Detection of High Mobility Group I HMGI(Y) Protein in the Diagnosis of Thyroid Tumors: HMGI(Y) Expression Represents a Potential Diagnostic Indicator of Carcinoma


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ABSTRACT

Hyperplastic or neoplastic proliferative lesions of thyroid follicular epithelium consist of a spectrum, ranging from nodular hyperplasia to undifferentiated (anaplastic) carcinoma, and usually present as palpable thyroid nodules. Thyroid nodules are a common occurrence in the general population, but only a small proportion of them are eventually diagnosed as carcinoma. The difficulty in objectively identifying those thyroid nodules that are malignant to avoid unnecessary surgery, combined with the range and effectiveness of the available therapeutic options in those patients who do, indeed, have thyroid carcinoma, has prompted the search for tumor markers and prognostic indicators. The high mobility group I (HMGI) proteins represent a class of nuclear proteins involved in the regulation of chromatin structure and function. HMGI(Y), one of the members of this class, is expressed at high levels during embryogenesis and in malignant tumors but at generally low levels in normal adult human tissues. Previous work on a limited number of thyroid samples suggested that the detection of the HMGI(Y) proteins may provide a clinically useful diagnostic tool. To verify this assumption, we analyzed HMGI(Y) expression by a combination of immunohistochemistry and reverse transcription-PCR in 358 thyroid tissue samples that were representative of the spectrum of thyroid tumor pathology. HMGI(Y) was detectable in 18 of 19 follicular carcinomas, 92 of 96 papillary carcinomas, and 11 of 11 undifferentiated (anaplastic) carcinomas but in only 1 of 20 hyperplastic nodules, 44 of 200 follicular adenomas, and 0 of 12 normal tissue samples. The correlation between HMGI(Y) expression and a diagnosis of carcinoma was highly significant (P < 0.0001). We also prospectively collected and analyzed for HMGI(Y) expression by immunohistochemistry and reverse transcription-PCR in 12 fine needle aspiration biopsies from 10 patients who subsequently underwent surgical removal of a solitary thyroid nodule. HMGI(Y) was detectable only in the four fine needle aspiration biopsies, corresponding to the thyroid nodules that were definitively diagnosed as carcinomas after surgery (two follicular carcinomas and two papillary carcinomas). The remaining eight samples (six follicular adenomas and two samples consisting of normal follicular cells) were negative. The findings of this study confirm the differential expression of HMGI(Y) in thyroid neoplasia and indicate the HMGI(Y) protein as a potential marker for thyroid carcinoma.

INTRODUCTION

Thyroid nodules represent a common occurrence in clinical practice. They are usually found by palpation, and their overall prevalence was 4.2% in a large epidemiological study of a nongeographical area in the United States (1). The large majority of these nodules are benign, and the identification of the 5–10% that are eventually diagnosed as carcinoma (2) relies on a combination of clinical evaluation, laboratory and radiographic studies, and, eventually, cytological examination of tissue samples (3). In the management of patients with solitary thyroid nodules, the search for thyroid carcinoma is fully justified because the majority of thyroid carcinomas can be effectively treated. On the basis of pathological examination and clinical features, thyroid tumors originating from the follicular epithelium are traditionally classified as papillary or follicular carcinomas. They also cover a wide spectrum of differentiation, ranging from well-differentiated phenotypes to highly malignant tumors, such as undifferentiated (anaplastic) carcinomas (4–6). The difficulty in objectively identifying those thyroid nodules that are malignant to avoid unnecessary surgical procedures, combined with the range and effectiveness of the available therapeutic options in those patients who do, indeed, have thyroid carcinoma, has prompted the search for tumor markers and prognostic indicators.

In a previous report, we showed that expression of the nuclear chromatinic protein HMGI(Y)3 generally correlates with the histological diagnosis of thyroid carcinoma (7). HMGI(Y) proteins are nuclear proteins that are involved in the regulation of chromatin structure and function (8, 9). The HMGI family is comprised of three proteins: HMGI, HMGY, and HMGI-C (10, 11). The first two proteins are products of the same gene generated through an alternative splicing mechanism (10). This gene is named HMGI(Y), and HMGI(Y) is commonly used to indicate both HMGI and HMGY proteins. Their expression is physiologically restricted to the embryological development (12); however, HMGI(Y) activation also occurs in several experimental and human malignant neoplastic diseases (13–19). High levels of HMGI(Y) expression are causally related to the development of a malignant phenotype because thyroid cells transfected with an antisense construct for the HMGI-C cDNA, in contrast with the untransfected ones, do not exhibit a malignant phenotype after infection with murine transforming retroviruses (20).

