Thyroid peroxidase immunodetection as a tool to assist diagnosis of thyroid nodules on fine-needle aspiration biopsy

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In a previous work we have reported the presence in 96.9% of malignant and 4.2% of benign thyroid tumors of an immunological abnormality of the enzyme thyroid peroxidase, impeding the fixation of the anti-thyroid peroxidase monoclonal antibody termed “MoAb47”. The present study has been designed to establish the ability of thyroid peroxidase immunodetection to assist the diagnosis of malignancy in fine-needle aspiration of thyroid nodules. The fixation of anti-thyroid peroxidase monoclonal antibody was investigated by immunohistochemistry on fine-needle aspirates of 150 surgically removed thyroid nodules (20 papillary carcinomas, five follicular carcinomas, 90 colloid adenomas, nine fetal adenoma, 13 atypical adenomas, five oncocyctomas, six Hashimoto’s thyroiditis and two Graves’ disease). The percentage of positive cells has been compared to the final histological diagnosis. In samples from 113/125 benign nodules 80–100% of cells presented a positive immuno reaction, whereas all samples from malignant tumors yielded less than 80% positive cells. Benign nodules exhibiting less than 80% positive cells corresponded to three degenerative colloid nodules, five atypical follicular adenomas, two oncocyctomas and two thyroiditis. According to results obtained in this series, with the value of 80% as the limit for discrimination between benign and high-risk nodules, the sensitivity of thyroid peroxidase staining for diagnosis of malignancy would be 100%, its specificity 90% and its overall accuracy 92%. Thyroid peroxidase staining with monoclonal antibody MoAb47 on fine-needle aspirates is a useful adjunct to conventional cytology for the investigation of patients with thyroid nodules.

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In countries where hyperplastic goiter is not endemic, solitary thyroid nodules occur in 2–6% of the population but only 5–20% of these nodules are malignant (1, 2). However, discrimination between malignant and benign lesions using scintigraphy, ultrasonography and other laboratory tests is not only costly but also unreliable.

Fine-needle aspiration biopsy (FNAB), well documented by Scandinavian authors (3), is widely accepted as the most effective procedure for detecting malignancy in thyroid nodules (4–7) and is often recommended as the initial diagnostic test for investigating these lesions. Nevertheless, many groups, especially in Europe, have been reluctant to use FNAB for several reasons: the quality of smears depends mainly on the experience of the operator and the accuracy of interpretation is determined by the experience of the cytologist (8, 9); the false negative rate in recent reports ranges from 2 to 18% (5, 10–15). Finally, with FNAB it is sometimes difficult to discriminate between the various types of follicular tumors (16–18).

These drawbacks could be obviated if a reliable marker for thyroid malignancy could be identified for use as an adjunct for cytological diagnosis. Encouraging attempts to discriminate benign from malignant thyroid tumors have been developed with image analysis (19). Immunohistochemistry with various substances, including lactoferrin (20), keratin, thyroglobulin (21) and carbohydrates (22), have been disappointing. Recently, encouraging results were obtained with dipeptidyl-amino-petidase IV (DAP IV) (23).

In a previous paper, we reported that immunohistochemical staining of thyroid peroxidase (TPO) with monoclonal antibody 47 (MoAb47) was significantly reduced or negative in 96.9% of malignant thyroid tumors as opposed to 4.2% of benign nodules (24). A preliminary report shows that this method could be of interest on cytological samples (25). In this work, we assessed the value of TPO immunodetection with MoAb47 in detecting malignancy on fine-needle aspirates of solitary or dominant thyroid nodules.
Material and methods

Patients
This study concerns 150 patients with thyroid nodules operated in the Department of Endocrine Surgery (Marseille). Choice of treatment has been made on the basis of physical examination, scintigraphy, ultrasonography and cytology. Histological classification was carried out according to the WHO criteria for thyroid tumors (26). The diagnosis of fetal adenoma was restricted to regular microfollicular tumors. Irregular microfollicular or trabecular nodules exhibiting cellular or architectural atypias (compact cellular areas, enlarged cells with high nuclear cytoplasmic ratio, stellate stromal or capsular fibrosis, and odd-shaped nuclei) were classified as atypical adenoma.

