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Immunohistochemical Biomarkers in Thyroid Pathology

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Abstract
The application of immunohistochemistry to the diagnosis of thyroid lesions has increased as new biomarkers have emerged. In this review, we discuss the biomarkers that are critical for accurate diagnosis, prognosis, and management. Immunohistochemical markers are used to confirm that an unusual tumor in the thyroid is indeed of thyroid origin, either of follicular epithelial or C-cell differentiation; the various mimics include nonthyroidal lesions such as parathyroid tumors, paragangliomas, thymic neoplasms, and metastatic malignancies. Tumors of thyroid follicular epithelial cells can be further subclassified using a number of immunohistochemical biomarkers that can distinguish follicular-derived from C-cell lesions and others that support malignancy in borderline cases. The use of mutation-specific antibodies can distinguish papillary carcinomas harboring a $BRAF^{V600E}$ mutation from RAS-like neoplasms. Immunostains have been developed to further identify molecular alterations underlying tumor development, including some rearrangements. Altered expression of several biomarkers that are known to be epigenetically modified in thyroid cancer can be used to assist in predicting more aggressive behavior such as a propensity to develop locoregional lymphatic spread. Immunohistochemistry can assist in identifying lymphatic and vascular invasion. Biomarkers can be applied to determine dedifferentiation and to further classify poorly differentiated and anaplastic carcinomas. The rare tumors associated with genetic predisposition to endocrine neoplasia can also be identified using some immunohistochemical stains. The application of these ancillary tools allows more accurate diagnosis and better understanding of pathogenesis while improving prediction and prognosis for patients with thyroid neoplasms.

Keywords Thyroid · Biomarkers · Immunohistochemistry

Introduction

The role of immunohistochemistry in thyroid pathology includes multiple aspects of diagnosis as well as biomarkers that serve to provide information about prognosis, prediction, and genetic predisposition.

In some instances, immunohistochemistry is required to prove the differentiation of a thyroid tumor; this entails identifying biomarkers of thyroid follicular cells or parafollicular C cells, or in contrast, proving differentiation as a distinct lesion, such as a parathyroid cell proliferation, a lesion derived from intrathyroidal thymic or salivary gland remnants, a stromal or lymphoid lesion, or metastatic malignancy.

Some biomarkers are useful to correctly classify a thyroid tumor of known cellular origin. With advances in molecular biology and changes in our understanding of follicular and papillary lesions derived from follicular epithelium, immunoprofiling can be used to distinguish benign from borderline or malignant lesions. Follicular epithelial-derived tumors with solid and trabecular growth pattern can also be appropriately classified based on loss of markers of differentiation and adhesion. The progress in these areas is yielding valuable information to correctly classify these tumors.

One of the major challenges in thyroid pathology is the ability to correctly identify tumors that are likely to behave aggressively and to distinguish them from tumors that will remain indolent. Many morphologic and functional features that can help to predict behavior have been described and several can be identified with immunohistochemistry [1, 2].

Finally, as the genetic basis of endocrine neoplasia is becoming clear, we have tools that can help the pathologist to identify patients who are candidates for genetic testing, an

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important aspect of diagnostic pathology that plays a critical role in prevention of disease for the patient and their family members.

In this review, we summarize the most important immunohistochemical biomarkers that can be used to address pitfalls in thyroid pathology [3]. In the interest of brevity, not all biomarkers will be reviewed. Discussion of stromal and lymphoid lesions of the thyroid is beyond the scope of this paper.

Confirmation of Cellular Origin of a Thyroid Lesion

Tumors in the thyroid can be derived from follicular epithelial cells, but they also can arise from parafollicular C cells and from other tissues in and around the thyroid, including parathyroid glands, thymus, and the various stromal elements. It is therefore important to have tools to ensure the correct classification of lesions. Here, we review the various thyroid-specific immunohistochemical biomarkers and those that identify other relevant lesions in the thyroid region.

Thyroid Transcription Factors

The discovery of thyroid transcription factors (TTFs) has made it possible to advance our understanding of thyroid biology. The parenchymal cells of the thyroid gland concurrently express four genes that encode the following TTF proteins: Nkx-2.1 encoded by NKX2-1, also known as thyroid transcription factor-1 (TTF-1) and homeobox protein, FOXE1 (also known as thyroid transcription factor-2 (TTF-2) and forkhead box protein, encoded by FOXE1), paired box protein PAX8 (encoded by paired box gene 8 (PAX8)), and homeobox protein Hhex (encoded by HHEX) [4–9]. These four transcription factors are expressed in thyroid follicular cells and selectively in other tissues. In the thyroid gland, TTFs modulate the development of the thyroid gland and also modulate the expression of thyroglobulin, thyroid peroxidase (TPO), the thyroid stimulating hormone receptor (TSHr), and the thyroid sodium/iodide cotransporter [6, 7, 9, 10]. Therefore, alterations in the functionality of the TTFs due to adverse genetic events such as mutations and epigenetic modifications can lead to thyroid dysgenesis and even development of tumors [4, 5, 11, 12].

TTFs are also expressed in other tissues throughout the body. The NKX2-1/TTF-1 is expressed in the fourth branchial pouch, ultimobranchial body, lung, trachea, posterior pituitary, hypothalamus, medial ganglionic eminence, C cells, and even parathyroid cells [13–15]. The FOXE1/TTF-2 is expressed in tissues derived from the pharyngeal arches and pharyngeal wall (tongue, palate, and esophagus), testis, epidermis, and hair follicles; additional expression is also noted in human thymus, brain, heart, placenta, lung, skeletal muscle, and kidney [16]. PAX8 expression is found in the kidney, endocervix, endometrium, ovary, fallopian tube, seminal vesicle, pancreatic islet cells, and lymphoid cells [17–20]. HHEX expression can be seen in the liver and hematopoietic cells in addition to thyroid follicular cells [4, 9, 15, 21–26].

