Retinoblastoma Expression in Thyroid Neoplasms

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Retinoblastoma (Rb) mutation in thyroid neoplasia has been identified in a few molecular studies; however, the utility of Rb immunohistochemistry in distinguishing benign and malignant thyroid lesions has not been documented in formalin-fixed, paraffin-embedded tissues. The present study investigated Rb immunohistochemistry in a series of 111 formalin-fixed, paraffin-embedded benign and malignant thyroid lesions. All of the major histologic subtypes were included to detect any heterogeneity in Rb-1 expression that might influence the diagnostic utility of this technique or further elucidate the pathogenesis of thyroid neoplasia among the categories. Altogether, 34 follicular adenomas, 9 follicular carcinomas, 7 Hürthle cell adenomas, 5 Hürthle cell carcinomas, 23 papillary carcinomas (8 of which were follicular variants), 4 insular carcinomas, 4 anaplastic carcinomas, 6 medullary carcinomas, and 19 nodular goiters were analyzed. Avidin-biotin immunohistochemistry was performed using the Dako Rb-1 clone. Pronase digestion was introduced into the epitope retrieval protocol to eliminate false-positive cytoplasmic staining. Nuclear immunoreactivity was assessed as positive if 10% or more of thyroid epithelial nuclei stained positively, and conversely as negative. The majority of benign non-Hürthle thyroid lesions, whether hyperplastic or neoplastic, retained Rb nuclear immunopositivity in most cells (51 of 53 cases [96%]). Conversely, malignant thyroid neoplasms lacked Rb immunoreactivity in the majority (42 of 51 cases [82%]), including all papillary carcinomas (23 of 23) and almost all follicular carcinomas (8 of 9 [89%]). Virtually all Hürthle cell neoplasms were negative (11 of 12 [92%]), whether benign or malignant. In conclusion, Rb immunohistochemistry can aid in the distinction between benign and malignant thyroid lesions in conjunction with morphology. This seems to be most applicable to the often problematic differentiation between follicular adenoma and the follicular variant of papillary carcinoma ($P < .0001$; sensitivity and specificity, 100%) or minimally invasive follicular carcinoma ($P = .0007$; sensitivity, 89%; specificity, 100%).

KEY WORDS: Immunohistochemistry, Nodular goiter, Paraffin-embedded tissue, Retinoblastoma, Thyroid neoplasm.

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Retinoblastoma (Rb) was the first discovered tumor-suppressor gene (1–3). It maps to chromosome 13q14 and encodes a 110–114 KD nuclear protein that plays a major role in the regulation of cell growth arrest (4–6). Rb protein product (P-Rb) is expressed in all cells, where it exists in an active hypophosphorylated and an inactive hyperphosphorylated state. In its active state, P-Rb serves as a brake on the advancement of cells from G1 to S phase of the cell cycle. When the cells are stimulated by growth factors, Rb protein is inactivated by phosphorylation, allowing the cells to transverse the G1–S checkpoint. Once cells enter the S phase, they are committed to divide. During the ensuing M phase, phosphate groups are removed from P-Rb by cellular phosphatases, thus regenerating the active hypophosphorylated form of the protein (5–8). The hypophosphorylated P-Rb achieves cell cycle arrest by forming a complex with the E2F family of transcription factors. These complexes bind to DNA and actively inhibit the transcription of S-phase genes, thereby preventing cell division (5–9).

Germline loss or mutation of the Rb gene predisposes to the development of retinoblastoma and to a lesser extent osteosarcoma. Its role in the pathogenesis of retinoblastoma has been elegantly explained by Knudson's two-hit hypothesis (2). Furthermore, somatically acquired Rb mutations have been described in glioblastomas; sarcomas; small cell and non–small cell carcinomas of the lung; and breast, prostatic, and bladder carcinomas (10–20).

