Diagnostic Usefulness of HBME1, Galectin-3, CK19, and CITED1 and Evaluation of Their Expression in Encapsulated Lesions With Questionable Features of Papillary Thyroid Carcinoma

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Abstract

We evaluated HBME1, galectin-3 (GAL3), cytokeratin (CK)19, and a new anti-CITED1 antibody in 127 follicular adenoma (FA) and papillary thyroid carcinoma (PTC) cases. The findings were used to evaluate 11 diagnostically challenging encapsulated follicular lesions with questionable features of PTC (FL/QPTC).

All 4 markers showed higher expression in PTC than FA. HBME1 was the most specific (96%), whereas CK19 was the most sensitive (96%). In addition, 100% specificity was seen with coexpression of HBME1/CK19. Negative expression of all 4 markers was 97% specific for FA. GAL3 and CITED1, less useful individually, could help in selective cases. FL/QPTC showed heterogeneous, often intermediate, staining patterns, implying that some FL/QPTCs may be biologically borderline lesions or represent a biologic spectrum of PTC. These antibodies can have a confirmatory role in distinguishing the follicular variants of PTC and FA. For FL/QPTC, these antibodies are helpful in some cases, their limitation perhaps suggesting the biologic ambiguity of these lesions.

Papillary thyroid carcinoma (PTC) is the most common thyroid malignancy and, in its classic form (PTCC), does not present a diagnostic difficulty. However, the follicular variant of PTC (PTCFV) often poses a diagnostic challenge in which the differential diagnosis includes other follicular patterned lesions such as follicular adenoma (FA) and follicular carcinoma. The distinction between these lesions is important because prognosis and management differ. Because PTCFV is diagnosed almost solely based on subjective cytologic criteria (nuclear grooves, nuclear clearing, nuclear overlap, and intranuclear pseudoinclusions) and no uniform minimal diagnostic criteria exist, it is not surprising that disagreements in the diagnosis of PTC have underscored the interobserver variability in the evaluation of these lesions.

In 1 study, Saxen et al found interobserver agreement for thyroid tumors to be only 58%, and similar findings have been documented repeatedly by additional studies. This interobserver variation is particularly true for encapsulated follicular lesions with partial or incomplete features of PTC. Williams proposed the term well-differentiated tumors of uncertain malignant potential (UMP) for such lesions in which the cytologic features are not developed enough to ensure an unequivocal diagnosis of PTC, and these tumors have been suggested as possible precursors to invasive PTC. However, this term has not been accepted universally as a diagnostic term, and uncertainty remains about the nature of these lesions and their relationship to PTC.

In an attempt to resolve this common diagnostic difficulty, many immunohistochemical markers have been evaluated for their potential in distinguishing PTC from other follicular lesions, the main ones including cytokeratin (CK)19, galectin-3 (GAL3), and HBME1.
HBME1 is a monoclonal antibody to an unknown microvillous surface antigen present on mesothelial cells. Its usefulness as a marker of thyroid malignancy in fine-needle aspiration and tissue specimens has been demonstrated in several studies, showing diffuse strong staining in the majority of PTCs. Similarly, CK19 has been shown to be consistently overexpressed in PTC, in both the classic and the follicular variant. GAL3, a member of the B galactosil binding lectin family, for which normal functions include cell-cell regulation, growth, and differentiation in some studies, seemed to be a discriminating marker of well-differentiated follicular-derived neoplasms.

In addition to HBME1, CK19, and GAL3, recent DNA microarrays have shown CITED1 (Cbp/p300-interacting transactivator 1, MSG1) gene overexpression in PTC, suggesting CITED1 as a new and potentially useful diagnostic marker. CITED1 protein belongs to a family of nuclear proteins presumably involved in the regulation of transcription factors. Of the limited studies designed to address the diagnostic usefulness of this protein, CITED1 was shown to be promising in the distinction of PTC from benign thyroid lesions. Our experience with the anti-CITED1 polyclonal antibody, however, has been suboptimal.