Thyroid Transcription Factor-1

In the thyroid gland, immunohistochemistry identifies TTF-1 as diffusely expressed in the nuclei of normal thyroid follicular cells and para-follicular cells. Thus, it is not surprising that TTF-1 shows diffuse expression in follicular cell-derived neoplasms such as follicular adenoma, well-differentiated follicular carcinomas (papillary and follicular), poorly differentiated carcinomas, and foci of C-cell hyperplasia and nearly all medullary thyroid carcinomas [7, 8, 27–29]. TTF-1 expression, usually focal, is retained in 5 to 15% of anaplastic carcinomas [30]. TTF-1 expression has also been reported in mucoepidermoid carcinomas of the thyroid gland and, in rare cases, of sclerosing mucoepidermoid carcinoma with eosinophilia [31–33]. TTF-1 is useful, except in cases of metastatic lung tumors (pulmonary adenocarcinoma, well-differentiated pulmonary neuroendocrine tumor) as well as in poorly differentiated neuroendocrine carcinoma of various sites, in cases where a metastatic tumor is considered in the differential diagnosis of a thyroid nodule, especially when secondary tumors present as a dominant thyroid nodule or metastasize to a primary thyroid neoplasm (“tumor to tumor metastasis”) [5, 11, 12, 34, 35]. Thyroglobulin immunoreactivity is frequently used to confirm follicular cell origin. However, one should be aware of the limitations of this biomarker as thyroglobulin can sometimes be negative in some thyroid follicular epithelial proliferations or diffusion-type staining can cause diagnostic challenge for diagnosticians (Fig. 1). Therefore, the use of TTF-1 and monoclonal PAX8 is advised. When distinguishing metastatic neuroendocrine tumors to the thyroid from primary thyroid neoplasms, calcitonin immunostaining may also be of limited value as it is expressed in nonthyroidal neuroendocrine tumors, especially those of pulmonary origin. Such instances will require comparison with patient’s primary tumor, careful determination of serum calcitonin levels (much elevated in medullary thyroid carcinoma as compared to other neuroendocrine tumors), immunostains for carcinoembryonic antigen (preferably monoclonal), and molecular genetics. When distinguishing metastatic carcinomas to the thyroid gland, it is also important to know that CDX-2 is expressed in columnar cell variant of papillary thyroid carcinoma [1] (Fig. 2).

TTF-1 expression in some lesions can also depend on the clone of antibody used for immunohistochemistry. For instance, the 8G7G3/1 clone has been reported to be either negative or variably/weakly positive in ultimobranchial body remnants of the thyroid gland known as solid cell nests [36–40]. However, a recent study demonstrated that the

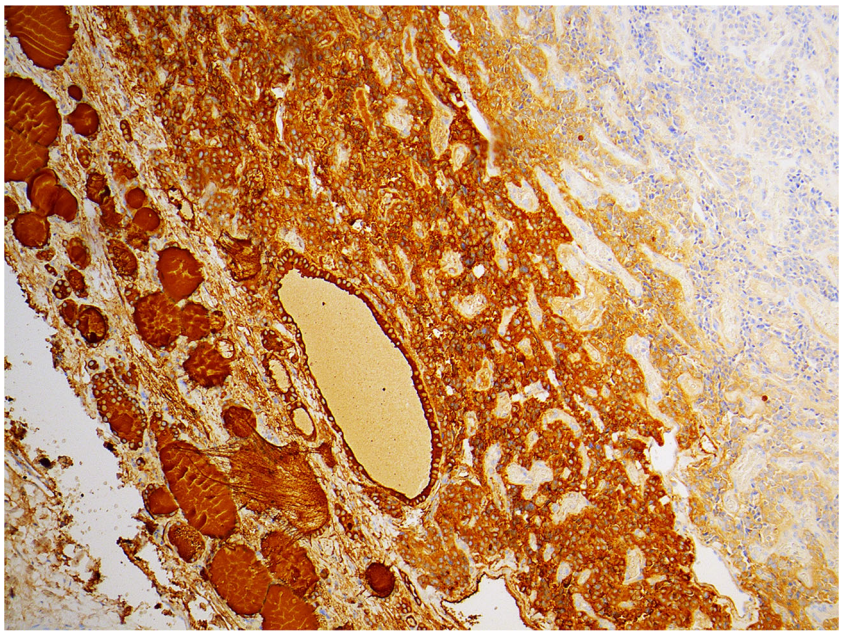

Fig. 1 Thyroglobulin immunohistochemistry. Thyroglobulin immunoreactivity is often considered to confirm follicular cell origin. However, thyroglobulin can sometimes be negative in some thyroid follicular epithelial proliferations, or diffusion-type staining can create a diagnostic challenge. This photomicrograph of the periphery of a medullary thyroid carcinoma that was positive for monoclonal CEA and calcitonin (not illustrated) illustrates true staining in the surrounding follicular epithelium (left) and diffusion-type false thyroglobulin reactivity at the periphery of the C-cell lesion (middle) that fades in the center of the lesion (right)

SPT24 clone of TTF-1 is diffusely and strongly expressed in ultimobranchial body remnants of the thyroid gland [41] (Fig. 3). The same study expanded the immunohistochemical profile of ultimobranchial body remnants by demonstrating positivity for GATA-3 and negativity for monoclonal carcinoembryonic antigen (CEA) in these structures that can simulate follicular epithelial proliferations in some cases [41].

TTF-1 expression in nonpulmonary neuroendocrine carcinoma has been described and can be encountered in high-grade tumors arising from the ovary, GI tract, pancreaticobiliary tract, and breast [11, 42, 43].

At present, the determination of thyroid follicular cell origin in cases of metastatic tumors is not only limited to formalin-fixed paraffin-embedded histopathology specimens; it can also be performed in cytologic preparations, such as cytospins, cell blocks, and smears. Studies have cautioned that the immunoreactivity of an antibody maybe compromised in cytologic specimens, especially those fixed in alcohol-based fixative. Therefore, the use of a TTF-1 antibody in cytologic specimens should only be employed after careful analytical validation [44].