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A number of studies have investigated the role of proto-oncogene activation in thyroid neoplasia. Activation of c-*erb*, c-*fos*, c-*myc*, and *ras*, among other genes, has been described (21–25). However, the diagnostic utility of these findings is limited by the occurrence of these abnormalities in both benign and malignant tumors (21–25). The role of tumor-suppressor gene loss in carcinogenesis has been documented in multiple tumors. p53 is the most notable example and has also been described in thyroid tumorigenesis (26–28).

The presence of Rb mutation in thyroid neoplasms has been documented in a small number of cases (29–31). Rb protein immunohistochemistry in malignant thyroid tumors has generated controversial results in the few published studies (29–31). The only study that has evaluated the diagnostic utility of Rb immunohistochemistry in the differential diagnosis of benign and malignant thyroid lesions analyzed only nine thyroid tumors by molecular and immunohistochemical assays limited to frozen sections and cytospin preparations (29). The usefulness of Rb-1 antibody in paraffin sections in distinguishing benign and malignant thyroid neoplasms has not been evaluated.

The objective of the current study was to evaluate Rb-1 immunohistochemistry in routine formalin-fixed, paraffin-embedded benign and malignant thyroid disorders. We introduce a modified technique that does not have the drawback of high cytoplasmic background as documented in the few studies evaluating Rb protein expression in paraffin sections (32–34). We stratified thyroid lesions into the major histologic subtypes to detect any heterogeneity in Rb-1 expression that might influence the diagnostic utility of this technique or further elucidate the pathogenesis of thyroid neoplasia.

MATERIALS AND METHODS

Case Selection

Representative sections of 10% neutral-buffered, formalin-fixed, paraffin-embedded tissue were obtained from 111 different patients' thyroid lesions, representing all of the major subtypes of thyroid neoplasms (Table 1). The cases included specimens from the archived files at the University of Washington Department of Pathology, as well as cases from the senior author's consultation service (MPB). All hematoxylin and eosin–stained slides on each case were reviewed to confirm the diagnoses using standard histologic criteria (35, 36) and to ensure that representative sections were submitted for immunohistochemical analysis. The diagnosis of medullary carcinoma *versus* follicular carcinoma was confirmed in each case by standard anticalcitonin and antithyroglobulin immunopositivity, respectively.

Immunohistochemistry

Analyses were performed using an avidin-biotin immunoperoxidase method. Four-micron-thick tissue sections were cut and placed on electrostatically charged slides and heated to 60° C for 15 min. The tissues were then deparaffinized, rehydrated, and incubated in 3% hydrogen peroxide for 5 min to block endogenous peroxidase and then washed in dH_2O. Heat-induced epitope retrieval was performed by microwaving for 18 min in 10 mM citrate buffer at pH 6.0. Sections were held in hot buffer for 20 min, washed in phosphate buffered saline (PBS) buffer, and then incubated for 10 min with pronase (0.1 gram/L at room temperature; Calbiochem, La Jolla, CA). The 10-min incubation time is the optimal time for digestion with this enzyme as determined from our laboratories' experience in our referral service and hospital practice. We selected this concentration and time after titration at different incubation times and with different concentrations of pronase. Using this combination of timing and concentration, we determined this to be optimal for preservation of tissue integrity and immunoreactivity. The sections were then washed in PBS, at which point the monoclonal primary antibody Rb-1 (Dako, Carpinteria, CA) was applied for 45 min at room temperature at a 1:50 dilution. Sections were then washed in PBS, and a biotinylated antimouse secondary antibody (Vector Laboratories, Burlingame, CA; 1:200) was applied for 25 min. Again the

TABLE 1. Rb-1 Immunohistochemical Results in Thyroid Neoplasms

Diagnosis	*N*	Rb Negative (<10% cells stained)	Rb Positive (≥10% cells stained)
Follicular adenoma	34	0 (0%)	34 (100%)
Nodular goiter	19	2 (11%)	17 (89%)
Follicular carcinoma	9	8 (89%)	1 (11%)
Papillary carcinoma follicular variant	8	8 (100%)	0 (0%)
Papillary carcinoma	15	15 (100%)	0 (0%)
Hürthle cell carcinoma	5	4 (80%)	1 (20%)
Hürthle adenoma	7	7 (100%)	0 (0%)
Insular carcinoma	4	4 (100%)	0 (0%)
Anaplastic carcinoma	4	0 (0%)	4 (100%)
Medullary carcinoma	6	3 (50%)	3 (50%)

sections were washed in PBS, and the avidin-biotin complex (Vector Laboratories; 1:100) was applied for an additional 25-min incubation. Diaminobenzidine tetrahydrochloride enhanced with 8% $NiCl_2$ was used for the chromogen black reaction product, followed by methyl green counterstaining, dehydration, and coverslipping.

