Galectin-3, fibronectin-1, CITED-1, HBME1 and cytokeratin-19 immunohistochemistry is useful for the differential diagnosis of thyroid tumors

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The diagnosis of thyroid tumors is critical for clinical management; however, tumors with follicular architecture often present problems. We evaluated the diagnostic use of the protein expression of four genes that were found to be upregulated in papillary thyroid carcinoma compared to normal thyroid (*LGALS3*, *FN1*, *CITED1* and *KRT19*), and of the mesothelial cell surface protein recognized by monoclonal antibody HBME1 in thyroid tumors. Tissues from 85 carcinomas (67 papillary, six follicular, eight Hürthle cell and four anaplastic) and 21 adenomas were evaluated by immunohistochemistry for the expression of these gene protein products, for example, galectin-3 (GAL3), fibronectin-1 (FN1), CITED1, cytokeratin-19 (CK19) and HBME1. Non-neoplastic thyroids (29 adenomatous and 14 thyrotoxic hyperplasia, and 59 normal) were also studied. The expression of all five proteins was significantly associated with malignancy, and highly specific ($\geq 90\%$) for carcinoma compared to adenoma. GAL3, FN1 and/or HBME1 expression was seen in 100% of carcinomas (85/85) and in 24% of adenomas (5/21). Coexpression of multiple proteins was seen in 95% of carcinomas and only 5% of adenomas ($P < 0.0001$). Coexpression of FN1 and GAL3 (FN1+GAL3+, 70/85) or FN1 and HBME1 (FN1+HBME1+, 53/85) was restricted to carcinomas, while their concurrent absence (FN1–GAL3– or FN1–HBME1–, 18/21 adenoma) was highly specific (96%) for benign lesions. Among non-neoplastic thyroids, adenomatous hyperplasia frequently expressed GAL3 ($n=16$), CK19 ($n=9$) and CITED1 ($n=7$), but the expression was predominantly focal in contrast to the diffuse expression in carcinomas. An immunohistochemical panel consisting of GAL3, FN1 and HBME1 may be useful in the diagnosis of follicular cell-derived thyroid tumors.

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The prognosis and management of thyroid nodules depends on their diagnoses. The current diagnostic 'gold standard' is pathologic evaluation using routine hematoxylin and eosin (H&E) stains. However, morphologic similarities between benign and

malignant lesions are frequent, and follicular and papillary architectures may be seen in both benign and malignant lesions. Several critical features of malignancy, for example, pale nuclei for papillary thyroid carcinoma are open to subjective interpretations, and interobserver disagreements among pathologists are well documented. Saxen *et al*¹ found only 58% agreement among five Nordic pathologists who tested the reproducibility of the WHO classification (1978) on 696 thyroid nodules. A more recent study compared the diagnoses of 21 follicular nodules by four American and four Japanese pathologists and showed an agreement of benign vs malignant in only 62% of the nodules.² A review of 200 thyroid tumors by seven Italian

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pathologists revealed good agreement for papillary and anaplastic thyroid carcinomas, moderate for medullary and poor for follicular thyroid carcinomas.³ In another review of 41 follicular carcinomas by five experienced French thyroid pathologists, the agreement for malignancy varied from 5% among all five pathologists to 56% between two pathologists.⁴ Of concern, the interobserver and intraobserver agreements for vascular invasion were only 20 and 68%, respectively. Thus, despite well-described criteria, the diagnostic agreement among pathologists remains poor. This underscores the need for additional diagnostic markers.

Recently, our group published a consistent gene expression profile for papillary thyroid carcinoma compared to normal thyroid.⁵ Here, we study the protein expression of four of the upregulated genes, *LGALS3*, *FN1*, *CITED1* and *KRT19*, and the expression of the mesothelial cell surface protein HBME1 in various thyroid nodules to evaluate their usefulness in differential diagnosis. *LGALS3* codes for galectin-3 (GAL3), a β -galactosil-binding lectin involved in regulating cell-cell and cell-matrix interactions. It is expressed in normal breast epithelial cells, inflammatory cells and various malignant cells. Several investigators have found GAL3 expression to be of value in discriminating between benign and malignant thyroid nodules.^{6–12} Fibronectin is an extracellular matrix protein produced by fibroblasts. Production of fibronectin by thyroid follicular cells is believed to be associated with transformation.^{5,13–15} Using the reverse transcription-PCR technique, Takano *et al*¹⁶ have suggested that fibronectin may be an accurate preoperative molecular diagnostic marker for papillary thyroid carcinoma. The *CITED1* gene encodes a 27-kDa protein belonging to the CITED (CBP/p300-Interacting Transactivators with glutamic acid [E] and aspartic acid [D]-rich C-terminal domain) family of nuclear proteins.¹⁷ The CITED proteins are believed to coregulate nuclear transcription proteins. Initial studies demonstrated a possible role in