The finding of a correlation between HMGI(Y) protein expression and thyroid carcinoma (7) was based on a study of a limited number of cases. Therefore, here, we have analyzed 358 tumor samples pooled from the pathological archives of five different institutions in Europe and the United States to verify whether HMGI(Y) might provide a clinically useful diagnostic marker. With the same aim, we have also

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3 The abbreviations used are: HMGI, high mobility group I; FNAB, fine needle aspiration biopsy; GAPDH, glyceraldehyde phosphate dehydrogenase.
DETECTION OF HMGI(Y) PROTEINS IN THYROID NEOPLASIAS

Table 1  \textit{HMG(Y)} protein expression in human thyroid tumors

<table>
<thead>
<tr>
<th>Histological type of thyroid specimens</th>
<th>No. of positive cases/no. of cases analyzed by immunohistochemistry$^a$</th>
<th>No. of positive cases/no. of cases analyzed by RT-PCR$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal thyroid</td>
<td>0/12 (0)</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>Goiters</td>
<td>1/20 (5)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Follicular adenomas</td>
<td>44/200 (22)</td>
<td>4/10 (40)</td>
</tr>
<tr>
<td>Follicular carcinomas</td>
<td>18/19 (95)</td>
<td>6/6 (100)</td>
</tr>
<tr>
<td>Papillary carcinomas</td>
<td>92/96 (96)</td>
<td>23/23 (100)</td>
</tr>
<tr>
<td>Undifferentiated carcinomas</td>
<td>11/11 (100)</td>
<td>2/2 (100)</td>
</tr>
</tbody>
</table>

$^a$ The numbers in parentheses indicate the percentage of positive cases. There was a strong correlation between a diagnosis of carcinoma and HMGI(Y) expression detectable by immunohistochemistry ($P < 0.0001$) or RT-PCR ($P = 0.0002$).

$^b$ RT-PCR was performed on selected cases that were also analyzed by immunohistochemistry. The cases that were positive by RT-PCR were also positive by immunohistochemistry.

prospectively collected and analyzed for HMGI(Y) 12 cytological samples from 10 patients who subsequently underwent surgery for the removal of a solitary thyroid nodule.

MATERIALS AND METHODS

**Immunohistochemical Analysis of Tissue Samples.** Thyroid specimens were collected at the "Istituto Nazionale dei Tumori di Napoli," Naples, Italy; Laboratoire d’Histologie et de Cytologie, Center Hospitalier, Lyon Sud, France; Laboratoire d’Anatomie Pathologique, Hôpital de L’Antiquaille, Lyon, France; the Hospital Universitario 12 de Octubre, Madrid, Spain; and Yale-New Haven Hospital, New Haven, CT. After formalin fixation, the tissue sections were routinely processed and embedded in paraffin for histological examination. For immunohistochemistry, 5–6-μm paraffin sections were deparaffinized and then placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min and then washed in PBS before immunoperoxidase staining. The slides were then incubated overnight at 4°C in a humidified chamber with the antibodies diluted 1:100 in PBS. The slides were subsequently incubated with biotinylated goat antirabbit IgG for 20 min (Vectastain ABC kits; Vector Laboratories) and then with premixed avidin-biotin reagent (Vector) for 20 min. The immunostaining was performed by incubating the slides in diaminobenzidine (DAKO, Glostrup, Denmark) solution containing 0.06 mM diaminobenzidine and 2 mM hydrogen peroxide in 0.05% PBS (pH 7.6) for 5 min, and after chromogen development, the slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips using a permanent mounting medium (Permount). Micrographs were taken on Kodak Ektachrome film with a photo Zeiss system. The antibodies used in this study were raised against the synthetic peptide SSSKQQPLASKQ specific for the HMGI(Y) proteins (7). They were affinity-purified against the synthetic peptide. Tissue samples were scored as positive for immunohistochemistry when tissue immunoreactivity was detected in at least 10% of the cells. As expected, immunohistochemical reactivity was predominantly localized to the cells nuclei. Negative controls were performed by omitting the first antibody. The specificity of the reaction was confirmed by the lack of tissue immunoreactivity after preincubation of the antibody with molar excess of the HMGI(Y) synthetic peptide.