Positivity was observed in the nuclei as black staining. The endothelial cells and lymphocytes served as positive internal controls as an additional step to monitor the retrieval and immunohistochemical techniques. The total number of positive epithelial cells in each tumor was estimated as a percentage of the total number of epithelial cells in the lesion, in 10% incremental semiquantitative assessments, by two observers independently. Reactivity in 10% or more of thyroid epithelial cell nuclei was considered positive, and less than 10% was considered negative, based on previous work using quantitative image analysis (29).

Statistical Analysis

Differences in percentage of tumors that were positive for immunoreactivity among the 10 subtypes of lesions were tested for significance using the likelihood ratio χ^2 statistic with a small constant (10^{-8}) added to every category to make numerical adjustment for sampling zeros (37). Because hundreds of comparisons are possible among the 10 subtypes, adjustment for multiple comparisons was done using the procedure of Gabriel (38). This procedure preserves the overall type 1 error rate (*i.e.*, false-positive rate or "alpha-level") at 0.05 so that the probability of making no type 1 errors in any of the comparisons remains at 95%.

RESULTS

The staining pattern with Rb-1 was a diffuse nuclear reactivity. We did not observe nonspecific cytoplasmic binding as reported previously (32–34). The cytoplasmic element of staining was eliminated by the addition of a 10-min incubation with pronase in the antigen retrieval procedure after microwaving. On comparing the quality of staining with and without pronase digestion, there was no change in the percentage of positive cells, but the cytoplasmic background staining was eliminated by pronase digestion. This rendered the ratio of noise to signal significantly lower and the task of evaluating the nuclear staining easier and more accurate.

Mesenchymal fibroblasts and endothelial cells and lymphoid cells within thyroid neoplasms and goiters consistently showed nuclear positivity but were not included in the percentages of positive cells in this study. Only thyroid epithelial cells were assessed using a 10% or greater cutoff for positivity. In addition and in all cases, internal control nonneoplastic thyroid tissue reacted positively in a portion of epithelial cell nuclei. This, along with the mesenchymal cell and lymphoid cell staining, served as internal staining quality control for all cases. In addition and in parallel with all experiments, an external positive control thyroid follicular adenoma reacted consistently with the antibody in the majority of the cells.

The majority of benign thyroid lesions (Table 1) were positive for Rb nuclear immunoreactivity, including 34 of 34 follicular adenomas (100%) and 17 of 19 nodular goiters (89%) (Fig. 1). The great majority of positive lesions reacted in most of the lesional nuclei. In all but two follicular adenomas and one nodular goiter nodule, more than 50% of lesional nuclei stained positively. In the three discrepant lesions, only 20% of the lesional cells in the two cases and only 10% in the third case showed nuclear immunoreactivity. Using a 10% cutoff, we assessed these three cases as positive. In general, however, using a 10% cutoff, the great majority of positive benign lesions (51 of 53 [95%]) were clearly and easily assessed as positive for Rb nuclear immunoreactivity because the majority (>50%) of cells were positive.

As for malignant neoplasms (Table 1), all 23 cases of papillary carcinoma showed lack of expression of P-Rb, as defined by reactivity in fewer than 10% of the tumor cell nuclei (Fig. 1). Our study also included nine cases of follicular carcinoma, of which only one showed Rb expression and eight were negative. Medullary carcinomas reacted positively in 3 of 6 (50%) cases, whereas the remaining three cases (50%) lacked expression of P-Rb. The four cases of anaplastic carcinoma we analyzed all showed strong positive staining (Fig. 2), in contrast to an adjoining insular carcinoma component in one case and a papillary carcinoma component in another case, both of which were negative.

