Expression of Thyroid-specific Transcription Factors TTF-1 and PAX-8 in Human Thyroid Neoplasms

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ABSTRACT

TTF-1 and PAX-8 are tissue-specific transcription factors expressed in the thyroid follicular cells, contributing to the maintenance of the differentiated phenotype. In fact, it has been demonstrated that TTF-1 and PAX-8 are able to activate transcription from thyroglobulin and thyroperoxidase (TPO) promoters, the transcriptional activity of which is in vivo restricted only to the thyroid follicular cell. In order to gain insight into how these transcription factors control in vivo the differentiation of the thyroid cell and to have a better molecular characterization of human thyroid tumors, TTF-1, PAX-8, thyroglobulin, and TPO mRNA levels were measured in nonmalignant and malignant human thyroid tissues. Results indicate that the expression of TTF-1 and PAX-8 is not sufficient per se for the expression of the thyroid-differentiated phenotype. Furthermore, in follicular adenomas, PAX-8 mRNA levels are strictly related to TPO mRNA levels, suggesting that the amount of PAX-8 could play a role in the modulation of TPO gene expression. TTF-1 mRNA is always well detectable in papillary carcinomas and, in contrast, always absent in anaplastic carcinomas. Identical results were obtained when the expression of TTF-1 protein was investigated using immunohistochemistry. Thus, TTF-1 gene expression could be a molecular marker in order to distinguish these two types of thyroid neoplasms.

INTRODUCTION

Transcription factors play a pivotal role in the determination and maintenance of cellular phenotype. The activity of transcription factors is in fact considered as the main switch to regulate gene expression (1). Transcription factors may be classified according to the localization of their expression. In this manner, these factors can be divided into ubiquitous and tissue-specific groups. Members of the latter group are present in a few cell types only and participate in the transcriptional regulation of genes expressed only in these cells (2–6). Hence, tissue-specific transcription factors can be very important for expression of the differentiated phenotype of each cell.

Two transcription factors, expression of which is restricted to the thyroid follicular cell, have been cloned thus far: TTF-1 (7) and PAX-8 (8). In addition to the follicular thyroid cell, in the adult animal, TTF-1 and PAX-8 are also expressed in the lung and kidney, respectively. However, the two factors are present together only in the thyroid follicular cell, suggesting that this unique combination could play a role in the expression of the thyroid-specific phenotype. This view is strengthened by molecular data; both TTF-1 and PAX-8 bind to sequences of Tg and TPO promoters and, albeit to a different extent, they are able to activate transcription of Tg and TPO genes (9–10). These data have been obtained by cotransfection of cell lines with both expression vectors of TTF-1 or PAX-8 and reporter genes transcriptionally controlled by Tg and TPO promoters. A main drawback of this experimental approach is the difficulty to quantitatively reproduce the real concentration of the expressed transcription factors. This problem is very important because, for several of these proteins, it has been clearly demonstrated that relatively small changes of their expression can have enormous biological effects (11–12). For this reason, the specificity of the phenotype observed using systems based on cell transfection is difficult to be clearly defined.

For these motivations, in order to better understand the role of TTF-1 and PAX-8 in the maintenance of the thyroid-differentiated phenotype, it would be relevant to use approaches able to complement findings obtained by in vitro studies. Thus, we decided to study the expression of TTF-1 and PAX-8 genes, together with the expression of their putative target genes Tg and TPO, both in normal and neoplastic human thyroid tissues. Thyroid tumors originating from the follicular cells comprise lesions with a broad spectrum of cell differentiation which include benign adenomas, differentiated (papillary and follicular) carcinomas, poorly differentiated carcinomas, and the highly undifferentiated anaplastic carcinomas (13). The degree of differentiation of these classes of neoplasms is heterogeneous. Often, heterogeneity in the expression of the differentiated phenotype is encountered for tumors of the same class (14).