melanocyte differentiation, hence the synonym *MSG1* (melanocyte-specific gene 1).¹⁸ Nuclear and cytoplasmic expression of CITED1 has been noted in melanocytes, breast epithelial cells, and testicular germ cells. *KRT19* encodes cytokeratin-19 (CK19), a cytoskeletal protein that is significantly increased in papillary thyroid carcinoma, and has been reported to be helpful in distinguishing it from other benign and malignant thyroid nodules.^{5,19–23} HBME1, a monoclonal antibody generated against a suspension of malignant epithelial mesothelioma cells, reacts with the microvillous surface protein of mesothelial cells. We included HBME1 because its expression has been reported in papillary and follicular thyroid carcinoma but not in normal thyroid cells.^{23–25}

Materials and methods

The study was comprised of 215 formalin-fixed paraffin-embedded thyroid tissues (Table 1). There were 67 papillary thyroid carcinoma, six follicular, eight Hürthle cell and four anaplastic carcinomas. The papillary thyroid carcinomas included 44 classic, 13 follicular, seven oncocytic, one columnar and two solid/poorly differentiated variants. Follicular and Hürthle cell carcinomas were diagnosed by the presence of complete capsular and/or vascular invasion, and the absence of nuclear features of papillary thyroid carcinoma. Hürthle cell carcinomas were composed of greater than 75% oncocytic cells having moderate to abundant eosinophilic granular cytoplasm.²⁶ Anaplastic thyroid carcinomas were comprised of high-grade pleomorphic spindle cells. Adenomas were defined as completely encapsulated follicular or Hürthle cell tumors with homogeneous architecture and morphology, lacking nuclear features of papillary thyroid carcinoma and without capsular and vascular invasion. Encapsulated follicular tumors showing atypical architecture (solid/trabecular pattern),

Table 1 Protein expression in thyroid

Diagnosis	GAL3	FN1	CITED1	HBME1	CK19	Coexpression ^a
Carcinoma						
Papillary (n = 67)	63 (94%)	61 (91%)	58 (87%)	57 (85%)	48 (72%)	65 (97%)
Follicular (n = 6)	4 (66%)	3 (50%)	3 (50%)	3 (50%)	3 (50%)	5 (83%)
Hürthle cell (n = 8)	7 (88%)	6 (75%)	2 (25%)	1 (13%)	4 (50%)	7 (88%)
Anaplastic (n = 4)	4 (100%)	4 (100%)	0 (0%)	0 (0%)	1 (25%)	4 (100%)
Neoplasms of undetermined malignant potential (n = 7)	6 (86%)	1 (14%)	3 (43%)	2 (29%)	1 (14%)	3 (43%)
Adenoma (n = 21)	2 (10%)	1 (5%)	2 (10%)	2 (10%)	1 (5%)	1 (5%)
Non-neoplastic thyroid						
Nodular goiter (n = 29)	16 (55%)	2 (7%)	7 (24%)	1 (3%)	9 (31%)	11 (38%)
Thyrotoxic hyperplasia (n = 14)	1 (7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Normal (n = 59)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (7%)	0 (0%)

GAL3 and/or FN1 and/or HBME1: 85/85 carcinoma and 5/21 adenoma.

^aCoexpression of ≥ 2 proteins.