Cytological material
Fine-needle aspiration biopsies of thyroid nodules were obtained using a 25-gauge needle and a 10-ml disposable syringe attached to a holder as described previously (3). Almost all the punctures were performed by the same surgeon. Slides were air dried and kept at -20°C until staining. As our goal was to compare immunochemical and histological diagnosis, only adequate samples, i.e. comprising more than 100 thyroid epithelial cells, were included in this study. Specimens were also performed by the pathologist on normal areas of the thyroid at the time of frozen section examination and processed in parallel with test slides for use as positive controls.

Antibodies
Monoclonal antibody 47 was raised by the hybridoma method using mouse lymphocytes immunized with purified human TPO (27). It recognizes an epitope located on the human TPO molecule and produces strong immunostaining in normal and almost all benign thyroid tissues. It does not react significantly with most malignant thyroid tumors or with non-thyroid tissue (24). Monoclonal antibody 63 was used as a negative control. It was raised using mouse lymphocytes immunized with adjuvant alone and does not react with any tissue. For immunostaining with both primary antibodies we used hybridoma tissue culture medium at dilution 1:10.

Staining procedure
As described previously (24), after an overnight incubation at +4°C with the two primary antibodies MoAb47 and MoAb63, immunostaining was performed with a universal avidin–streptavidin–peroxidase kit according to the manufacturer’s instructions (Immuno- tech, Marseille, France). Peroxidase activity was revealed in 0.2% diaminobenzidine tetrahydrochloride (Sigma), with 0.02% H2O2 in 0.1 mol/l TRIS buffer (pH 7.2).

Expression of results
All cytological samples were analysed at the same time by three cytologists without knowledge of histological diagnosis. Histological diagnoses were recorded from the files of the Department of Pathology. Results of TPO immunostaining were expressed as the percentage of positive thyroid cells. For calculation of sensitivity, specificity, predictive value and accuracy, the following formulae were used

Sensitivity = TP/TP + FN
Specificity = TN/TN + FP
Positive predictive value = TP/TP + FP
Negative predictive value = TN/TN + FN
Accuracy = (TP + TN)/(TP + TN + FP + FN)

Table 1. Thyroid peroxidase immunostaining with monoclonal antibody 47 (MoAb47) in fine-needle aspiration biopsy of thyroid nodules according to histological type.

<table>
<thead>
<tr>
<th>Histological diagnosis</th>
<th>No. of cases</th>
<th>0–19%</th>
<th>20–39%</th>
<th>40–59%</th>
<th>60–79%</th>
<th>80–100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrofollicular adenoma</td>
<td>90</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td>2</td>
<td>87</td>
</tr>
<tr>
<td>Fetal adenoma</td>
<td>9</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>9</td>
</tr>
<tr>
<td>Atypical adenoma</td>
<td>13</td>
<td>1*</td>
<td>2</td>
<td>–</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Oncocytomas</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Thyroiditis</td>
<td>6</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Graves’ disease</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>Papillary cancer</td>
<td>20</td>
<td>13</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Follicular cancer</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>–</td>
<td>1</td>
<td>–</td>
</tr>
</tbody>
</table>

*Number of cases.
Results

With MoAb47, 80–100% of the epithelial cells from normal thyroids displayed significant brown, slightly granular staining. This reaction was located throughout the cytoplasm. In the perinuclear area, a well-defined dark ring was observed in most of the cells. Staining was not obtained with MoAb63 on any thyroid cell smears.

Table 1 shows the percentage of positive TPO immunostaining obtained with MoAb47 according to histological type. In most colloid nodules and macrofollicular adenomas, more than 80% of the cells showed dark staining (Fig. 1). The perinuclear ring often was preserved in naked nuclei that are associated frequently with colloid nodules. This feature allows discrimination from lymphocytes. In three degenerative nodules, a varying number of negative cells corresponding either to small atrophic cells or large dystrophic cells were observed.

