samples with TA <10 Units did not correlate with cytology. Cytological diagnosis showed 13% indeterminate cases that corresponded to follicular adenomas or follicular carcinomas, later confirmed by surgery. On the other hand, there were no indeterminate results for TA: carcinoma samples showed values ≥10 Units, whereas benign nodules had no detectable TA.

Surgery samples from 26 patients (16 papillary carcinomas, 4 follicular carcinomas, and 6 follicular adenomas) were examined and assayed for TA, p53 protein, and Tg mRNA. Telomerase activity was significantly different in carcinomas and adenomas (papillary and follicular carcinomas: 55.3 ± 12.0 Units, follicular adenomas: no detectable activity, p < 0.01). Statistical analysis [31] was carried out with results obtained from the densitometric tracings of dot blot Tg mRNA assays (papillary and follicular carcinomas: 0.80 ± 0.21 Units, follicular adenomas: 2.53 ± 0.27 Units, p < 0.01). Differentiated papillary and follicular carcinomas as well as follicular adenomas were p53 negative. Three undifferentiated carcinomas were also studied; results showed that they were positive for p53 protein (Table 3), but negative for Tg mRNA. P53 staining was confined to tumor cell nuclei in thyroid carcinomas, whereas the adjacent normal thyroid tissue was negative. It is worth noting that patients with differentiated carcinomas (follicular and papillary) showed up to 5 y lifespan without disease.

Table 2
Final histopathological and preoperative FNAB cytological diagnosis from 85 thyroid nodule/tumor samples obtained for telomerase activity analysis

<table>
<thead>
<tr>
<th>FNAB cytology</th>
<th>Telomerase activity</th>
<th>Surgery/final histopathology</th>
</tr>
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<tbody>
<tr>
<td>Colloid goiter (total: 53)</td>
<td>No detectable*</td>
<td>Not operated</td>
</tr>
<tr>
<td>Colloid goiter (total: 3)</td>
<td>3–10 Units</td>
<td></td>
</tr>
<tr>
<td>Hashimoto thyroiditis (total: 2)</td>
<td>No detectable*</td>
<td></td>
</tr>
<tr>
<td>Graves’ disease (total: 1)</td>
<td>No detectable*</td>
<td></td>
</tr>
<tr>
<td>Indeterminate (total: 6)</td>
<td>No detectable*</td>
<td>6 follicular adenomas</td>
</tr>
<tr>
<td>Indeterminate (total: 5)</td>
<td>&gt;10 Units</td>
<td>4 follicular carcinomas</td>
</tr>
<tr>
<td>Papillary carcinomas (total: 15)</td>
<td>&gt;10 Units</td>
<td>Papillary carcinomas</td>
</tr>
</tbody>
</table>

FNAB: fine needle aspiration biopsy. Units of telomerase activity were determined as described in Materials and methods; *no detectable means <0.1 Units of telomerase activity.

Table 3
P53 expression in undifferentiated thyroid carcinomas

<table>
<thead>
<tr>
<th>Presentation</th>
<th>Histopathology</th>
<th>P53 positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1: Thyroid hard nodule (6×3 cm)*</td>
<td>Anaplastic giant cells</td>
<td>55%</td>
</tr>
<tr>
<td>No. 2: Thyroid hard nodule (4×4 cm)*</td>
<td>Anaplastic with follicular differentiated component</td>
<td>7%</td>
</tr>
<tr>
<td>No. 3: Thyroid hard nodule (4×4 cm)*</td>
<td>Anaplastic spindle cells</td>
<td>80%</td>
</tr>
</tbody>
</table>

* All of these tumors were rapidly growing, patients died during this study (5 y). Patients with differentiated carcinomas (follicular and papillary) showed up to 5 y lifespan without disease.
mentioning that the follicular residual component of sample No. 2 (Table 3) was negative for p53.

4. Discussion

One of the critical issues in the diagnosis of thyroid cancer is to determine whether the nodule is benign or malignant. Neoplasm discrimination is usually carried out by fine needle aspiration biopsy followed by cytological examination. However, this method is not always conclusive. Thyroidectomy is used in cytological diagnosis of indeterminate cases, 80% of which turn out to be benign on histological analysis [6]. Therefore, it is clear that diagnostic methods must be improved. Since the possibility of obtaining enough material through fine needle aspiration biopsy is relatively simple in the case of thyroid nodules, our aim was to study new markers such as telomerase activity.

In order to be useful as a diagnostic tool in the determination of benign and malignant tissue, telomerase assays must be feasible in cytological samples. The development of the telomeric repeat amplification protocol (TRAP) assay [25] improved telomerase analysis in FNAB samples. Our FNAB study showed no detectable telomerase activity in Graves’ disease, Hashimoto’s thyroiditis, or follicular adenomas. Nevertheless, 3 of 56 goiter FNAB samples showed telomerase values lower than 10 Units, other authors also found values from 0 to 9.3 Units of TA in thyroid benign nodules [30]. Whereas previous reports showed 8.3% false positives in benign FNAB samples [31], we only found 4% cases, possibly as a consequence of the reagents used. In the case of FNAB from papillary and follicular carcinomas, we detected values higher than 10 Units (17.6–133.4 Units). Similar values were reported by other authors in prostate, thyroid and bladder carcinomas [22,31,32]. Therefore, if a 10-Unit cut off is established, as in the case of bladder cancer studies [32], detectable TA would be confined only to thyroid carcinomas. As regards FNAB from papillary and follicular carcinomas, most cases were accurately diagnosed by cytology, but in FNAB from follicular carcinomas, cytology was not helpful and telomerase was better to indicate abnormal proliferation. This suggested that TA assessment in indeterminate FNAB samples should be indicated prior to possible surgery, since this assay would be a useful complement to cytology in the diagnosis of cancer.

Our study showed that differentiated carcinomas were negative for p53, while undifferentiated carcinomas had detectable p53 expression. Although the number of undifferentiated carcinomas studied was small due to the low incidence of this disease, p53 accumulation was observed in all of them, thus suggesting that p53 would be a differentiation marker in thyroid tumors. Other authors reported that this protein might be related to a more advanced disease status and to the development of undifferentiated carcinomas [11,33]. Thyroglobulin mRNA levels were significantly lower in differentiated carcinomas than in benign thyroid nodules, in agreement with reports from other authors [12,34]. Therefore, Tg mRNA levels may serve as an index of thyroid cell differentiation. We concluded that these two markers correlate with the degree of differentiation observed in thyroid tumors, but their expression is not useful to discriminate between benign nodules and differentiated malignant tumors.

Results clearly show that telomerase activity measured by TRAP assay in fine needle aspiration biopsy samples could be a useful marker in the diagnosis of thyroid cancer, especially in FNAB cases with indeterminate cytological examination.

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