Paired Box Gene 8

In the thyroid gland, strong and diffuse expression of PAX8 is observed in thyroid follicular epithelium and its associated neoplasms [16, 45]; weak and focal expression has also been reported in cases of medullary thyroid carcinoma (41 to 75% of cases showing weak immunostaining). Nonthyroidal tumors that stain positive with PAX8 antibodies include renal tumors, ovarian neoplasms of Müllerian origin, and endometrioid carcinoma and seminoma [46]. Interestingly, diffuse to weak immunostaining has also been reported in rare cases of parathyroid lesions and nonneoplastic thymic tissue. In the experience of some authors of this paper (OM, SLA), PAX8 reactivity in some sites is identified with polyclonal antisera [47]; in contrast, the use of monoclonal antisera yields negative reactivity in parathyroid gland and thymus, suggesting that the reported reactivity may be nonspecific.

It has been shown that PAX8 expression is often retained in cases of anaplastic thyroid carcinoma and is helpful in cases with limited to no expression of TTF-1 and cytokeratins (Fig. 4), especially those which lack an associated component of well-differentiated thyroid carcinoma [2, 43, 48]. Bishop et al. have shown that PAX8 immunostaining is also helpful in distinguishing between anaplastic thyroid carcinoma showing squamous differentiation and squamous cell carcinoma of the head and neck, as it is negative in the latter [48].

Thyroid Transcription Factor-2 (FOXE1)

The literature is limited on the expression of TTF-2 in thyroid and nonthyroidal lesions. It is diffusely and strongly expressed in all follicular-cell-derived well-differentiated and poorly differentiated carcinomas. It has been reported to be weakly and focally expressed in a few cases of anaplastic carcinoma and in up to 75% of cases of medullary thyroid carcinoma. It is not expressed in other organs where TTF-1 expression is seen, especially tumors of pulmonary origin [16, 43].

Thyroglobulin

Thyroglobulin (TG) is one the largest proteins in the vertebrate proteome; it is exclusively synthesized by thyroid

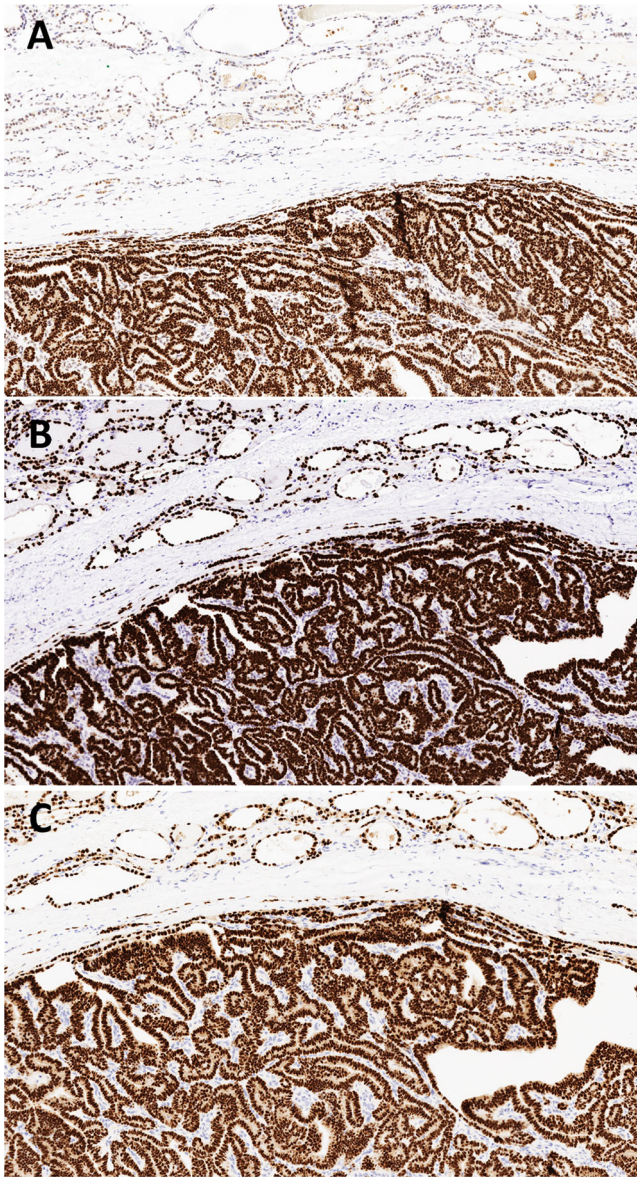

Fig. 2 CDX-2 reactivity in columnar cell variant papillary thyroid carcinoma. When distinguishing metastatic carcinomas to the thyroid gland, it is important to know that CDX-2 is expressed in columnar cell variant of papillary thyroid carcinoma. This photomicrograph illustrates CDX-2 reactivity in a columnar cell variant papillary thyroid carcinoma (**a**) that was negative for thyroglobulin (not illustrated). Positivity for TTF-1 (**b**) and monoclonal PAX8 (**c**) confirmed thyroid follicular epithelial origin of this tumor