Table 2 Antibodies used in this study

<i>Antibody</i>	<i>Clone & source</i>	<i>Dilution</i>	<i>Source</i>
GAL3	Monoclonal, NCL-Gal3	1:300	Novocastra, UK
HBME1	Monoclonal, HBME-1	1:200	Dako, Carpinteria, CA, USA
CK19	Monoclonal, RCK108	1:100	Dako, Carpinteria, CA, USA
FN1	Polyclonal, A0245	1:2000	Dako, Carpinteria, CA, USA
CITED1	Polyclonal, J72220K	1:2000	T Shioda, Boston, MA, USA

Dako's Target Retrieval Solution was used to carry out the heat-induced antigen retrieval for GAL3, FN1 and CITED1.

severe cytologic atypia or incomplete capsular invasion without definite vascular invasion were classified as follicular neoplasms of uncertain malignant potential.^{27,28} We also included 29 nodular goiters, 14 diffuse thyrotoxic hyperplasia and 59 normal thyroid tissues to study the protein expression in non-neoplastic thyroid lesions. All cases were initially diagnosed by a pathologist (not a coauthor) and then reviewed by a second pathologist (MLP).

Immunohistochemistry was performed on 4 μ m thick sections using the labeled streptavidin–biotin peroxidase complex system (LSAB2) in a Dako Autostainer (DakoCytomation, Carpinteria, CA, USA). Heat-induced antigen retrieval was carried out for GAL3, FN1 and CITED1 (Dako's Target Retrieval solution, pH 6.1, steaming for 30' at 94°C) and sections were incubated with primary antibodies (Table 2) for 30'–60' at room temperature. After primary antibodies, all sections were blocked for endogenous avidin and biotin by incubating with avidin solution for 20' followed by biotin solution for 20' (Dako's avidin/biotin blocking system, X0590). Positive controls were normal breast for GAL3 and CITED1, tonsil for FN1, small intestinal mucosa for CK19 and an epithelioid mesothelioma for HBME1. Appropriate negative controls by substituting primary antibody with isotype-matched mouse or rabbit IgG were also included.

Interpretation and Analysis

CITED1 and GAL3 expressions were nuclear and cytoplasmic. CK19 expression was cytoplasmic only. HBME1 and FN1 expressions were cytoplasmic and membranous with luminal accentuation. Normal FN1 expression in fibrous tissue and basement membrane served as internal control. The protein expression was initially assessed along a scale of 0–100% of tumor cells showing immunoreactivity. After evaluating normal and hyperplastic thyroid tissues, positive (increased) expression was defined as presence of $\geq 10\%$ immunoreactive thyrocytes. Sensitivity (true positive/true positive + false negative), specificity (true negative/true negative + false positive) and accuracy (true positive + true negative/all positive + all negative) of the markers and their combinations were compared.

Two-sided Fisher's exact test was used to determine statistical significance with α level set at ≤ 0.05 .

Results

The expression of GAL3, FN1, CITED1, HBME1 and CK19 in various thyroid tissues is shown in Table 1 (Figures 1–3). In general, the expression of the proteins in malignant tumors was diffuse, while in benign tumors and non-neoplastic thyroid tissues, there was no expression or focal expression, usually in less than a third of the tumor cells.

Carcinomas

Of 85 carcinomas, 81 (95%) coexpressed multiple proteins (Tables 1 and 3, Figures 1 and 2). There was no significant difference in protein expression among the variants of papillary thyroid carcinoma. All anaplastic thyroid carcinomas diffusely and strongly expressed GAL3 and FN1 (100%), and one additionally showed focal CK19 expression (Figure 2). The expression was more frequently diffuse in papillary and anaplastic carcinomas compared to others.

Adenomas

There were 19 follicular and two Hürthle cell adenomas. Two adenomas showed expression of GAL3 in approximately 10% of tumor cells (Figure 3). One adenoma each showed diffuse expression of FN1, CITED1, HBME1 and CK19, while a single adenoma (5%) coexpressed two proteins (CITED1 + HBME1 +). Hürthle cell adenomas did not express any of the proteins.