The studies presented in this paper could also provide clinically relevant information. In fact, differentiated thyroid carcinomas (which represent over 90% of the total malignancies in thyroid) are a heterogeneous disease: some of them are a mortal disease while others do not affect life expectancy (15–17). Thus far, clinical and histomorphological characteristics are often inadequate to predict their clinical behavior. Therefore, new parameters, such as molecular characterization, could help to better classify them and to more accurately predict their prognosis. Because in tumor cells differentiation markers and malignant phenotype are very often inversely related, the expression of tissue-specific transcription factors controlling the differentiated phenotype could be an additional tool in the evaluation of cancer aggressiveness.

MATERIALS AND METHODS

Plasmids. The DNA fragments used as probes in Northern analysis were: for Pax-8, a HindIII/EcoRI fragment of human Pax-8 complementary DNA contained in plasmid H26P/S3; for Tg, plasmid prTGF2 containing a 5′ coding fraction of Tg complementary DNA (18); for TPO, the SalI/EcoRI coding fragment of pTPO 5′c (18); for TTF-1, the 0.7-kilobase SacI fragment of plasmid prTTF-1/4 (7); for GAPDH, a 1.3-kilobase PstI fragment of plasmid pGAPDH1 containing the coding region of GAPDH (from H. Francis-Lang, Imperial Cancer Research Fund, Oxford, United Kingdom).

Tissue Collection, RNA Extraction, Northern Blots, and Values Normalization. Thyroid tissue was obtained from patients undergoing surgery for clinical indications. After surgery, thyroid pieces were cut into fragments of about 1 cm³ and quickly frozen. Histological diagnosis was obtained for all tissues. Total RNA from frozen tissues and FRTL-5 cells was prepared by the acid guanidinium thiocyanate-phenol chloroform procedure (19). Northern blots were performed using standard procedures (formaldehyde/agarose gel and filter hybridization using the protocol described in Ref. 20), and at the end, filters were exposed at -80°C for autoradiography. The intensity of the signals was quantitated by scanning densitometry of the autoradiograms. Each value back of this experimental approach is the difficulty to quantitatively reproduce the real concentration of the expressed transcription factors.
was normalized using GAPDH as a reference mRNA. For this gene, mRNA levels do not significantly vary in the samples studied (data not shown). Furthermore, in order to compare samples run in different gels, each value was further normalized for Tg, TPO, TTF-1, and PAX-8 gene expression of FRTL-5 cells. To perform this normalization, in each gel a same amount of FRTL-5 RNA was run. In this manner, the values of the thyroid-specific gene expression in human tissue do not indicate the absolute amounts but the ratio between the gene expression of human tissues and the gene expression of FRTL-5 cells.

**Immunohistochromy.** Immunohistochemical detection of TTF-1 was performed using a polyclonal antibody to TTF-1 produced in rabbit. Specimens were fixed in 4% buffered formaldehyde and were routinely embedded in paraffin. The streptavidin-biotinylated alkaline phosphatase technique was applied. Briefly, after rehydration, the sections were incubated with normal swine serum. Subsequent incubations were as follows (all at room temperature): antibody to TTF-1 (1:200 dilution) for 2 h; biotin-labeled swine anti-rabbit for 30 min; streptavidin-biotinylated alkaline phosphatase complex (Dakopatts A/S, Glostrup, Denmark) for 30 min; and incubation in new fuchsin substrate solution. The sections were counterstained with hematoxylin and mounted with Glycergel. Negative controls were carried out. Cells were considered positive when red staining of the nucleus was identified. The extent of TTF-1 positivity in each case was evaluated by determining the percentage of positivity in at least 5000 cells.