It is interesting that almost all of the Hürthle cell neoplasms lacked Rb-1 expression, whether benign or malignant. Only one of five carcinomas was positive, and seven adenomas were negative (Fig. 3).

Together, these results have significant implications for improving the accuracy of difficult differential diagnoses in thyroid pathology. One hundred percent of follicular adenomas were positive (95% confidence interval [CI] = [85%, 100%]). This was in marked contrast to the 11% positive in follicular carcinomas (95% CI = [0.3%, 48%]) and the 0% positive found in papillary carcinoma including the follicular variant (95% CI = [0%, 13%]. When the percentage positive for immunoreactivity in follicular adenoma was compared with the percentage positive in each of the other subtypes, it was found that the percentage positive in follicular adenoma was significantly greater than in follicular carci-

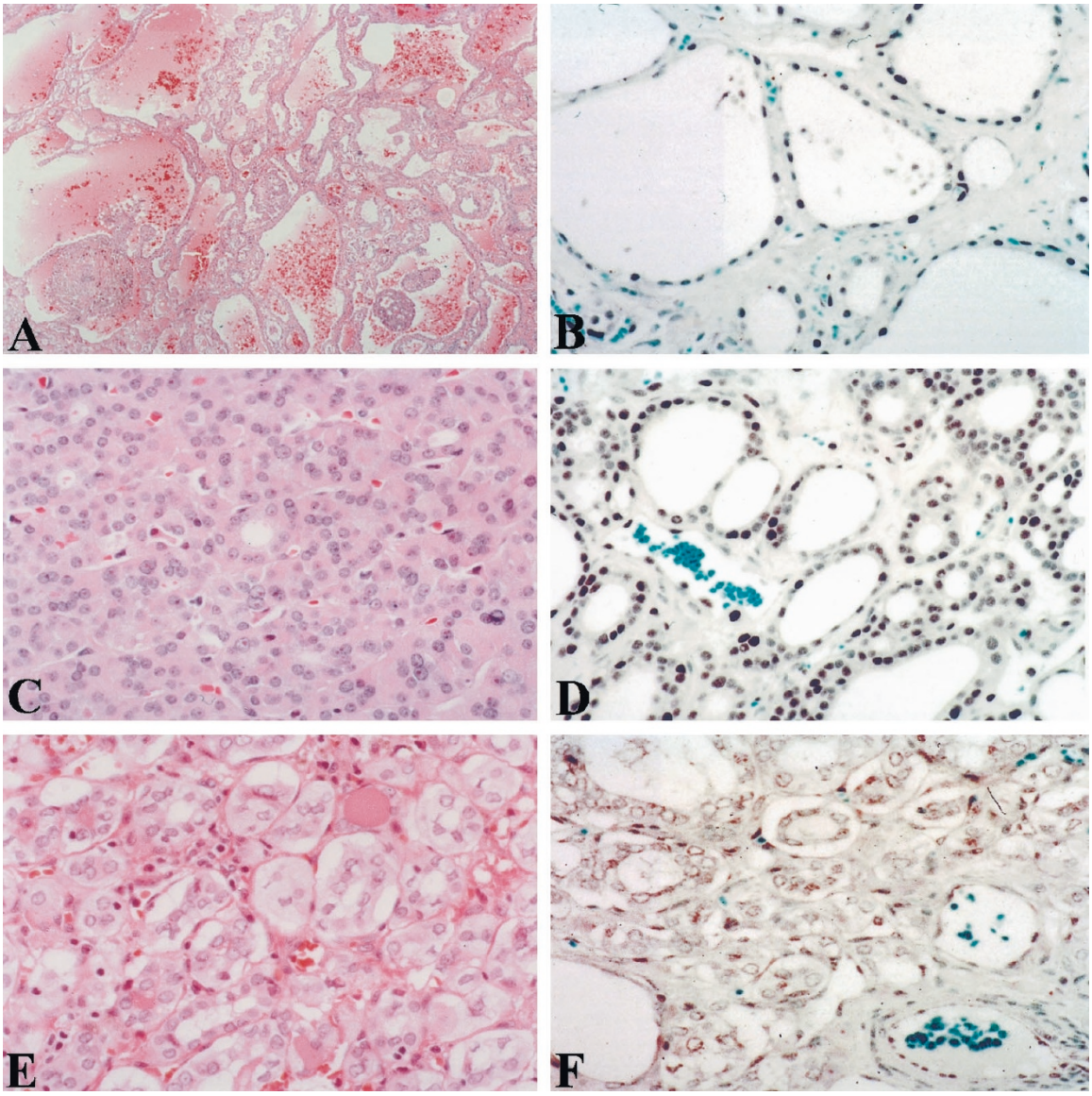

FIGURE 1. **A**, nodular goiter (hematoxylin and eosin). **B**, same nodular goiter showing retinoblastoma (Rb) immunoreactivity in almost all nuclei (Rb immunohistochemistry with diaminobenzidine (DAB) chromogen and nickel chloride enhancement to produce a black positive signal against methyl green counterstaining). **C,** follicular adenoma (hematoxylin and eosin). **D**, same follicular adenoma showing Rb immunoreactivity in almost all nuclei (Rb immunohistochemistry with DAB chromogen and nickel chloride enhancement to produce a black positive signal against methyl green