Cells from regular fetal adenomas consistently exhibited 80–100% positive immunostaining (Fig. 2). In contrast, staining of more than 80% of the cells was observed in only 8/13 fetal adenomas with atypical features. In other cases, nests of positive and negative cells in varying proportions were noted.

Oncocytomas displayed a pattern of TPO immunostaining that was slightly different from normal cells. Staining of the cytoplasm was lighter and the perinuclear ring often was absent. In some cases only the periphery of the cell beneath the cytoplasmic membrane was stained. Accurate determination of the percentage of positive cells often was difficult because the results of staining were not clear-cut.

In cells from thyroiditis-related lesions, TPO staining with MoAb47 depended on the state of tissue degradation. In one case in which the gland had been replaced by a sclerotic inflammatory mass containing isolated nests of degenerative follicular cells, the percentage of staining was low. In most cases, clumps of unstained atypical or degenerative cells were scattered among TPO-positive cells.

In Graves' disease, large cells with abundant granular cytoplasm were always 100% TPO-positive.

Most papillary cancers were completely negative (Fig. 3). However, in a few cases, positive cells were observed within otherwise negative clusters. In one case, the percentage of positive cells was higher but the pattern of reactivity was abnormal in that the perinuclear ring had disappeared and the staining was concentrated on the edges of the cytoplasm.

In four cases of follicular carcinoma, the positive immunostaining involved less than 40% of cell clusters.

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Fig. 1. Thyroid peroxidase immunostaining with monoclonal antibody 47 (MoAb47) on thyroid cells from a colloid nodule. All the cells exhibit slightly granular cytoplasmic staining with a perinuclear reinforcement (×400).

Fig. 2. Thyroid peroxidase staining on aspirate from a regular microfollicular tumor. All the cells are stained significantly (×400).
most of which appeared transparent (Fig. 4). In one case, staining was observed in 75% of the cells but it was lighter than in normal cells and the perinuclear ring appeared faded. In this case it was difficult to evaluate the percentage of positive cells.

The expression of these results in terms of histological diagnosis is shown in Table 2. All 25 malignant nodules had less than 80% positive cells and 113 out of the 125 benign nodules had more than 80% positive cells. Taking this value as the end point, in this series there were no false negative and 12 false positive results for the diagnosis of malignancy. The sensitivity of TPO immunostaining was 100%, its specificity 90%, positive predictive value 67.6%, negative predictive value 100% and overall accuracy 92%.

Discussion
In this study we used an anti-TPO monoclonal antibody called MoAb47 as a marker of malignancy to assist cytological diagnosis on FNAB of 150 histologically proven thyroid nodules. As reported previously, MoAb47 fails to react with TPO in malignant thyroid tissues.

The limit for discrimination between benign and high-risk nodules was positive staining on 80% of cells. This value was chosen retrospectively because most benign nodules yielded more than 80% positive cells and the highest positive percentage of positive cells observed in malignant tumors was 75%. Obviously this method of analysis is bound to give the best possible impression of the discriminatory power of the technique. Using the 80% threshold in a prospective study of a larger set of cases, the definite value of TPO staining might, therefore, prove slightly different: the number of carcinomas in this study is still too small to exclude the possibility of finding some cases with more than 80% TPO positive cells. Keeping these limitations in mind it should, however, be stressed that 21/25 (84%) malignant tumors produced staining percentages lower than 40% and that 106/125 (84.8%) benign nodules exhibited percentages of 90% or more. Thus, in nearly 85% of cases the response of TPO staining was

<table>
<thead>
<tr>
<th>Histological class</th>
<th>No. of cases</th>
<th>Percentage of TPO positive cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>≥ 80%</td>
</tr>
<tr>
<td>Benign</td>
<td>125</td>
<td>113</td>
</tr>
<tr>
<td>Malignant</td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Interpretation of thyroid peroxidase (TPO) immunostaining in thyroid nodules according to potential for malignancy.

Fig. 3. Thyroid peroxidase staining on an aspiration smear from a papillary carcinoma. No staining in any of the cells (x400).