Although the aforementioned immunohistochemical markers have been evaluated singly and in various combinations, the sensitivity and specificity for each marker varied among studies, and consensus of how to best use these markers is lacking. In the present study, using tissue microarray (TMA) technology, we first evaluated the diagnostic use of these 4 antibodies, including a new polyclonal anti-CITED1 antibody that we produced, in unequivocal cases of FA and PTC (including PTCFV). The findings from this “training set” then were used to evaluate encapsulated follicular lesions with questionable features of PTC (FL/QPTC), and the value and limitations of this antibody panel were assessed.

Materials and Methods

Case Selection and TMA

The files of New York Presbyterian Hospital Department of Pathology, New York, NY, were reviewed for benign and malignant thyroid lesions from January 2002 through December 2004. A total of 127 cases were included in the study. Formalin-fixed, paraffin-embedded blocks were retrieved. The histologic diagnosis for each case was reviewed, and only cases for which a consensus diagnosis was reached by 2 pathologists (T.S. and Y.-T.C.) were used.

Thyroid tumors were classified according to the current World Health Organization classification. The diagnosis of PTC was based on characteristic cytologic features, which included nuclear irregularity (grooves, indentations, clearing, and increased size) and pseudoinclusions. PTCFVs included tumors with follicular architecture, no papillary structures, and the presence of characteristic PTC nuclei. FAs were well-circumscribed, encapsulated lesions, lacking PTC nuclei and without evidence of capsular and/or vascular invasion. By these criteria, there were 49 cases of PTCC, 29 unequivocal cases of PTCFV, and 49 FAs.

Eleven cases were well-encapsulated follicular lesions in which the morphologic signs of PTC were qualitatively incomplete and that did not demonstrate evidence of capsular and/or vascular invasion. In essence, these cases could be classified as well-differentiated tumors of UMP as proposed by Williams. TMAs were constructed from formalin-fixed, paraffin-embedded tissue samples using a Beecher tissue microarray instrument (Beecher Instruments, Sun Prairie, WI). Each case was represented by three 1.0-mm tissue cores from neighboring areas on the same block. Complete tissue loss was not seen for any sample, allowing evaluation of all tissue samples for each antibody studied.

Production of CITED1 Recombinant Protein and Polyclonal Antibody

Expression cloning and production of recombinant CITED1 protein and rabbit polyclonal anti-CITED1 antibody was performed as previously described. Briefly, the full-length CITED1 complementary DNA sequence was obtained by reverse transcription–polymerase chain reaction using RNA extracted from a case of PTC. The complementary DNA was cloned into the BamHI-HindIII sites of pQE30 (Qiagen, Chatsworth, CA), a plasmid expression vector that contained a 6x-histidine tag, and the inserted CITED1 sequence was confirmed by DNA sequencing. The CITED1 protein then was produced in Escherichia coli and purified using Ni2+ ion affinity chromatography, following the manufacturer’s protocol (Qiagen). The CITED1 protein was used to immunize an NZW rabbit, and IgG antibody was purified from hyperimmune serum by protein A affinity chromatography (Covance, Denver, PA).

Immunohistochemical Staining

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded, 5-µm tissue sections using a Techmate automated immunostainer (Ventana Medical Systems, Tucson, AZ). The staining was performed according to a modified monoimmunoperoxidase protocol using the Envision+ horseradish-peroxidase mouse (or rabbit) detection system (DakoCytomation, Carpinteria, CA). The following mouse monoclonal antibodies were used: anti–galectin-3 (clone 9C4, dilution 1:200; Novocastra Laboratories, Newcastle upon Tyne, England), anti-HBME1 (clone HBME1, dilution 1:50; DakoCytomation),
and anti-CK19 (clone BA17, dilution 1:100; DakoCytomation). The CITED1 rabbit polyclonal IgG was used at a concentration of 1.0 µg/mL. Before staining, paraffin tissue sections were incubated with DAKO Target Antigen Retrieval Solution in a water bath for 95°C for 40 minutes. A case of PTCC positive for all 3 antibodies served as the positive control, and appropriate negative isotype control experiments also were run in parallel.

The staining intensity for each antibody was scored as follows: 0, negative; 1+, weak; 2+, moderate; and 3+, strong. Positive immunostaining was defined as 2+ to 3+ staining intensity in more than 10% of the cells. All 3 cores (when present) from the same lesion were taken into account in determination of protein expression.