counterstaining). **E**, follicular variant of papillary carcinoma (hematoxylin and eosin). **F**, same follicular variant of papillary carcinoma showing complete absence of Rb immunoreactivity (Rb immunohistochemistry with DAB chromogen and nickel chloride enhancement to produce a black positive signal against methyl green counterstaining).

noma, papillary carcinoma (follicular variant), papillary carcinoma, Hürthle adenoma, and insular carcinoma ($P = .0007$, $P < .0001$, $P < 10^{-7}$, $P = .0003$, and $P = .02$, respectively, after adjustment for multiple comparisons). When viewed as a diagnostic test for differentiating follicular adenoma from follicular carcinoma, the percentage negative in follicular carcinoma corresponds directly to the test sensitivity (89% sensitivity with 95% CI = [52%, 99.7%]), whereas the percentage positive in follicular adenoma (97%) corresponds to the specificity (95% CI = [85%, 99.9%]). Similarly, the estimated sensitivity for diagnosing papillary carcinoma (follicular variant) *versus* follicular adenoma is 100% (95% CI = [69%, 100%]) with the same specificity as in the previous differential diagnosis.

DISCUSSION

The classification of thyroid neoplasia relies predominantly on morphology; however, difficult cases that defy precise classification on morphologic grounds are not infrequently encountered. More recent, immunohistochemistry has been recognized as an adjunct to the classification of thyroid neoplasia. The most striking example is the use of calcitonin and neuroendocrine marker immuno-