Neoplasms of Uncertain Malignant Potential

There were six follicular and one Hürthle cell tumor of undetermined malignant potential. All tumors were encapsulated follicular neoplasms showing incomplete capsular invasion ($n=5$) and/or some nuclear atypia that did not meet the criteria for papillary thyroid carcinoma ($n=3$). The expression of proteins was usually focal with diffuse expression

Figure 1 (a) Follicular variant of papillary thyroid carcinoma showing expression of (b) CITED1, (c) HBME1 and (d) CK19.

of GAL3 and CITED1 being seen in two tumors each. Three tumors (43%) showed coexpression of multiple proteins (GAL3 + HBME1 + CITED1 + in two

and GAL3 + FN1 + CITED1 + CK19 + in one tumor). No correlation was noted between their morphology and the protein expression.

Figure 3 Expression of proteins in benign thyroid nodules: (a) Follicular adenoma showing (b) focal GAL3 expression, but (c) no expression of FN1, (d) HBME1, and (e) CITED1. (f) Nodular goiter with cystic degeneration and papillary hyperplasia showing (g) GAL3, (h) weak CITED1, and (i) CK19 expression.

Non-Neoplastic Thyroids

Nodular goiters frequently showed follicular and focal papillary hyperplasia associated with fibrosis, hemorrhage, inflammation and cystic degeneration, and in addition to thyrocytes, several other cells expressed one or more proteins. Fibroblasts and inflammatory cells including macrophages expressed GAL3. Solid cell nests and metaplastic squamous cells expressed GAL3 and CK19. GAL3 was the most frequently expressed protein (55%) followed by CK19 (31%) and CITED1 (24%) in the thyrocytes (Table 1, Figure 3). However, the protein expression was usually focal with only three goiters (10%) showing diffuse expression of GAL3 and one each (3%) showing diffuse expression of CK19 and CITED1. FN1 expression was observed in extracellular fibrosis in most goiters but was considered positive in only two cases that demonstrated

cytoplasmic and membranous expression of FN1 in thyrocytes in association with fresh hemorrhage and fibrin deposition. Coexpression of multiple proteins, usually GAL3 and/or CK19 and/or CITED1 was seen in 11 goiters (38%).

All 14 *diffuse thyrotoxic hyperplasia* showed focal or diffuse papillary hyperplasia and did not express any protein except one case that focally expressed GAL3 in approximately 10% of cells. Four *normal thyroid* tissues focally expressed CK19 in approximately 10–20% of thyrocytes. Normal thyroids did not express any other proteins. Thyrotoxic hyperplasia and normal thyroid tissues did not show diffuse expression or coexpression of proteins.

Statistical Analysis

The significance of these markers in differentiating malignant from benign tumors is shown in Tables 3

Figure 2 Expression of proteins in thyroid carcinomas: (a) Follicular thyroid carcinoma showing vascular invasion in a capsular blood vessels, (b) GAL3 expression in the tumor and the intravascular tumor emboli, and (c) CITED1 expression in tumor cells invading capsule. (d) Hürthle cell carcinoma showing (e) diffuse FN1 and (f) focal HBME1 expression. (g) Anaplastic thyroid carcinoma showing diffuse (h) GAL3 and (i) FN1 expression in malignant spindle cells.

Table 3 Coexpression and concurrent absence of expression

Coexpression	Carcinoma (n = 85)	Adenoma (n = 21)	Sensitivity for carcinoma (%)	Specificity for carcinoma (%)
FN1+GAL3+	70	0 (0%)	82	100
FN1+HBME1+	53	0 (0%)	62	100
> any two proteins ^a	81	1 (5%)	95	95
Concurrent absence			Sensitivity for adenoma (%)	Specificity for adenoma (%)
FN1- GAL3- ^b	3 (4%)	18	86	96
FN1-HBME1- ^b	3 (4%)	18	86	96

FN1+GAL3+: 59 papillary, two follicular, five Hürthle cell and four anaplastic carcinoma.

FN1+HBME1+: 52 papillary and one follicular carcinoma.

FN1-GAL3-: two papillary and one follicular carcinoma.

FN1-HBME1-: one papillary, one follicular and one Hurthle cell carcinoma.

^a $P < 0.0001$, accuracy for carcinoma—95%.

^bAccuracy for adenoma—94%.