**Results**

Clinicopathologic characteristics of the patients with the tumors included in the study are summarized in Table 1. No differential staining patterns were identified for the varying clinicopathologic parameters examined within individual histologic groups, eg, lymph node-positive vs lymph node-negative PTC cases.

HBME1 showed a predominantly membranous pattern. CK19 and GAL3 showed cytoplasmic staining mainly, with GAL3 also showing occasional nuclear staining. CITED1 showed a mixed nuclear and cytoplasmic staining pattern, with cytoplasmic expression the weaker component of the two in most cases. Of 78 PTC cases, 68 (87%) were CITED1+, showing a spectrum of staining intensity with heterogeneous, patchy staining seen in most of the weaker positive cases. Of 49 cases of FA, 8 (16%) showed positive CITED1 staining, 3 in a diffuse pattern. Adjacent normal thyroid glandular and stromal tissues were negative for CITED1.

The immunohistochemical results for HBME1, GAL, CITED1, and CK19 in the various tumors are summarized in Table 2.

**Papillary Thyroid Carcinoma**

Positive immunostaining was observed in a higher percentage of PTCs than in FAs for all 4 markers (Table 2). Of 78 PTCs, 75 (96%) showed positive staining with CK19. Positive staining for GAL3 was seen in 73 (94%) of 78. Expression of CITED1 and HBME1 was seen in fewer PTCs, each showing positivity in 68 (87%) of 78 cases. All PTC cases, with the exception of 1 PTCFV, showed staining with at least 1 of the 4 antibodies.

All 49 PTCs stained strongly with CK19, and the 3 tumors negative for CK19 were all PTCFVs. GAL3, CITED1, and HBME1 stained 47 (96%), 44 (90%), and 43 (88%) of 49 PTC cases, respectively. Frequencies of positive staining were lower for the 29 PTCFVs, with GAL3, HBME1, and CITED1 showing positive staining in 26 (90%), 25 (86%), and 24 (83%), respectively. Coexpression of 2 or more markers was observed in 74 (95%) of 78 PTCs. Of the 4 tumors that did not show coexpression of 2 or more markers, 1 was a PTC that showed only staining for CK19, and the 3 remaining cases were PTCFVs. Two of the PTCFVs stained with CK19, and 1 was negative for all 4 antibodies. Coexpression of HBME1, GAL3, and CK19 was seen in 42 (86%) of 49 PTCs and 23 (80%) of 29 PTCFVs. Coexpression of all 4 antibodies was seen in 38 (78%) of 49 PTCs and 21 (72%) of 29 PTCFVs.

The specificity and sensitivity of different antibody and antibody combinations were calculated. For a single antibody, HBME1 was the most specific (96%), and GAL3 had the lowest specificity among the 4 markers. In contrast, CK19 was the most sensitive for the diagnosis of PTC vs FA (96%) and PTC vs FA (100%). However, when comparing PTCFV vs FA, CK19 and GAL3 showed equal sensitivity (90%), higher than CITED1 or HBME1. For antibody combinations, coexpression of any 2 antibodies raised the specificity of diagnosing PTC to more than 90% (range, 92%-100%), with coexpression of HBME1 and CK19 being 100% specific. This antibody combination has a sensitivity of 83% (65/78).
Image 1 Characteristic immunohistochemical expression profile in the classic form of papillary thyroid carcinoma. A, Classic cytologic features: nuclear grooves, clearing, elongation, and overlapping (H&E, x400). B, HBME1 shows diffuse membranous positivity (x400). C, Cytokeratin 19 shows strong, predominantly cytoplasmic staining (x400). D, Galectin-3 shows predominantly cytoplasmic staining with occasional nuclear staining (x400). E, CITED1 (Cbp/p300-interacting transactivator 1, MSG1) shows nuclear and cytoplasmic staining (x400).
Follicular Adenomas

The majority of FAs (31/49 [63%]) showed negative staining for all 4 antibodies (Image 3). Of the 49 FA cases, CK19 was positive in 7 (14%), GAL3 in 9 (18%), and CITED1 in 8 (16%). In comparison, HBME1 staining was present in only 2 (4%) of 49 FAs. One showed focal staining, and the other showed strong, diffuse positivity. Coexpression of 2 or more antibodies was identified in only 5 (10%) of 49 cases. Two cases stained with 2 antibodies (one with GAL3 and CK19, the other with GAL3 and CITED1), and 3 stained with 3 antibodies (2 with HBME1, GAL3, and CITED1 and one with GAL3, CK19, and CITED1). Coexpression of HBME1, GAL3, and CK19 was not seen in any FA.