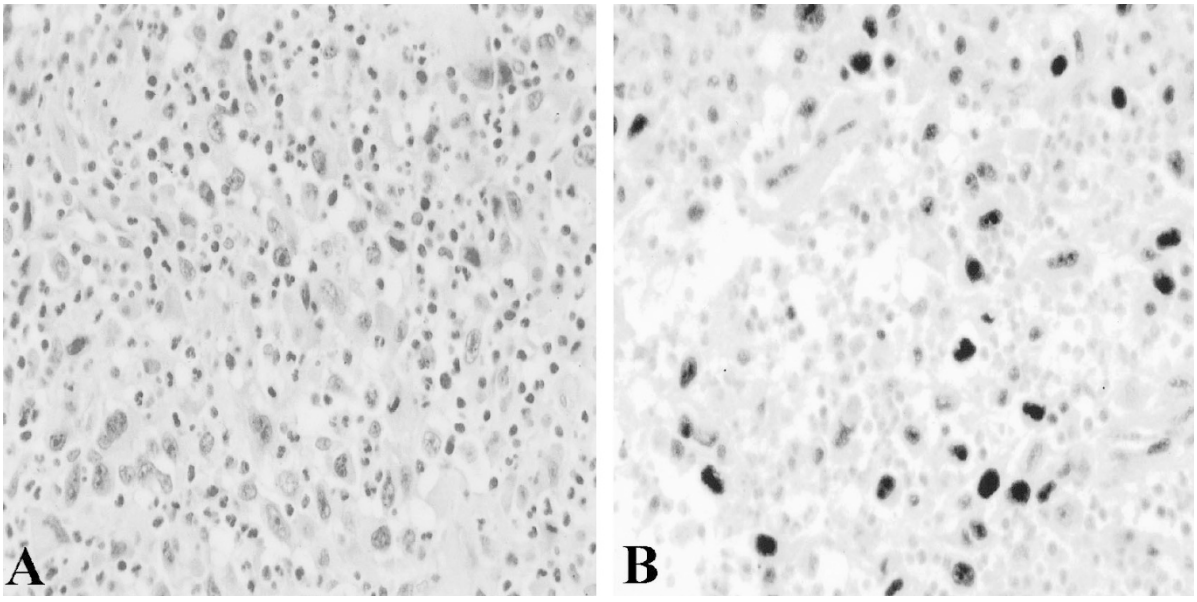

FIGURE 2. **A**, anaplastic thyroid carcinoma (hematoxylin and eosin). **B**, same anaplastic carcinoma showing retinoblastoma immunoreactivity in most nuclei. Note that prominent infiltrating lymphocytes are also occasionally positive (retinoblastoma immunohistochemistry with diaminobenzidine chromogen and nickel chloride enhancement to produce a black positive signal).

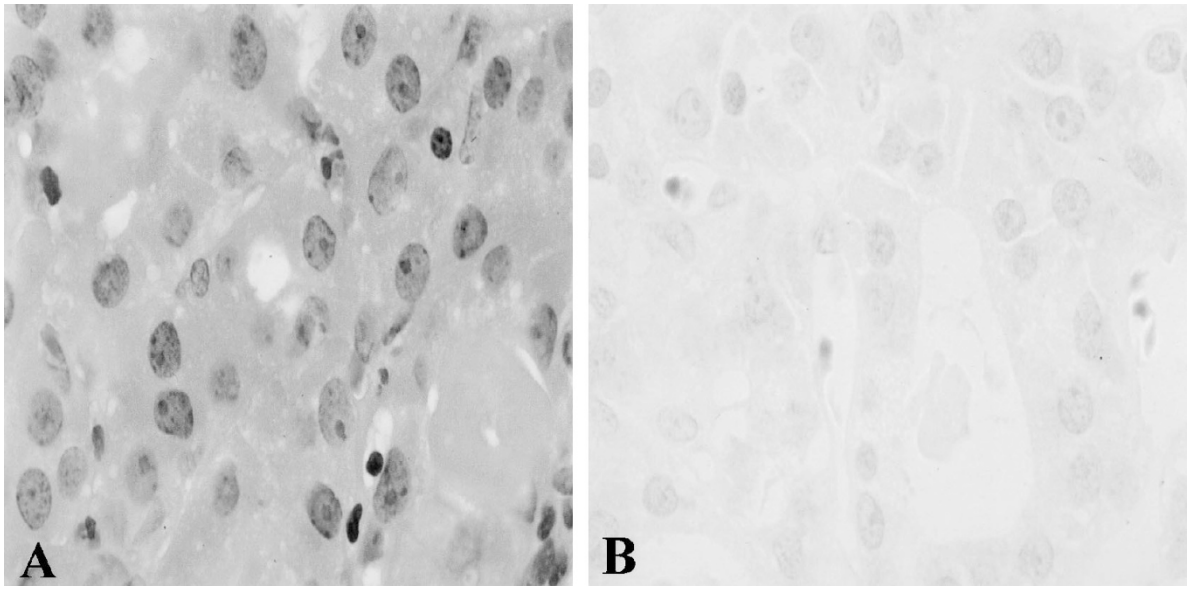

FIGURE 3. **A**, Hürthle cell adenoma showing characteristic granular eosinophilic cytoplasm and prominent nucleoli (hematoxylin and eosin). **B**, same Hürthle cell adenoma showing absence of retinoblastoma immunoreactivity (retinoblastoma immunohistochemistry with diaminobenzidine chromogen and nickel chloride enhancement to produce a black positive signal).