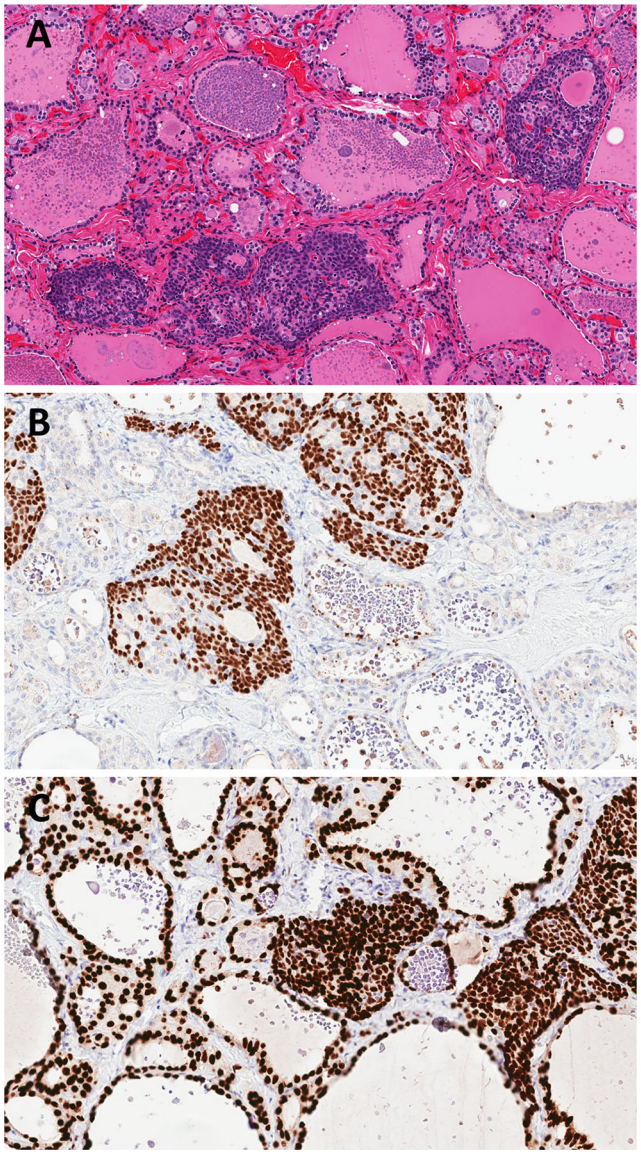

Fig. 3 Ultimobranchial body remnants (solid cell nests). This composite figure illustrates solid cell nests (**a**) that are positive for p63 (**b**) and TTF-1 (**c**). It is important to know the clone used for TTF-1 immunohistochemistry, as the 8G7G3/1 clone has been reported to be either negative or variably/weakly positive in solid cell nests. However, the SPT24 clone of TTF-1 (**c**) is diffusely and strongly expressed in ultimobranchial body remnants

follicular cells. Human TG is encoded by chromosome 8q24.2-8q24.3 and has a monomeric molecular mass of 330 kDa and approximately consists of 2750 amino acids. The expression of TG genes is controlled by TTF-1 and PAX8. TG is stored in a highly concentrated form within the extracellular colloid of thyroid follicles. This efficient storage system is a unique feature of the thyroid gland which provides on-demand availability of thyroid hormones and avoids the disastrous aftermaths of iodide deficiency. The posttranslational modification of TG is iodination which leads to synthesis of T3 and T4; this step is carried out upon stimulation by thyroid-stimulating hormone (TSH) by an orchestrated effort of the sodium iodide symporter (NIS), dual function oxidase (DUO), and TPO [49, 50].

The reference range of serum TG is 1.40–29.2 ng/ml for males and 1.50–38.5 ng/ml with a half-life of 65 h [51]. The serum levels of TG can be elevated in both nonneoplastic and neoplastic lesions of the thyroid gland [49]. A significant increase can be observed in patients with follicular-derived thyroid cancers as compared to those with benign conditions [51].