For the diagnosis of FA, negative staining with all 4 antibodies is highly specific (98%) but low in sensitivity (63%). In comparison, the HBME1-CK19 combination seemed to be a more effective antibody combination, and negative staining for both markers was highly indicative of FA (99% specificity and 82% sensitivity).

Encapsulated Follicular Lesions With Questionable Features of PTC

Eleven cases were identified in which questionable features of papillary carcinoma were present, i.e., the cytologic features of PTC were qualitatively incomplete. The immunohistochemical results for these cases are listed in Table 3.
### Table 2
**Expression of Four Immunohistochemical Markers in Thyroid Tumors**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>PTCC (n = 49)</th>
<th>PTCFV (n = 29)</th>
<th>FA (n = 49)</th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
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<td>HBME1</td>
<td>43 (88)</td>
<td>25 (86)</td>
<td>2 (4)</td>
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<td>87</td>
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<td>CK19</td>
<td>49 (100)</td>
<td>26 (90)</td>
<td>7 (14)</td>
<td>86</td>
<td>96</td>
</tr>
<tr>
<td>CITED1</td>
<td>44 (90)</td>
<td>24 (83)</td>
<td>8 (16)</td>
<td>84</td>
<td>87</td>
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<td>GAL3</td>
<td>47 (96)</td>
<td>26 (90)</td>
<td>9 (18)</td>
<td>82</td>
<td>94</td>
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<tr>
<td>HBME1/CK19</td>
<td>43 (88)</td>
<td>23 (79)</td>
<td>0 (0)</td>
<td>100</td>
<td>83</td>
</tr>
<tr>
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<td>44 (90)</td>
<td>22 (76)</td>
<td>1 (2)</td>
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<tr>
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<td>25 (86)</td>
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<td>HBME1/CITED1</td>
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<td>23 (79)</td>
<td>2 (4)</td>
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<tr>
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<td>24 (83)</td>
<td>2 (4)</td>
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<td>24 (83)</td>
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<td>23 (79)</td>
<td>0 (0)</td>
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<td>HBME1/CITED1/CK19</td>
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<td>21 (72)</td>
<td>0 (0)</td>
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<td>77</td>
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<tr>
<td>CK19/CITED1/GAL3</td>
<td>43 (88)</td>
<td>22 (76)</td>
<td>1 (2)</td>
<td>99</td>
<td>83</td>
</tr>
<tr>
<td>HBME1/GAL3/CITED1</td>
<td>38 (78)</td>
<td>23 (79)</td>
<td>2 (4)</td>
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<td>78</td>
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</table>

CITED1, Cbp/p300-interacting transactivator 1, MSG1; CK, cytokeratin; FA, follicular adenoma; GAL3, galectin-3; PTC, papillary thyroid carcinoma; PTCC, classic PTC; PTCFV, follicular variant of PTC.

* Data are given as number (percentage) positive unless otherwise indicated.