**Table 1.** Tg, TPO, TTF-1, and PAX-8 mRNA levels in thyroid follicular adenomas

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Tg (kilobases)</th>
<th>TPO (kilobases)</th>
<th>TTF-1 (kilobases)</th>
<th>PAX-8 (kilobases)</th>
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<td>4</td>
<td>8.5</td>
<td>3.2</td>
<td>2.4</td>
<td>3.1</td>
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<tr>
<td>5</td>
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<td>7</td>
<td>3.09</td>
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<td>3.79</td>
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<td>19.4</td>
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<tr>
<td>15</td>
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<td>46</td>
<td>1.63</td>
<td>8.0</td>
<td>41.6</td>
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</tr>
<tr>
<td>51</td>
<td>1.36</td>
<td>10.1</td>
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<td>65</td>
<td>0.82</td>
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<td>66</td>
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<td>72</td>
<td>0.4</td>
<td>15.4</td>
<td>13.5</td>
<td>753</td>
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</table>

**Fig. 2.** Relationship between the mRNA levels of the thyroid-specific transcription factors (TTF-1 and PAX-8) and target genes (Tg and TPO) in thyroid follicular adenomas. Cases and mRNA levels are those numerically presented in Table 2.
TTF-1 AND PAX-8 EXPRESSION IN THYROID NEOPLASMS

RESULTS

Tg, TPO, TTF-1, and PAX-8 Gene Expression in Normal and Nonmalignant Thyroid Tissues. We determined whether the variability of our experimental approach (described in “Materials and Methods”) allowed a reliable comparison between values obtained from samples run in different gels and those independently processed. Several samples were run in two different gels that were independently blotted and further processed. Tg, TPO, TTF-1, and PAX-8 mRNA levels were detected and normalized as described in Methods. Successively, the differences of values obtained for each sample in the two different blots were calculated. For each gene, the mean difference was not over 10% of the mean value. Hence, Tg, TPO, TTF-1, and PAX-8 gene expression was evaluated in normal thyroids (n = 3) and in differentiated, nonneoplastic pathological tissues such as goiters (n = 13) and follicular adenomas (n = 12). In all of these tissues, Tg, TPO, TTF-1, and PAX-8 gene expression was detectable (Fig. 1), although at variable levels. The levels of expression of the mRNAs studied were especially variable in thyroid adenomas. (Values are shown in Table 1.) An attempt was made to correlate the expression of Tg and TPO with that of their regulators PAX-8 and TTF-1 (Fig. 2). Weak correlations were found between Tg and TTF-1 mRNA levels and TPO and TTF-1 mRNA levels. Conversely, a highly significant correlation was found between TPO and PAX-8 mRNA levels, suggesting that the amount of PAX-8 in the thyroid follicular cells could be a limiting factor for TPO gene expression. No correlation was found between levels of PAX-8 and the expression of Tg. These data indicate that PAX-8 mostly acts on TPO gene expression. The weak correlations between TTF-1 and target genes suggest that the amount of TTF-1 plays only a marginal role for the modulation of Tg and TPO gene expression.

Tg, TPO, TTF-1, and PAX-8 Gene Expression in Malignant Thyroid Tissues. The expression of thyroid-specific genes was investigated in 21 differentiated carcinomas (20 papillary and 1 follicular), in 1 poorly differentiated carcinoma (insular carcinoma), and in 3 undifferentiated (anaplastic) carcinomas. In differentiated carcinomas, Tg, TPO, TTF-1, and PAX-8 mRNA gene expression was highly heterogeneous. In Fig. 3, a representative sample of the results obtained is shown. In some cancers, all four genes were expressed at levels similar to normal tissues, while in some others, the expression

Fig. 3. Tg, TPO, TTF-1, and PAX-8 gene expression in malignant thyroid tissues. RNA extraction and Northern blot were performed as described in “Materials and Methods.” Autoradiograms of exemplificative cases only are shown.