**Immunohistochemical Analysis of Thyroid FNABs.** The FNABs were performed at the “Istituto Nazionale dei Tumori di Napoli” according to established procedures using a 25-gauge needle attached to a 20-ml syringe in a plastic syringe holder. Alcohol-fixed and air-dried smears were immediately immunostained using the same antibody as described above. Samples were obtained from 10 patients with solitary thyroid nodules who subsequently underwent surgery because examination of the FNABs yielded cytological diagnoses that were suspicious for carcinoma (the diagnoses were follicular neoplasms for eight of the cases and features suggestive of papillary carcinoma for the remaining two cases). Normal thyroid follicular cells obtained after FNAB of the contralateral lobe in two of the patients were used as controls.

**RT-PCR Analysis of the HMGI(Y) Gene Expression.** RNA was extracted from paraffin-embedded blocks on 30 cases that were analyzed in parallel for HMGI(Y) expression by immunohistochemistry. RNA extraction
was performed as described (21). Briefly, single 6–8-μm tissue sections, cut from paraffin blocks, were stirred for 20 min in 1.5-ml tubes with 1 ml of xylene. After centrifugation, the pellet was washed with 0.5 ml of ethanol and air-dried. The dried pellet was resuspended in 200 μl of 6 mg/ml proteinase K (Sigma Chemical Co., St. Louis, MO), 1 m guanidinium thiocyanate, 25 mM 2-mercaptoethanol, 0.5% Sarkosyl, and 20 mM Tris-HCl (pH 7.5) and incubated at 37°C for 18 h. RNA was then extracted with phenol and precipitated with ethanol following a standard procedure (22). Fine needle aspiration samples were washed twice with 1 x PBS and then processed for RNA extraction following the same procedure (22). Fine needle aspiration samples were washed twice with 1 x PBS and then processed for RNA extraction following the same procedure (22). Fine needle aspiration samples were washed twice with 1 x PBS and then processed for RNA extraction following the same procedure (22).

RESULTS

Immunohistochemical Analysis of HMGI(Y) Gene Expression. Using the antibodies against a HMGI(Y)-specific peptide located at the NH2 terminus of the proteins, 358 thyroid samples were analyzed by immunohistochemistry for HMGI(Y) protein expression. The results of the immunohistochemical study of the tissue samples are summarized in Table 1 and illustrated in Figs. 1 and 2. No immunoreactivity was observed in the sections of normal thyroid tissue (Fig. 1A). This result was also confirmed using more sensitive immunohistochemical procedures such as the Catalyzed Signal Amplification system (DAKO; data not shown). Almost all of the nodular hyperplastic nodules were negative. About 80% of adenomas also scored negative. One representative case is shown in Fig. 1B. On the contrary, the large majority of thyroid carcinomas (121 of 126, corresponding to 95% of cases) exhibited tissue immunoreactivity for HMGI(Y) localized in the cell nuclei (Fig. 1, C and D). There was a strong association between HMGI(Y) expression and a diagnosis of carcinoma (P < 0.0001). Positive cells were observed regardless of the degree of differentiation and were usually homogeneously distributed throughout the tumor. Precise quantification of gene expression levels is not possible using immunohistochemical methods. However, a greater proportion of neoplastic cells positive for HMGI(Y), as well as a stronger intensity of the immunohistochemical reaction, was generally observed in the undifferentiated (anaplastic), compared to the well-differentiated carcinomas (papillary or follicular; Fig. 2). The
higher HMG\(\text{I}(Y)\) expression level in undifferentiated tumors noted after immunohistochemistry was confirmed at the mRNA level by the RT-PCR assay (see below). There was no difference in tissue immunoreactivity or mRNA expression levels among the positive adenomas, follicular carcinomas, and papillary carcinomas. Six cases of morphological variants of papillary carcinoma, such as those designated follicular variants of papillary carcinoma and encapsulated variants of papillary carcinoma, were included in the study. All of them were positive for the presence of the HMG\(\text{I}(Y)\) proteins. Among thyroid adenomas analyzed, 11 follicular adenomas showed focal and imperfectly developed cytological features reminiscent of those described for papillary carcinoma. Five of these cases were positive for HMG\(\text{I}(Y)\) expression. In the other five follicular adenomas, there were focal cellular areas with occasional mitotic figures in the absence of capsular or vascular invasion. Two of these cases were positive for HMG\(\text{I}(Y)\). In five follicular adenomas, there was incomplete penetration to tumor capsule; two of these cases were positive. In addition, positive HMG\(\text{I}(Y)\) reactivity was observed in all of the eight minimally invasive follicular carcinomas, including four carcinomas diagnosed as such on the basis of only focal definite evidence of capsular penetration. Positivity for HMG\(\text{I}(Y)\) in the follicular adenomas did not correlate with any particular histological growth pattern (e.g., micro- versus macrofollicular). Follow-up (range, 2–4 years) of 10 HMG\(\text{I}(Y)\)-positive and 10 HMG\(\text{I}(Y)\)-negative follicular adenomas has not demonstrated any difference in clinical behavior in the adenomas, regardless of their HMG\(\text{I}(Y)\) expression status.