Table 4 Differential diagnosis of benign and malignant tumors with follicular architecture

Proteins	Carcinoma ^a n = 28 (sensitivity)	Adenoma n = 21	Specificity (%)
GAL3 ^b	25 (89%)	2 (10%)	90
FN1	21 (75%)	1 (5%)	95
CITED1	19 (68%)	2 (10%)	90
CK19	18 (64%)	1 (5%)	95
HBME1	17 (61%)	2 (10%)	90

^aFollicular variants of 14 papillary carcinoma, six follicular carcinoma and eight Hürthle cell carcinoma.

^bAccuracy of GAL3 was the highest (90%).

and 4 and Figure 4. All five proteins were highly specific ($\geq 90\%$) for carcinomas. GAL3 was the most sensitive but failed to detect seven carcinomas (8% false negative). Tables 1 and 3 show that coexpression of multiple proteins was significantly more frequent in carcinomas than adenomas ($P < 0.0001$). Coexpression of some proteins, for example, FN1 + GAL3 + or FN1 + HBME1 + immunophenotype was observed only in carcinomas (100% specific), while their concurrent absence, that is, FN1-GAL3- or FN1-HBME1- immunophenotype was highly specific (96%) for adenomas (Table 3). Table 4 compares the expression of these proteins in benign and malignant tumors with follicular architecture, that is, 21 adenomas vs 14 follicular variants of papillary carcinoma, six follicular and eight Hürthle cell carcinomas. All five markers were highly specific ($\geq 90\%$) for carcinoma and GAL3 was the most sensitive (89%) and accurate (90%). CITED1 and/or

Figure 4 Differential expression of proteins in 85 carcinomas and 21 adenomas. GAL3: sensitivity 92%, specificity 90%, FN1: sensitivity 87%, specificity 95%, CITED1: sensitivity 74%, specificity 90%, HBME1: sensitivity 72%, specificity 90%, CK19: sensitivity 66%, specificity 95%.**Table 5** Follicular variant of papillary carcinoma vs follicular and Hürthle cell carcinoma

Protein	Papillary carcinoma (n = 14)	Follicular+ Hürthle cell carcinoma (n = 14)	P-value
CITED1	14 (100%)	5 (36%)	≤ 0.001
HBME1	13 (93%)	4 (29%)	≤ 0.01
CITED1+HBME1	13 (93%)	2 (14%)	< 0.0001
GAL3	14 (100%)	11 (79%)	0.22
FN1	12 (86%)	9 (64%)	0.38
CK19	11 (79%)	7 (50%)	0.23

HBME1 were significantly more associated with papillary than follicular or Hürthle cell carcinoma ($P < 0.01$, Table 5).

GAL3, FN1 and/or HBME1 were expressed in 100% of carcinoma, 86% of tumors of undetermined malignant potential (6/7) and 24% adenomas (5/21). GAL3, FN1 and/or CITED1 also revealed similar frequency of expression. All other triple or double antibody combinations were less than 100% sensitive for carcinoma.

When the differentiation of carcinoma from benign nodules included goiters, GAL3 was the least specific followed by CK19 and CITED1 (55, 31 and 24% false-positive goiters, respectively), whereas HBME1 and FN1 were most specific (3 and 7% false-positive goiters, respectively).

The thyroid tumors of undetermined malignant potential showed expression profiles intermediate between adenomas and carcinomas. Three tumors showed coexpression of multiple proteins (43 vs 5% adenoma vs 95% carcinoma). One tumor showed

coexpression of FN1 + GAL3 + (14 vs 0% adenoma vs 82% carcinoma), but none showed coexpression of FN1 + HBME1 + (0 vs 0% adenoma vs 62% carcinoma). One tumor of undetermined malignant potential lacked expression of FN1 and GAL3 (14 vs 86% adenoma vs 4% carcinoma), and four tumors lacked expression of FN1 and HBME1 (57 vs 86% adenoma vs 4% carcinoma).

Discussion

We selected four genes from among several genes found to be consistently upregulated in papillary thyroid carcinoma compared to normal thyroid because of the availability of antibodies against their protein products that gave robust immunoreaction in formalin-fixed paraffin-embedded tissues allowing their evaluation in archival tissues. The monoclonal antibody HBME1 was included for comparison because of its reported specificity for papillary thyroid carcinoma.^{23–25} Our study shows that these proteins were not only expressed in papillary thyroid carcinomas but also in other follicular cell-derived thyroid carcinomas. Indeed, this relative lack of specificity for malignant tumors was very useful in differentiating all carcinomas from adenomas.