histochemistry to discriminate medullary carcinoma from thyroid neoplasms of follicular origin (38). Other antibodies have been investigated for their ability to discriminate benign and malignant thyroid pathology. Although a plethora of studies have been published recently, only a few antibodies have demonstrated reasonable accuracy in discriminating benign from malignant thyroid neoplasms. The reported potentially beneficial antibodies include HBME-1 (39, 40), galectin-1 and galectin-3 (41), and now Rb-1 on the basis of this report.

In the present study, we detected Rb-1 antibody reactivity in the majority of the benign non-Hürthle thyroid lesions (51 of 53 [96%]), whether hyperplastic nodular goiters or presumably neoplastic solitary follicular adenomas. The one notable exception to this pattern in benign lesions was observed in Hürthle cell neoplasms, which lacked Rb-1 immunoreactivity in both benign and malignant lesions. Malignant thyroid neoplasms in general and conversely lacked reactivity with the antibody in the majority (42 of 51 [82%]). These overall results indicate the potential utility of the Rb-1 antibody in conjunction with morphology in the distinction between benign and malignant thyroid neoplasms in general surgical pathology practice.

The most promising application of Rb immunohistochemistry, based on our results, relates to the distinction of the follicular variant of papillary carcinoma or minimally invasive follicular carcinoma on one hand from follicular adenoma on the other. Not infrequently, these particular differential diagnoses can be problematic. The difficulties concern the potential focality of the nuclear changes in papillary carcinoma and that the typical scarring or infiltrative growth patterns of the follicular variant of the papillary carcinoma may be inconspicuous. Similarly, discriminating true capsular and vascular invasion of minimally invasive follicular carcinoma from benign epithelial herniation into or trapping of benign tissue within the capsule of an adenoma can also be challenging. Rb-1 nuclear immunoreactivity in such lesions would provide adjunctive data in favor of a follicular adenoma, whereas absence of staining would favor malignant alternatives, in conjunction with the morphologic findings. Based on our results, a negative Rb immunostain had a sensitivity of 89% for differentiating follicular carcinoma from adenoma (95% CI = [52%, 99.7%]), whereas the specificity for a positive Rb immunostain in this differential diagnosis was 100% (95% CI = [85%, 99.9%]). Similarly, the estimated sensitivity and specificity for differentiating a papillary carcinoma (follicular variant) from follicular adenoma on the basis of a negative Rb immunostain are 100% (95% CI = [69%, 100%]).

Another potentially useful application of Rb immunohistochemistry is the distinction between changes after fine-needle aspiration of follicular adenomas in the form of scarring, hemorrhage, and vertical capsular pseudoinvasion, as described previously (42), from true invasion in follicular carcinoma. In the last case, lack of Rb immunoreactivity would favor the diagnosis of carcinoma, whereas a high percentage of positive cells, along with a history of fine-needle aspiration biopsy, would support a benign process.

Reactivity of Rb-1 with follicular adenomas and nodular goiter was detected in the majority of the cells in all cases that we studied. None of 34 cases of follicular adenoma and 2 of 19 cases of nodular goiter lacked Rb expression, all with internal positive control staining of adjacent thyroid epithelial and mesenchymal cells. These findings suggest that the loss of protein expression can be seen in benign lesions. An alternative may be that these lesions were in the process of malignant evolution.

The intensity of staining for P-Rb within some thyroid lesions varied from cell to cell in our study, also similar to previous reports (29, 33, 43). Despite this variability, the distinction between positive and negative cases was always easy to assess at the 10% cutoff level that we used.