**RT-PCR Analysis of the HMG\(\text{I}(Y)\) Gene Expression.** To validate the immunohistochemical data and to objectively compare the level of HMG\(\text{I}(Y)\) expression, we analyzed, in parallel, 50 samples, representative of the cases studied by immunohistochemistry, for HMG\(\text{I}(Y)\) mRNA levels by a semiquantitative RT-PCR assay. The results are shown in Table 1 and illustrated in Fig. 3. Like the immunohistochemical findings, they demonstrate a strong association between detectable HMG\(\text{I}(Y)\) mRNA and a diagnosis of carcinoma \((P = 0.0002)\). The highest HMG\(\text{I}(Y)\) mRNA levels were detectable in undifferentiated (anaplastic) tumors and in the anaplastic thyroid cell line ARO, thus supporting the impression based on the immunohistochemical findings that the degree of HMG\(\text{I}(Y)\) expression is inversely related to tumor differentiation.

**Analysis of FNABs.** FNAB is an integral part of the diagnostic evaluation of patients with thyroid nodules. Therefore, we prospectively analyzed 10 cases of FNABs performed on patients who presented with solitary thyroid nodules and who, after the cytological diagnosis and clinical work-up, underwent surgical removal of the thyroid mass. The histological diagnoses were carcinoma in four patients (two papillary carcinomas and two follicular carcinomas) and follicular adenoma in six patients. In addition, FNABs from the contralateral lobe were performed in one patient with follicular adenoma and one with follicular carcinoma. These two additional FNABs yielded normal thyroid follicular cells that were used as controls. All cytological specimens were analyzed for HMG\(\text{I}(Y)\) expression by immunohistochemistry and RT-PCR. The results are summarized in Table 2 and illustrated in Figs. 4 and 5. There was full concordance between the immunohistochemical and RT-PCR findings. Detectable HMG\(\text{I}(Y)\) expression with either technique correlated with a diagnosis of carcinoma in all cases analyzed \((P = 0.002)\).

**DISCUSSION**

Here, we describe the analysis of 358 thyroid specimens, compatible with the spectrum of hyperplastic or neoplastic proliferative lesions of thyroid follicular epithelium, for HMG\(\text{I}(Y)\) expression. The study was performed by immunohistochemistry, and the results were confirmed in a selection of representative cases by RT-PCR. A strong correlation was found between HMG\(\text{I}(Y)\)-positive samples and a diagnosis of carcinoma, regardless of the technique used. These findings are consistent with our previous observations in a limited number of thyroid neoplasms \((7)\) and with the activation of HMG\(\text{I}(Y)\) during the process of neoplastic transformation in the thyroid gland, as well as in other tumor models \((13–19)\). The purpose of this study was to retrospectively evaluate the stringency of the association between HMG\(\text{I}(Y)\) and a diagnosis of malignancy in thyroid neoplasia by analyzing a large number of cases retrieved from the pathological
archives of five different institutions in Europe and the United States. Despite the immunohistochemical detection of HMG(Y) in approximately one-fifth of the 200 follicular adenomas analyzed in this series, the strong correlation between HMG(Y) expression and a histological diagnosis of malignancy \( (P < 0.0001) \) indicates that immunohistochemistry for HMG(Y) may be a useful diagnostic indicator.