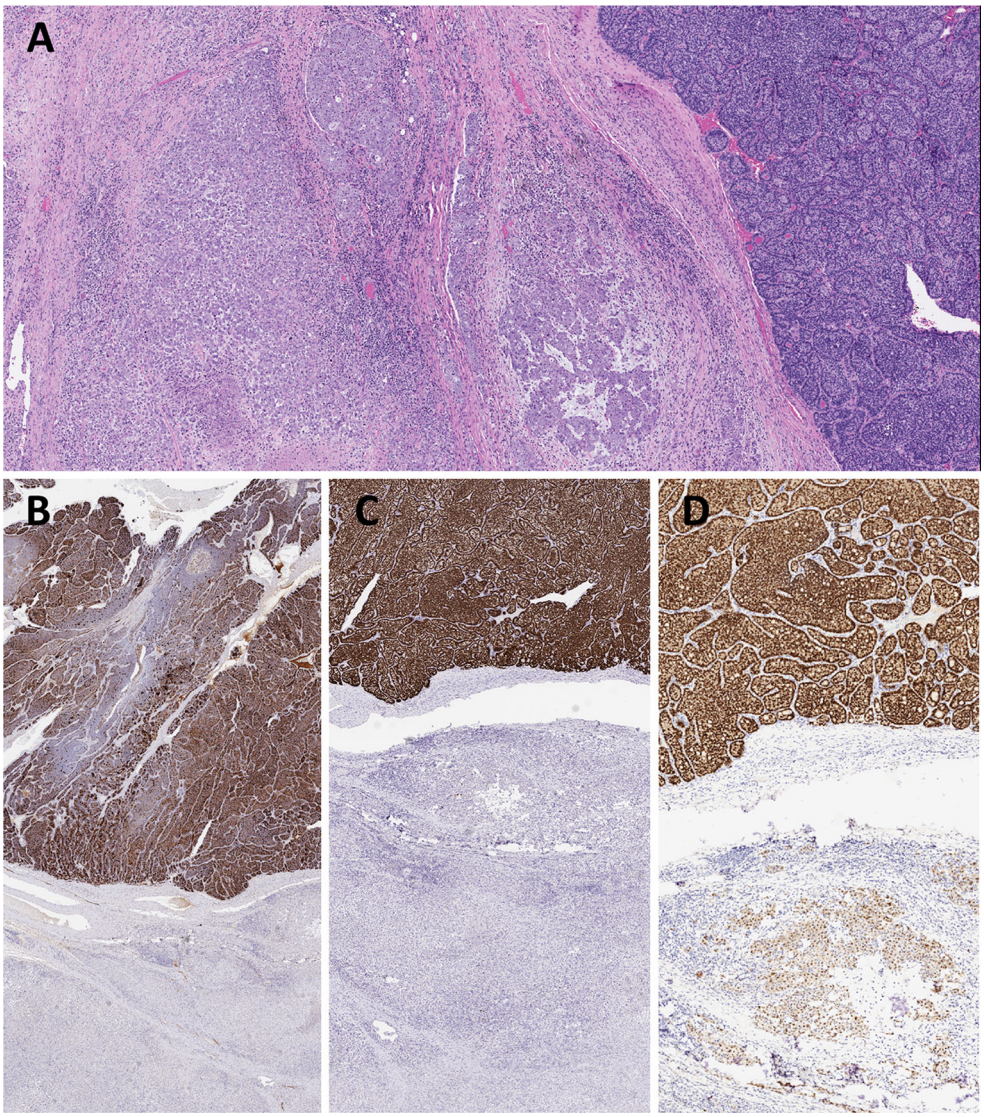

Fig. 4 The role of PAX8 in the diagnosis of anaplastic thyroid carcinoma. This composite photomicrograph illustrates an anaplastic thyroid carcinoma arising in the background of poorly differentiated thyroid carcinoma (**a**—left side: anaplastic thyroid carcinoma). The anaplastic carcinoma component is negative for thyroglobulin (**b**) and TTF-1 (**c**) and is positive for monoclonal PAX8 (**d**)

Postthyroidectomy levels of TG can be utilized to monitor locoregional tumor recurrence or distant metastasis; however, this is highly dependent on the presence of residual thyroid tissue after surgery and the degree of differentiation of the malignant tumor [52, 53].

By immunohistochemistry, TG expression is indicative of thyroid follicular origin; however, the intensity and type of immunostaining is highly dependent on the tumor type [42, 43]. Most follicular and papillary thyroid carcinomas show strong and diffuse cytoplasmic expression with intense staining of the luminal colloid. In case of oncocytic (Hürthle cell) tumors, TG immunostaining can appear as dense granular deposits localized to the perinuclear area, whereas some cases may show weak and focal expression. TG expression is relatively decreased in poorly differentiated thyroid carcinomas and always absent in cases of anaplastic carcinoma. Most C-cell-derived lesions do not express TG; however, rare cases of medullary thyroid carcinomas may show focal immunostaining with TG antibody, a feature which most likely is either due to entrapped follicular epithelium within the tumor or diffusion-type staining including TG sequestered within macrophages and vascular channels [42, 43, 54]. Rare thyroid tumors known as mixed or composite medullary and follicular/papillary carcinomas will genuinely express both calcitonin and TG in C-cell and follicular cell-derived components, respectively [55–58]. In the experience of some authors of this paper (OM, SLA), the use of monoclonal PAX8 and calcitonin can be used to confirm the diagnosis of composite medullary thyroid carcinoma and follicular epithelial-derived carcinomas (Fig. 5).

TG expression in fine-needle aspiration specimens can also be utilized to confirm follicular cell origin, especially in cases of secondary tumors clinically mimicking a solitary thyroid

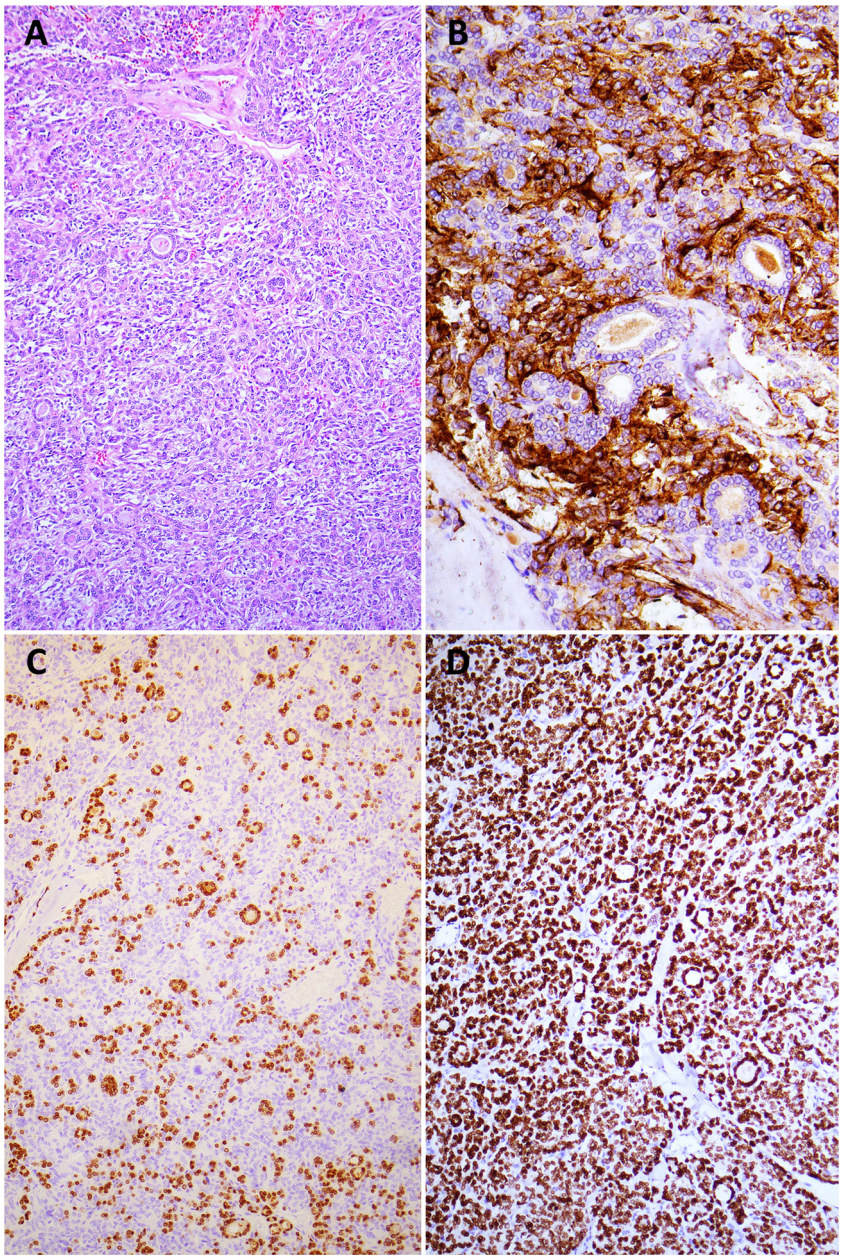

Fig. 5 Composite medullary thyroid carcinoma and follicular variant papillary thyroid carcinoma. The tumor nodule is composed of a dual cell population (**a**). C cells are positive for calcitonin (**b**) and the follicular component is positive for monoclonal PAX8 (**c**). Both components are positive for TTF-1 (**d**)