The series of Hürthle cell neoplasms examined herein lacked expression of Rb-1 in both benign and malignant lesions, except for a single Hürthle cell carcinoma that reacted with the antibody in ~50% of the cells' nuclei. This staining pattern for Rb-1 in Hürthle neoplasms suggests that mechanisms other than Rb alteration are involved in this category of thyroid neoplasia, similar to many other biologic and molecular aspects that distinguish this subset of thyroid neoplasms (44). This same pattern of absence of Rb-1 staining was observed in Hürthle cell metaplasia in a case of Hashimoto's thyroiditis that we also stained (data not shown). This case also harbored an Rb-positive follicular nodule, in contrast to the negative metaplastic Hürthle cells in the adjacent thyroid tissue.

All cases of insular and papillary carcinoma were negative for Rb-1, including both the classic type of papillary carcinoma and the follicular variant of papillary carcinoma. As for follicular carcinomas, eight of nine cases lacked expression of Rb. In our series, only three of six cases of medullary carcinoma (50%) showed loss of expression of Rb, suggesting varied biologic mechanisms of tumorigenesis in these neoplasms. All four cases of anaplastic carcinoma included in this study reacted strongly and positively with Rb-1 antibody, again suggesting either that other mechanisms operate in the transformation to anaplastic carcinoma or that the mutant Rb protein is immunoreactive as has been documented by Geradts *et al.* (32).

The study by Holm and Nesland (31) reported the presence of Rb protein product in the majority of their thyroid carcinomas in paraffin sections. This discrepancy in comparison to our results may be ascribed to four potential factors. First, the definition of positive results in Holm's study was based on *any* degree of reactivity with Rb antibody, whereas in our study, positivity is defined by Rb-1 staining in 10% or more of the epithelial lesional cells. Figge and colleagues (29) also used a 10% cutoff, based on positive nuclear surface area quantitated by image analysis. Assessing a neoplasm as positive for Rb protein expression when in fact only a small minority of the cells are reacting discounts that normal non-neoplastic tissue may express Rb protein at any given time. There may be contamination of neoplasms by a minority of non-neoplastic epithelial cells, or a minority of positive neoplastic cells may reflect tumor heterogeneity or oligoclonality. Thus, accepting any reactivity does not seem to be the best approach for distinguishing benign from malignant neoplasms. This is particularly true given that in the setting of neoplasia, a significantly larger number of cells may be in late G1–M phase (45) as compared with normal tissue, and in these phases of cell cycle, the total level of P-Rb is higher compared with G0/mid G1, as shown

by Xu *et al.* (43). The best arbitrary cutoff between benign and malignant would be a percentage taking into account the background level of positivity in cycling cells. In our study and in the study by Figge *et al.* (29), a 10% cutoff proved to have excellent discriminatory power. In addition, when the tissues were positive, they were consistently significantly higher than the 10% cutoff. In fact, 48 of 51 positive benign lesions (94%) were positive in more than 50% of nuclei, making the distinction between positive and negative cases easily achievable. Second, Holm *et al.* used a different Rb antibody clone that may bind to different epitopes in the target Rb antigen that might be associated with nonspecific binding to other nuclear antigens. Differences between Rb clones have been documented previously by Geradts *et al.* (33) and confirm our personal observations in our referral service. Third, interlaboratory differences in the handling of tissues and quality control procedures might be responsible for the differences in the results. Fourth, endogenous biotin activity in the thyroid tissue has been documented by Kashima *et al.* (34). This factor makes evaluating nuclear positivity in the midst of dark false-positive cytoplasmic background challenging and increases the likelihood of false positives. We observed this same artifact when we tried using the microwave only method of antigen retrieval; this yielded a high cytoplasmic background that prohibited accurate estimation of the number of positive nuclei in any single section. Subsequent pronase digestion eliminates this problem.

Although the gold standard for classifying follicular cell-derived thyroid lesions as benign or malignant is based on morphology, the future may continue to add adjunctive techniques, such as immunohistochemistry, into the diagnostic triage. Rb immunohistochemistry is a promising candidate.

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