One of the most frequent difficulties in thyroid pathology is differentiating adenomas from carcinomas, especially those with follicular architecture, for example, follicular variant of papillary thyroid carcinoma, follicular and Hürthle cell carcinomas.^{2,4} This differentiation is critical for the treatment and long-term management of the tumors. We found that all five markers were highly specific for carcinoma, but GAL3 was the most sensitive and accurate. Only two adenomas showed focal expression of GAL3 in approximately 10% of tumor cells. Other investigators have used GAL3 in differentiating follicular carcinomas from adenomas in fine-needle aspirates.^{9,11} However, certain problems emerged with GAL3. First, GAL3 expression was also seen in several nonthyroidal cells, for example, squamous cells, fibroblasts and inflammatory cells including macrophages. These nonthyroidal cells were often associated with hemorrhage, fibrosis and cystic degeneration, which are frequently present in nodular goiter. Indeed, GAL3 expression was seen in 55% of the goiters in the current series making it the least-specific marker if the differential diagnosis included goiters. Other investigators have also reported GAL3 expression in benign thyroid lesions by various techniques including reverse transcription-PCR.^{22,29–32} The second problem with GAL3 was that seven carcinomas (8%) were false negative.

Therefore, we next attempted to identify a combination of markers with no false-negative results. We found that all carcinomas were positive for GAL3, FN1 and/or HBME1. Thus, an immunohistochemical diagnostic panel comprising of these

three markers may improve sensitivity for carcinomas. This panel had other advantages as well, for example, coexpression of FN1 and GAL3 or FN1 and HBME1 (FN1 + GAL3 + or FN1 + HBME1 + phenotype) was diagnostic of carcinoma, whereas their concurrent absence (FN1–GAL3– or FN1–HBME1– phenotype) was highly specific for benign lesions. FN1 was first reported to be overexpressed in thyroid tumors by immunohistochemistry in 1988, but we are unaware of any subsequent studies comparing it to other markers of thyroid malignancy or evaluating its expression in hyperplastic nodules.¹⁴ We found FN1 expression by tumor cells to be nearly as sensitive as GAL3, and highly specific for all follicular cell-derived thyroid carcinomas. Several recent studies have reported HBME1 expression to be diagnostically useful in papillary thyroid carcinoma.^{23,25} However, to the best of our knowledge, no single marker by itself is 100% sensitive for malignancy. By immunohistochemistry, ret/PTC and PPAR γ are reported in <70% of papillary and follicular thyroid carcinomas, respectively, while CD10 is expressed in follicular carcinoma and follicular variant of papillary carcinoma, but not in conventional papillary thyroid carcinoma.^{33–36} Therefore, a combination of multiple markers may be more sensitive than any single marker. Our study shows that a diagnostic immunohistochemical panel comprising of GAL3, FN1 and HBME1 was 100% sensitive for all follicular cell-derived carcinomas. Although GAL3, FN1 and/or CITED1 were also expressed in all carcinomas, we preferred not to include CITED1 due to its higher false-positive results in goiters compared to FN1 and HBME1. In addition, unlike CITED1, antibodies to GAL3, FN1 and HBME1 are commercially available and can be readily used. No other combination of two or three proteins in our study proved to be 100% sensitive. HBME1 and CITED1 were highly specific for papillary thyroid carcinoma, especially when concurrently expressed, and were helpful in differentiating follicular variant of papillary from follicular and Hürthle cell carcinomas. Although this differentiation may not be critical for treatment, the precise classification of carcinoma is helpful in predicting outcome, and the route of metastasis. The association of HBME1 with papillary thyroid carcinoma in the current series is consistent with the observations made by other investigators.^{23,25}

The current study included seven tumors classified as neoplasms of uncertain malignant potential, a concept initially proposed by Rosai *et al*²⁷ and later recommended by Williams²⁸ following his examination of thyroid nodules that developed in the exposed population of the Chernobyl accident. The tumors were encapsulated, had a follicular architecture, did not show definite capsular or vascular invasion or showed some nuclear atypia and pallor that did not quite meet the diagnostic criteria for papillary thyroid carcinoma. Their

protein expression pattern was intermediate between adenoma and carcinoma. Interestingly, GAL3 expression was seen in six of seven neoplasms of uncertain malignant potential, which may support the proposal made by some investigators that GAL3 is a marker of early malignant transformation and minimally invasive carcinoma.^{11,22} Our results suggest the 'indeterminate or borderline' nature of these lesions. Although these markers fail to classify these tumors as clearly malignant or benign, they may help in selecting tumors for prolonged follow-up in order to determine their true biologic nature.