### Image 3
**Immunohistochemical protein expression profiles in representative follicular patterned lesions.** Follicular variant of papillary thyroid carcinoma (PTC) (A, H&E, ×400) showing strong and diffuse expression for HBME1 (B, ×400), cytokeratin 19 (CK19) (C, ×400), galectin-3 (GAL3) (D, ×400), and CITED1 (Cbp/p300-interacting transactivator 1, MSG1) (E, ×400). Follicular lesion with questionable features of PTC (FL/QPTC) (F, Case 4, H&E, ×400) negative for HBME1 (G, ×400) and CK19 (H, ×400) and positive for GAL3 (I, ×400) and CITED1 (J, ×400). FL/QPTC (K, Case 7, H&E, ×400) positive for HBME1 (L, ×400), CK19 (M, ×400), and CITED1 (O, ×400) and negative for GAL3 (N, ×400). Follicular adenoma (P, H&E, ×400) showing characteristic negative staining for HBME1 (Q, ×400), CK19 (R, ×400), GAL3 (S, ×400), and CITED1 (T, ×400).
cases stained for at least 1 antibody. Four cases were positive for all 4 antibodies. Two cases stained for 3 of 4 antibodies: one stained with a combination of GAL3, CITED1, and HBME1, and the other stained with CK19, CITED1, and HBME1. Of the 5 remaining cases, 4 stained for 2 antibodies, and 1 stained for CK19 only. CITED1 showed the highest frequency of staining—positive in 10 (91%) of 11 cases. In comparison, CK19, GAL3, and HBME1 staining each were present in 7 (64%) of 11 cases. Qualitatively, however, the staining of cells in this group of lesions tended to be more heterogeneous and lower in the percentage of positive cells than in PTC cases with complete cytologic features, as exemplified in Image 3.

**Discussion**

Although CK19, HBME1, GAL3, and CITED1 all have been shown to have higher expression in PTC than in benign follicular lesions of the thyroid, the results have varied among studies. In addition, how to best use these antibodies in routine practice, particularly when dealing with encapsulated lesions with questionable PTC features, has not been addressed fully. This study was designed to address these issues, using TMAs that contained cases of unequivocal PTC and FA, as well as histologically equivocal cases. The use of TMA for evaluating protein expression has been shown to be a valid and acceptable technique with excellent concordance between TMAs and full sections.35,38-40

The usefulness of CK19 in diagnosing PTC has been studied extensively, with most studies showing strong diffuse expression of CK19 in the majority of PTCs. In our study, CK19 was the most sensitive marker among the 4 evaluated. On the other hand, CK19 was expressed in approximately 14% of FAs, making it one of the less specific markers. CK19 expression in FA also has been reported by others,14-16,19,22,23,25 and most authors agree that the usefulness of this marker for distinguishing PTC from FA lies in the intensity and distribution of staining, with most adenomas tending to have more focal and weak staining and carcinomas showing strong, diffuse expression. Because focal or weak expression has been seen in nodular hyperplasia and normal tissue,14,16,19,22,23,25 comparison between lesional and adjacent normal thyroid tissue also is crucial in assessing CK19 expression.

In contrast with CK19, HBME1 was the most specific among the 4 markers analyzed, and we concluded that diffuse and intense membranous staining with HBME1 strongly supports the diagnosis of PTC. The sensitivity of HBME1 varied in different studies, and although it was the most sensitive marker in some studies,16,41 in our study, it actually was one of the least sensitive markers along with CITED1. Therefore, the high sensitivity of CK19 and high specificity of HBME1 can be used in a complementary manner. In our series of unequivocal cases, tumors showing diffuse strong positivity for both were exclusively PTCs, and the ones negative were almost exclusively benign (one PTCFV was negative for both).

In addition to CK19 and HBME1, we also evaluated CITED1 and GAL3 as potential markers. The CITED1 messenger RNA transcript has been shown repeatedly to be among the most highly differentially expressed genes in PTC, distinguishing it from other benign and malignant thyroid lesions.33,34 Studies so far to evaluate CITED1 expression immunohistochemically have relied on a rabbit antipeptide antibody.19,33,35 Although most studies reported good distinction between PTC and FA using this antibody, 1 recent report claimed positive staining in most benign thyroid lesions,42 and we also have found previously that this antipeptide antibody tended to generate higher background (usually cytoplasmic) staining than CK19, HBME1, and GAL3 antibodies, and the interpretation was difficult in many cases.36 For that reason, we produced and tested a rabbit polyclonal anti-CITED1 recombinant protein antibody for this study. As would be anticipated for polyclonal antibodies against multiple epitopes, this antibody showed an improved signal/background ratio over the one used in previous studies (data not shown). With this new antibody, however, we still found CITED1 to be less sensitive than CK19 and less specific than HBME1, staining 87% of PTCs and 16% of FAs. Therefore, the value of CITED1 as a first-line antibody for this differential diagnosis is limited.