Fig. 4. a, Tg, TPO, TTF-1, and PAX-8 mRNA levels of differentiated thyroid carcinomas. Values were obtained as described in “Materials and Methods” and for each gene normalized for respective mean values of normal tissues. b, relationship between TPO and PAX-8 values of papillary carcinomas. a and b: ○, mRNA levels of papillary carcinomas; ○, gene expression of a papillary cancer where an anaplastic focus was evident. a, the insular carcinoma (crosses) and the follicular carcinoma (C) mRNA levels are shown.
of one or more thyroid-specific gene(s) was undetectable or very low. In order to better analyze the results, Tg, TPO, TTF-1, and PAX-8 gene expression levels were quantitated as described previously. Results of differentiated carcinomas are shown in Table 2 and Fig. 4. TTF-1 and PAX-8 gene expression was detected in all papillary carcinomas. TTF-1 mRNA was always well detectable, except in one case (case 18C, see Table 2 and Fig. 4) where anaplastic foci were carcinomas. TTF-1 mRNA was always well detectable, except in one case (case 18C, see Table 2 and Fig. 4) where anaplastic foci were carcinomas. TTF-1 and PAX-8 gene expression was slightly reduced or slightly increased compared to normal tissues in 6 of 20 cases, whereas in the remaining cases, it was markedly reduced, reaching levels as low as 5% of the normal levels. Tg mRNA was undetectable in 5 of 20 (25%) cases, and TPO mRNA was undetectable in 7 of 20 (35%) cases. The levels of expression of these two genes were not correlated. These data suggest that Tg and TPO gene expression is controlled by, at least in part, independent mechanisms.

In general, a good correlation was present between TPO and PAX-8 mRNA levels in papillary carcinomas (Fig. 4b). However, in three cases (nos. 41, 57, and 94 in Table 2), there was a dramatic reduction in TPO mRNA levels, in spite of only an about 50% reduction of PAX-8 mRNA levels. TTF-1 and PAX-8 mRNA levels were not correlated to each other, suggesting that these two genes are, at least partially, independently controlled.

As shown in Table 2, in order to investigate whether the level of TTF-1 and PAX-8 mRNAs was related to the different aggressiveness in papillary carcinomas, these 20 neoplasms were subdivided into a high risk group (8 cases with a maximal diameter ≥1.5 cm, extrathyroidal tumor extension, and nodal metastases) and a low risk group (12 cases without extrathyroidal tumor extension and nodal metastases). Values of Tg, TPO, and PAX-8 but not TTF-1 were lower in carcinomas of the high risk group. Only two cases of the high risk group (cases 21C and 11C) showed overlapping values with the low risk group; in case 21C, however, TPO was undetectable, and PAX-8 was low. The only carcinoma classified at histology as poorly differentiated (insular carcinoma) unexpectedly showed a good expression of all differentiation markers except Tg that was quite reduced compared to control values (Fig. 4). Analysis of anaplastic carcinomas showed a complete shut-off of all thyroid-specific genes in the three cases studied.

### Table 2 Thyroid-specific gene expression in human papillary cancers

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Tg</th>
<th>TPO</th>
<th>TTF-1</th>
<th>PAX-8</th>
</tr>
</thead>
<tbody>
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<td>Low-risk papillary carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
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<td>102</td>
<td>126</td>
<td>107</td>
</tr>
<tr>
<td>41</td>
<td>12</td>
<td>0</td>
<td>84</td>
<td>58</td>
</tr>
<tr>
<td>57</td>
<td>79</td>
<td>5</td>
<td>63</td>
<td>66</td>
</tr>
<tr>
<td>94</td>
<td>22</td>
<td>0</td>
<td>94</td>
<td>48</td>
</tr>
<tr>
<td>100</td>
<td>106</td>
<td>87</td>
<td>115</td>
<td>132</td>
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<tr>
<td>120</td>
<td>25</td>
<td>0</td>
<td>84</td>
<td>16</td>
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<tr>
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<td>63</td>
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<td>161</td>
<td>39</td>
<td>2</td>
<td>84</td>
<td>13</td>
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<td>High-risk papillary carcinoma</td>
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<tr>
<td>40</td>
<td>0</td>
<td>0</td>
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<tr>
<td>163</td>
<td>2</td>
<td>73</td>
<td>15</td>
<td></td>
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<tr>
<td>11C</td>
<td>112</td>
<td>107</td>
<td>94</td>
<td>120</td>
</tr>
<tr>
<td>12C</td>
<td>0</td>
<td>0</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>18C</td>
<td>10</td>
<td>10</td>
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<td>38</td>
</tr>
<tr>
<td>21C</td>
<td>40</td>
<td>2</td>
<td>84</td>
<td>20</td>
</tr>
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</table>