Significant levels of HMG(Y) expression are detectable in embryonal tissues \( (12) \) and malignant tumors \( (13-19) \). However, recent studies have shown that HMG(Y) may be also expressed in a variety of benign mesenchymal tumors as a result of specific chromosomal rearrangements involving the HMG(Y) gene, located at 6p21 \( (24-27) \). Cytogenetic analysis of thyroid neoplasms has revealed at least one case of thyroid adenoma with a translocation in the chromosome region 6p21.\(^4\) This chromosome alteration may induce expression of the HMG(Y) gene by several molecular mechanisms (such as promoter alteration, fusion with other genes, and deletions of the 3' end of the untranslated gene), similar to what already demonstrated in the other benign neoplasms \( (24-27) \), and, therefore, may account for the occasional detection of HMG(Y) in benign thyroid tumors. The alternative explanation that the thyroid nodules expressing HMG(Y) have already transformed and are, in fact, biologically if not yet clinically malignant must be taken into account. However, the HMG(Y)-positive tumors classified as adenomas underwent careful pathological evaluation. They were not histologically different from the HMG(Y)-negative adenomas, and the clinical follow-up available has shown no difference in behavior between HMG(Y)-positive and -negative adenomas. To verify the potential use of HMG(Y) as marker for thyroid carcinoma, we also prospectively analyzed its expression in 12 FNAB samples from 10 patients with solitary thyroid nodules who subsequently underwent surgery because of the cytological diagnosis. Results of FNABs usually fall into four different categories: probably benign, suspicious, malignant, and inadequate for diagnosis. Although papillary carcinoma can be easily diagnosed when the characteristic cytoarchitectural features are present in the FNAB, it is virtually impossible to distinguish benign from malignant among well-differentiated follicular neoplasms for which the ultimate diagnosis depends on the identification, in the surgically resected specimen, of vascular and/or capsular invasion by the tumor. The cytological diagnosis of follicular neoplasm is, therefore, included in the suspicious category and is an indication for surgical removal of the node. Up to 23% of thyroid FNABs are diagnosed as suspicious, but only an average of 20% of the cytologically suspicious nodules are eventually confirmed to be malignant \( (3) \). The search for adjunctive methods to improve diagnostic efficacy has included tumor DNA content and nuclear ploidy analysis in FNABs but has, thus far, given conflicting results \( (3) \). In this continuous effort, we have found HMG(Y) to be of potential diagnostic value. HMG(Y) expression could not only be easily detected on cytological specimens by both immunohistochemistry and RT-PCR, but there was full correlation with the histological diagnosis. These results indicate the promising potential of HMG(Y) as a diagnostic tool, despite the presence of its expression in a minority of histologically and clinically benign thyroid nodules. Recent data from our laboratory suggest that additional transcription factors may demonstrate differential expression in thyroid tumors. In fact, our group has recently shown that the fra-1 gene is activated in human thyroid neoplasias. However, its expression is not restricted to malignant histotypes because it was also detected in the majority \( (80\%) \) of thyroid adenomas \( (28) \).\(^5\) In contrast, Ets protein family members \( (Ets-1, Ets-2, and Elf-1) \) are abundantly present only in the malignant forms of thyroid neoplasias.\(^6\) Other genes coding for proteins other than transcriptional factors are differentially expressed in normal thyroid and in benign and malignant thyroid tumors. In fact, the thymosin \( \beta-10 \) gene is overexpressed in thyroid carcinomas but not in adenomas \( (29) \). Moreover, recent results indicate that expression of the oncofetal fibronectin is restricted to papillary carcinoma \( (30) \). Therefore, the analysis of the expression of all these proteins in conjunction with HMG(Y) may contribute to the diagnosis of thyroid neoplastic diseases.

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