nodule or metastatic thyroid tumors at other body sites. Additionally, the determination of TG levels on aspirated material from a neck lymph node can confirm the thyroid follicular cell origin of metastatic carcinoma. This is extremely beneficial in cases of nondiagnostic fine-needle aspiration (FNA) specimen from cystic metastasis of papillary thyroid carcinoma [59, 60]. However, the use of TG in association with TTF-1 and monoclonal PAX8 is advised.

Thyroid Peroxidase

TPO is an enzyme expressed specifically by differentiated thyroid cells [61]. It is useful to define cells as of thyroid follicular differentiation.

Calcitonin

In the early 1960s, two major developments occurred in the understanding of C-cell disorders. These included the discovery of the hormone calcitonin and the description of the pathology of medullary thyroid carcinoma (MTC) [62, 63]. In 1966, Williams postulated that medullary carcinoma could be derived from the C cells and, if so, could produce calcitonin, which might potentially serve as an elegant tumor biomarker [63]. This concept was proven by Bussolati and Pearse in 1967 by demonstrating the presence of calcitonin in C cells using immunofluorescence techniques [64].

Calcitonin (CT) is a highly effective hypocalcemic polypeptide hormone comprised of 32 amino acids. The *CALC-1*

gene encodes human CT; it is a member of the CALC gene family and is housed on the tip of the short arm of chromosome 11 (11p15.3-15.5). Calcitonin exerts its function by binding to specific CT receptors which are widely expressed in adult tissues [65, 66]. Interestingly, CT receptor expression can also be seen in neuroendocrine tumors of the lung as well as in malignant tumors of the breast and prostate [65, 67]. Demonstration of elevated serum calcitonin levels (basal and stimulated) remains a sensitive and specific test for diagnosing both sporadic and familial forms of medullary thyroid carcinoma [68]. It has been shown that serum calcitonin correlates with the size of the MTC. However, with dedifferentiation, CT can decrease while CEA rises. Similarly, the morphologic diagnosis of MTC in pathologic specimens (fine-needle aspiration and histopathology) is usually confirmed by using immunohistochemistry to demonstrate CT expression. Up to 95% of the MTC show positive immunostaining with CT; however, the staining pattern can vary from diffuse to focal staining, and up to 25% of cases will show only focal expression [54].

Undetectable serum calcitonin levels can be seen in a few cases of MTC; however, by immunohistochemistry, these tumors will show diffuse and strong to focal and weak calcitonin expression [69–71]. Some experts have suggested that loss of CT is indicative of poor prognosis; however, others have not been able to corroborate this association [69, 72, 73]. It has been shown that procalcitonin can serve as a useful marker in calcitonin-negative tumors [84–86]. Similarly, calcitonin gene-related peptide can also serve as a biomarker in CT-negative MTC [76].

Calcitonin Gene-Related Peptide

The calcitonin/calcitonin gene-related peptide (CGRP) gene is responsible for both producing both calcitonin and α-CGRP. CT is the major peptide synthesized in thyroid C cells, whereas α-CGRP is widely expressed in neural tissues including perivascular nerve fibers, trigeminal ganglia, nonvascular elements of dura, and cerebellum. A similar peptide known as β-CGRP encoded by a separate gene has also been identified [74–76]. Expression of α-CGRP can be detected by immunohistochemistry and in situ hybridization techniques in MTC. Furthermore, it can also be detected in the serum of patients with MTC [76].

Other Biomarkers of MTC

In addition to CT and CGRP, C cells also express CEA, chromogranin, and TTF-1. CEA staining is seen in foci of C-cell hyperplasia and is very helpful in cases of MTC which show focal weak or negative CT expression. Since monoclonal CEA is virtually positive in all C-cell proliferations, it is considered the best biomarker of MTC [1, 2] (Fig. 6). It has been shown that CEA maybe a superior biomarker of MTC as compared to calcitonin for long-term follow-up. Metastases to thyroid from neuroendocrine neoplasms arising elsewhere in the body (usually lung and GI tract) can morphologically mimic MTC. Therefore, it is important to stain for CT, CGRP, CEA, and TTF-1 as well as chromogranin A and S100 [42, 43, 54].

Immunoprofiles of Primary Thyroid Neoplasms of Nonfollicular and C-Cell Origin

Thyroid Paraganglioma

Primary thyroid paragangliomas (TPGL) are rare and morphologically can mimic MTC or even follicular cell-derived tumors with solid and insular growth pattern [77–79]. Based on the findings of the European and American Head and Neck Paraganglioma Registry, the prevalence of TPGL is 0.5% (5 TPGL out of 944 head neck paraganglioma cases), and except for one case, all were likely to carry germline *SDHX* mutations. In this analysis, most cases stained for chromogranin A, synaptophysin, and S100, and almost all were negative for calcitonin and TTF-1 [78]. Castelblanco et al. suggested an immunohistochemical panel comprised of biomarkers based on cDNA results that included NDUFA4L2, COXIV2, and VMAT2 in addition to CGRP/calcitonin, CEA, and TTF-1 to diagnose TPGL [79]. According to these authors, a combination of low to negative expression of CT or CGRP and any expression of NDUFA4L2, COXIV2, or VMAT2 was most suggestive of TPGL. An easier approach is to use GATA-3 and tyrosine hydroxylase, since a significant proportion of TPGL will also demonstrate expression of these two biomarkers that are perhaps more readily available [77]. Importantly, lack of keratin reactivity is important, since GATA-3 is also expressed in parathyroid tumors that stain for chromogranin A.