TTF-1 Protein Detection by Immunohistochemistry. Data obtained using Northern blots indicate that TTF-1 could be used as a molecular marker to distinguish differentiated and undifferentiated thyroid carcinomas. In order to further support this finding, the immunohistochemistry detection of TTF-1 in paraffin-embedded sections was performed. Malignant and nonmalignant thyroid tissues were studied. Results are expressed in Table 3, and example cases are shown in Fig. 5. All papillary and follicular carcinomas showed a high percentage of nuclear positivity, superimposable to that observed in goiters and follicular adenomas. In contrast, in anaplastic cancers, no staining for TTF-1 was observed.

### DISCUSSION

The data obtained in this study strongly support the notion that TTF-1 and PAX-8 play a major role in controlling the tissue-specific gene expression of the follicular thyroid cells. In fact, in none of the tissues studied could the presence of Tg or TPO mRNAs be observed in the absence of TTF-1 or PAX-8 mRNAs.

TTF-1 is a very strong activator of Tg promoter transcriptional activity, as demonstrated by cotransfection experiments. Moreover, during development, TTF-1 mRNA appears before Tg mRNA (21). These data suggest that TTF-1 is an important determinant for Tg gene expression in follicular thyroid cells. Accordingly, in the present study, we found that, in anaplastic thyroid carcinomas, the absence of TTF-1 is associated to the absence of Tg gene expression. This finding

* M. De Felice, G. Damante, R. Di Lauro, unpublished data.
By cotransfection experiments, it has been demonstrated that PAX-8 is a dose-dependent activator of the TPO promoter and, but to a much lesser extent, of the Tg promoter (10). Present data show a tight correlation between TPO and PAX-8 mRNA levels in all thyroid tissues examined, especially in follicular adenomas. Lack of correlation between TPO and PAX-8 mRNA levels was observed in three papillary carcinomas and could indicate that the regulatory effect of PAX-8 on TPO gene expression is coupled to at least another independent regulatory event. It is important to note that, in FRTL-5 cells, TPO mRNA levels are controlled by thyroid-stimulating hormone, at least partially, through nontranscriptional mechanisms (24). Nonetheless, our data suggest that the amount of PAX-8 present in the cell is a major determinant for the levels of TPO gene expression; therefore, variations in the amount of PAX-8 levels could play a role not only in the determination and maintenance of the differentiated phenotype but also in controlling the functional behavior of the thyroid follicular cell (10).

From the clinical point of view, the markers studied could certainly differentiate between differentiated and anaplastic thyroid carcinomas. The diagnosis of anaplastic thyroid carcinomas usually can be achieved by clinical and histopathological criteria. However, the differentiation markers can be useful, in addition to morphological parameters, in very early stages of anaplastic changes, like in case 18C of our series.

When we grouped papillary carcinomas into high risk (diameter ≥1.5 cm, extrathyroidal invasion, and metastases) and low risk (absence of these characteristics), we found significantly lower values in the expression of PAX-8, Tg, and TPO in the high risk group, with value overlap present only in two cases.

Due to the indolent evolution of most papillary carcinomas, the duration of follow-up did not allow us to correlate differentiation markers to tumor relapse and mortality rates. Insular carcinomas are classified at histology in the class of less differentiated cancers and are usually aggressive. There is increasing evidence, however, that they are a heterogeneous group (like papillary carcinomas are), and some of them may concentrate iodine (25–26). This characteristic is clinically important because it allows us to treat metastases with radioiodine therapy. Accordingly, the case described here showed a good correlation of all differentiation markers.

It is likely that these markers, in addition to a panel of other molecular markers (e.g., oncogene and tumor suppressor gene mutations, expression of growth factors, and their receptors; Refs. 27–36) will, in the future, help more accurately in classifying both differentiated and less differentiated thyroid carcinomas.

ACKNOWLEDGMENTS

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