Fig. 4. Thyroid peroxidase staining on an aspirate from a case of follicular carcinoma. Some cells show a coarse staining predominating at the periphery of the cytoplasm. The perinuclear ring has disappeared in almost all the cells (x400).
clear-cut and the assessment of TPO immunostaining was simple and reliable.

Using these criteria, in all 25 cases of thyroid carcinoma the diagnosis of malignancy was suspected or asserted by TPO-MoAb47 immunostaining. Although FNAB is generally considered to be a reliable technique for diagnosis of papillary cancer (28, 29), there is great uncertainty about the incidence of overlooked malignancy among nodules diagnosed as benign by standard cytology: published false negative error rates derived from series of surgically treated patients range between 2.3% (7) and 16.6% (14), with an average of 5% (12). Actually, typical morphological features of papillary cancers, i.e. large, oval, overlapping nuclei with dusty chromatin, intranuclear cytoplasmic invaginations and nuclear grooves (30), are inconsistent. They are present in 80–85% (31) of the cases but can be missed when samples are poorly cellular: this is a frequent event, because obtaining satisfactory aspirates is one of the main difficulties of FNAB. In the remaining cases, features are more or less atypical and the cells can be confused easily with dystrophic cells of thyroiditis, regressing colloid nodules or even benign follicular cells. Thyroid peroxidase immunostaining is of great value in all of these cases because it arouses suspicion of malignancy even if only a few cellular clusters are available.

The number of cases of follicular cancer in this series, i.e. five, was too low to allow definite conclusions to be drawn about the value of TPO immunodetection for this entity. However, we should stress that normal TPO staining was not observed in any of the present follicular malignant tumors or any subsequent cases in our experience. In one case, 75% of cells were TPO positive, but, as in other malignant or atypical tumors, changes were observed in the location of TPO staining, which disappeared from the perinuclear cisternae and concentrated beneath the cytoplasmic membranes on the periphery of the cells: although fading of perinuclear staining proved to be another reliable marker of malignancy, we felt that it was unnecessary to take this variation into account in the calculation of staining because most malignant tumors display less than 40% positive cells.

Specificity is extremely difficult to define, as shown by previous studies on thyroid FNAB, because it depends on whether equivocal cytological findings and diagnosis of follicular tumors are included within benign or malignant results (16). When they are excluded from calculations, values for specificity range from 79.9% to 100% (7, 12, 14). Taking into account that nearly all patients with such diagnosis undergo surgery and that the goal of cytology is to select thyroid nodules for surgery, it seems more correct to put these results together with malignant responses in the evaluation of accuracy. The specificity of cytology for the diagnosis of malignancy fluctuates then from 45% to 70.6% (7, 13). The specificity of TPO immunostaining with MoAb47 for the diagnosis of malignancy in these 150 thyroid nodules, including 32 follicular tumors, was 90%, which is improved significantly as compared with standard cytology. The false positive results of TPO detection were due to five degenerative colloid nodules or thyroiditis and seven atypical follicular or oncocytic tumors. Discrimination of malignancy in microfollicular tumors and oncocytomas is not possible with standard cytology (5, 22–24). For this reason, most practitioners group them under the designation of “follicular tumors” and advise surgery for histological diagnosis. In this study, TPO staining successfully identified five out of five malignant follicular lesions and 20 out of 27 benign follicular lesions for an overall success rate of 25 out of 32 (78%). It must be stressed that all fetal adenomas with small, well-stained, rounded and distinct nuclei were more than 80% TPO positive. In contrast, TPO staining on microfollicular tumors with atypical cytological features was less regular and these tumors were considered frequently as “suspicious”.

We conclude from this study that TPO staining on FNAB of thyroid nodules is highly accurate in separating benign from malignant thyroid tumors. The limitations of TPO staining are not the same as those of standard cytology: standard cytology achieves better results for thyroiditis and dystrophic colloid nodules, whereas TPO staining is more effective for follicular tumors and papillary cancers. Thus, these methods appear to be complementary. Based on our data, the combination of standard cytology with MoAb47 immunostaining on fine-needle aspirates appears to be the method of choice to select thyroid nodules for surgery.

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