GATA-3 is a member of dual zinc-finger transcription factors which is involved in the development of multiple organs including parathyroid glands, kidney, Th2 subset of helper T cells, mammary glands, sympathetic nervous system, and epidermal keratinocytes. To date, pathology studies employing GATA3 antibodies have shown its expression in many tissues including parathyroid, breast, urothelium, germ cell tumors, paragangliomas, and a subset of kidney tumors [80, 81]. GATA-3 immunoreactivity is not seen in normal thyroid follicular epithelial cells, C cells, or ultimobranchial body remnants [41, 43], or in benign and malignant thyroid neoplasms except a few cases of anaplastic carcinoma [82] because of its propensity to be epigenetically dysregulated in highly proliferative tumors.

Tyrosine hydroxylase is an aromatic amino hydroxylases and catalyzes the conversion of tyrosine to dopamine. It is a rate-

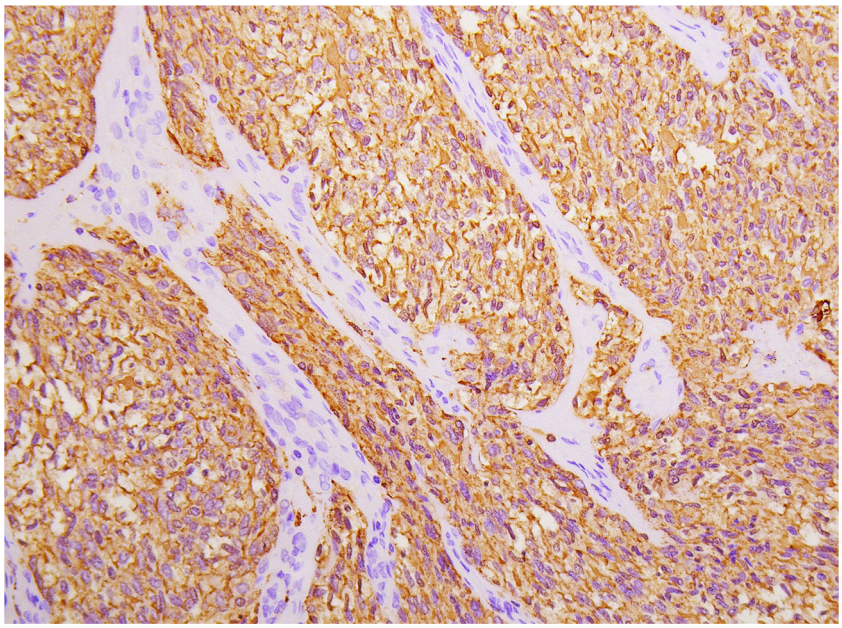

Fig. 6 CEA is the best biomarker of medullary thyroid carcinoma. This photomicrograph illustrates monoclonal CEA reactivity in a medullary thyroid carcinoma that is variably positive for calcitonin (not illustrated)

limiting step in catecholamine synthesis. Immunoexpression of tyrosine hydroxylase in combination with positive chromogranin immunostaining and morphology is considered diagnostic of paraganglioma. However, immunoreactivity for tyrosine hydroxylase can be weak and variable and rarely even negative in paragangliomas of parasympathetic origin (head and neck paragangliomas) as compared to their sympathetic (thoracolumbar paragangliomas) counterparts [77, 78, 83, 84]. Of note, tyrosine hydroxylase is also expressed in TT (MTC) cell lines and it has been reported in a case of keratin- and TTF-1 expressing medullary thyroid carcinoma arising in a patient with MEN2 syndrome [85]. Therefore, tyrosine hydroxylase reactivity supports paraganglial differentiation in conjunction with GATA-3 reactivity and the absence of keratin and other transcription factor reactivity [86].

Intrathyroidal Parathyroid Adenoma

Parathyroid neoplasms are encountered involving the parathyroid glands at their normal locations. However, these neoplastic proliferations can also affect intrathyroidal or juxtathyroidal parathyroid glands, resembling a thyroid nodule [2]. The incidence of intrathyroidal parathyroid gland ranges from 1.4 to 3.2% (0.2% in autopsy studies) [87, 88]. The presence of intrathyroidal parathyroid may be detected by radiologic studies (especially ultrasound) during workup of hyperparathyroidism or, less reliably, during evaluation of thyroid nodules. An intrathyroidal parathyroid neoplasm can be diagnosed by ultrasound due to its imaging characteristics and further confirmed by Technetium-99m-sestamibi scintigraphy [87].

FNA has proven to be helpful in the diagnosis of intrathyroidal parathyroid lesions when the possibility is considered [2]. This procedure is most effective in this regard when either a parathyroid hormone (PTH) assessment is performed on an aliquot of the FNA specimen or immunohistochemical studies are carried out on cell block preparations [89]. The diagnostic immunopanel to confirm parathyroid origin and distinguish a parathyroid from a thyroid neoplasm includes antibodies to PTH, chromogranin, GATA-3, TTF-1, and thyroglobulin [88, 89]. The utility of GATA-3 in the diagnosis of parathyroid lesions in both cytology and histopathology specimens carries a higher specificity than PTH immunostaining which can show focal and weak positivity in a subset of parathyroid neoplasms [88–90]. However, it must be remembered that GATA-3 is also expressed by other tumors including renal and breast carcinomas, paragangliomas, and lymphomas. The use of GCM2, one of the master regulators of the parathyroid, has also been shown to be reliable in the distinction of parathyroid origin [91, 92].