GAL3 initially was found in some studies to be a good marker of malignancy, distinguishing PTC and follicular carcinomas from benign conditions.29,30,43,44 However, several additional studies have shown GAL3 expression in benign conditions and FAs, in addition to staining inflammatory cells and reactive normal epithelium, dampening the usefulness of this marker in discriminating benign from malignant.19,25,45,47 In the present series, we found GAL3 to be slightly more sensitive than CITED1, but it had a similar lack of specificity as a first-line marker for PTC.

**Table 3**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>CK19</th>
<th>GAL3</th>
<th>CITED1</th>
<th>HBME1</th>
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<tr>
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</table>

CITED1, Cbp/p300-interacting transactivator 1, MSG1; CK, cytokeratin; GAL3, galectin-3.

*Staining was scored as follows: –, negative; 1+, weak; 2+, moderate; 3+, strong.
Equipped with these findings, we studied the diagnostic usefulness of these antibodies in FL/QPTC. Of 11 cases, 4 stained with all 4 antibodies and 2 stained with 3 antibodies, leading us to conclude that these 6 cases are bona-fide PTC cases, despite their incomplete histologic features. However, the 5 remaining cases of FL/QPTC showed histologic features and expression profiles intermediate typical PTC and FA, often with heterogeneous immunohistochemical staining patterns. This finding suggests that these lesions may be biologically borderline lesions that are not fully malignant, corresponding to the proposed UMP category. Similar immunohistochemical observations recently have been made. In the study by Papotti et al, the expression of GAL3 and HBME1 was studied in 13 so-called well-differentiated tumors of UMP, and some degree of staining with either antibody was noted in 12 of 13 cases, which the authors concluded as evidence supporting a relationship between these lesions and PTC and that the nuclear features observed were not mere artifacts.

Alternatively, it also is possible that these lesions represent a biologic spectrum of PTC with lesions showing well-developed features at one end and those with incomplete features at the opposite end and, therefore, that these lesions all should be treated as PTCs clinically. Consistent with this notion of the biologic spectrum of PTC, it is worth noting that a difference in expression of these 4 markers also was seen between PTCC and PTCFV, with PTCFV showing a lower frequency of positive staining and less intense staining in general. Whether this diversity in morphologic and phenotypic findings is of clinical significance and whether one should identify UMP as a distinctive category can be validated only by long-term follow-up studies of a larger number of these lesions. On the other hand, this morphologic diversity of PTC most likely parallels the molecular changes in these tumors. In a recent study, for example, Adeniran et al showed that mutational status (BRAF, RET/PTC, and RAS) correlated with morphologic features, with PTCFV showing less prominent nuclear features and harboring a higher frequency of RAS mutations. Anticipating that such molecular studies might help shed light on these borderline or UMP lesions, we are pursuing mutational analysis (BRAF, RET/PTC, and RAS) and DNA microarrays, and preliminary results support the notion that these lesions are biologic intermediates (data not shown).

The identification of this borderline group of tumors also means that an unambiguous diagnosis of PTC and FA that will be agreed on by all pathologists will not be possible for every FL/QPTC case, even with the help of immunohistochemical markers. Therefore, morphologic diagnosis remains the “gold standard” for PTC, particularly in these cases. Immunohistochemical findings, if in conflict with the histologic interpretation, could be included in the diagnosis as a cautionary note to suggest the possible borderline nature of these lesions.

Despite this limitation, however, we would conclude that immunohistochemical markers that include HBME1, GAL3, CK19, and CITED1 are helpful in the diagnosis of PTC and its follicular variant. Strong expression of 2 or more markers supports the diagnosis of PTC, particularly if HBME1 is one of the positive antibodies. Negative staining with 3 or 4 antibodies, in contrast, strongly supports the diagnosis of FA. Using these antibodies can have a confirmatory role in distinguishing PTCFV and FA, being most helpful in preventing overdiagnosis and underdiagnosis of PTCFV by less experienced pathologists. For FL/QPTC lesions, this panel of antibodies also can be helpful in a proportion of cases, with its limitation likely reflecting the biologic ambiguity of these lesions.

References