The role of CK19 in the diagnosis of thyroid carcinoma has been controversial. This may be partially due to the subjectivity involved in assessing positive expression. Sahoo *et al*²¹ found CK19 expression in all benign tumors although the majority of them expressed CK19 in <5% of tumor cells. Other investigators have reported diffuse expression in papillary thyroid carcinoma compared to focal expression in other tumors and nodular goiters.^{19,20} Although we used $\geq 10\%$ immunoreactive cells as the criteria for protein overexpression, we agree that sometimes benign and malignant tumors may show only borderline positive or negative reactions. Despite its apparent shortcoming, $\geq 10\%$ was a useful cutoff value in differentiating carcinomas from adenomas.

Lack of expression of the papillary thyroid carcinoma-associated proteins in normal thyroid and diffuse thyrotoxic hyperplasia shows that their expression is not associated with papillary formation or hyperplasia. Interestingly, nodular goiters expressed several of these proteins, in thyrocytes and in other reactive cells. GAL3, CK19 and CITED1 expression was noted in 55, 31 and 24% of goiters respectively. GAL3 was expressed in squamous cells, proliferating fibroblasts and inflammatory cells, for example, macrophages. Metaplastic squamous cells in benign thyroid and the embryonic remnant ultimobranchial body expressed CK19, as has been also reported by others.²³ Although only two goiters showed FN1 expression by thyrocytes, FN1 (being an extracellular matrix protein synthesized by fibroblasts) was increased in hemorrhage and fibrosis, often associated with goiters. Using $\geq 10\%$ immunoreactive thyrocytes as the criteria for protein overexpression helped avoid overinterpretation of nonspecific immunoreactivity. False-positive protein expression in benign lesions was usually focal and in less than one-third of the thyrocytes. Multiple protein markers in conjunction with careful morphologic examination would help resolve problems associated with borderline expressions.

A limitation of all thyroid studies is that the 'gold standard' (H&E examination) used to classify thyroid neoplasms as benign or malignant is flawed. Approximately half of our carcinomas had demonstrated metastases, and there was no difference in the immunohistochemical profile of those with

metastases or without metastasis. To the best of our knowledge, none of the adenomas or tumors of 'undetermined malignant potential' have metastasized (data not shown). While presence of metastasis confirms malignancy, lack of metastasis does not rule it out. Thus, benign lesions may be difficult to differentiate from malignant tumors that did not metastasize and may have been cured by treatment. In the current study, all thyroid lesions were initially diagnosed by a staff pathologist and then reviewed by one of the authors (MLP). We made an attempt to include unequivocal cases, reserving the term 'undetermined malignant potential' for tumors that could not be definitively categorized as benign or malignant.

In conclusion, GAL3, FN1, HBME1, CITED1 and CK19 were expressed in all follicular cell-derived carcinomas. Their expression was helpful in differentiating adenomas from carcinomas, especially those with follicular architecture, that is, follicular variants of papillary carcinoma, follicular carcinoma and Hürthle cell carcinoma. An immunohistochemical panel consisting of GAL3, FN1 and HBME1 was 100% sensitive for carcinomas. The phenotype FN1 + GAL3 + or FN1 + HBME1 + was diagnostic of carcinoma, while the phenotype FN1 – GAL3 – or FN1 – HBME1 – was highly specific of adenoma. CITED1 and HBME1 were significantly associated with papillary thyroid carcinoma and were helpful in differentiating follicular variants of papillary carcinoma from follicular carcinoma and Hürthle cell carcinoma. However, many of the proteins, for example, GAL3, CITED1 and CK19 were expressed focally in goiters. These markers need to be used in combination with one another, and in conjunction with careful morphologic evaluation.

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