Intrathyroidal Thymic Rests and Neoplasms

The thymus and inferior parathyroid gland develop from the third and fourth branchial pouches, respectively, and migrate caudally together from the 2nd to the 6th week of gestation to settle at their normal anatomical locations. During the course of migration, thymic precursors can become embedded into various tissues in a region known as the thymopharyngeal tract, which usually regresses later [91]. Ectopic thymic tissue can develop due to aberrant migration, sequestration, or persistent thymic tissue along the thymopharyngeal tract. Ectopic intrathyroidal thymic rests are rare in adults, usually are

asymptomatic and discovered incidentally either during radiologic workup of a thyroid nodule or on histopathologic examination of surgically removed thyroid glands [91, 93]. Intrathyroidal thymic rests can pose diagnostic difficulties on fine-needle aspiration specimens and can be either mistaken for chronic lymphocytic thyroiditis or a lymphoproliferative lesion. Tumors arising from intrathyroidal thymic rests are rare and include thymoma, spindle epithelial tumor with thymus-like differentiation (SETTLE), and thymic carcinoma of the thyroid (formerly known as carcinoma showing thymus-like differentiation "CASTLE") [94, 95]. Thymic differentiation in an intrathyroidal tumor can be confirmed by performing a battery of immunostains. These tumors are usually negative for TTF-1, PAX8, TG, and CT. Intrathyroidal thymomas are positive for pancytokeratins, CK5/6, EMA, p63, CD5, and C-kit. The lymphocytic component seen in thymoma usually shows expression of CD3 and TdT and stains negative for B-cell markers. SETTLE stains positive for AE1/AE3, CAM5.2, EMA, CK7, vimentin, and CD117 (C-kit). Thymic carcinoma of the thyroid, which is easily identified as a malignant tumor, stains positive for pancytokeratin (panCK), CD5, BCL2, p63, and CD117 (C-kit) [95–98].

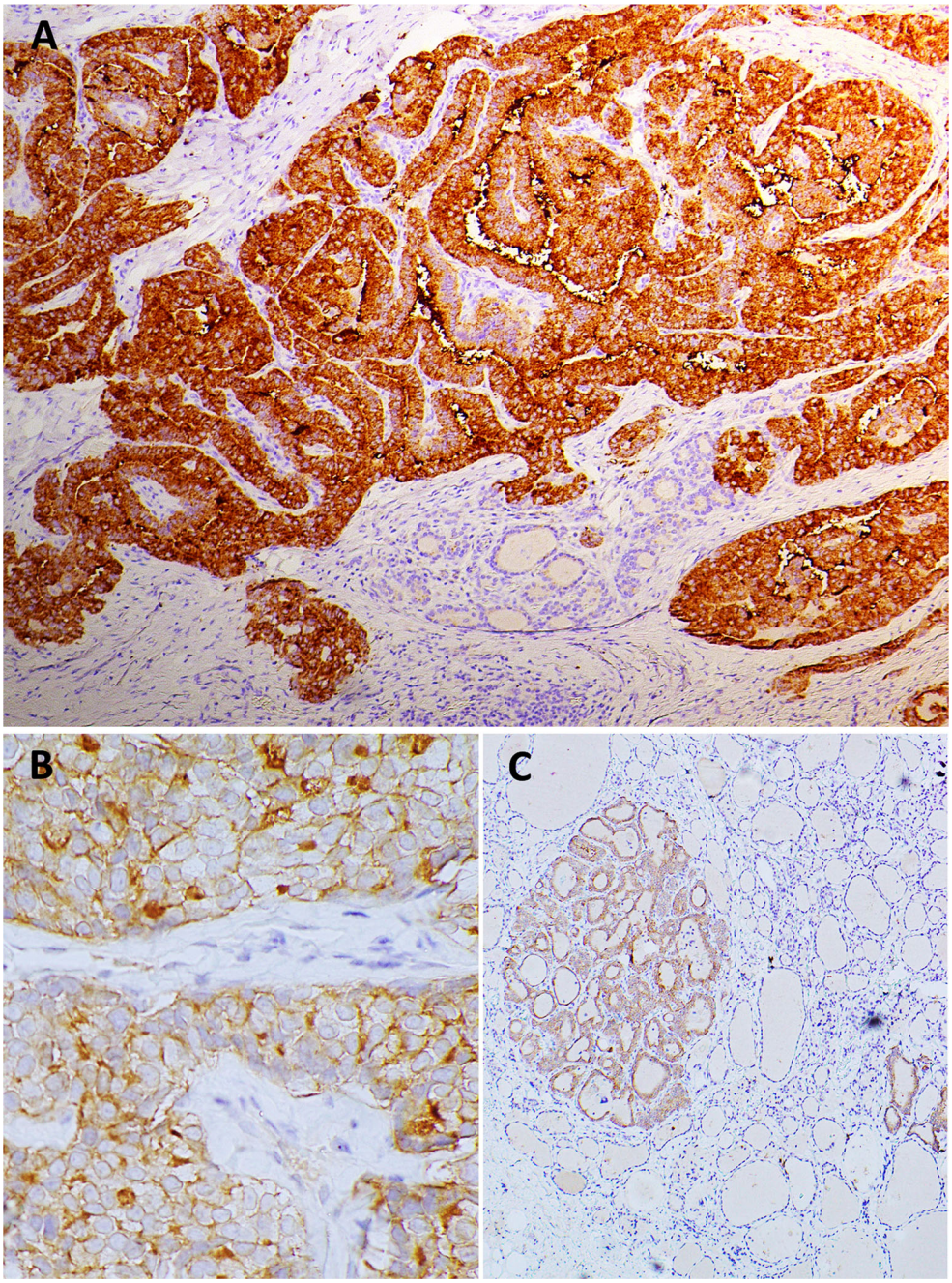

Fig. 7 Molecular immunohistochemistry in thyroid pathology. The VE1 antibody can be used to distinguish thyroid carcinomas driven by *BRAFV600E* (**a**). The *RAS* mutation-specific antibodies can also be used in various thyroid neoplasms including sporadic RAS-driven medullary thyroid carcinomas. Cytoplasmic staining is seen in a *RAS*-mutant thyroid carcinoma using NRAS61QR immunohistochemistry (**b**). ALK immunohistochemistry also gained recent popularity in the detection of *ALK* fusion-driven papillary thyroid carcinomas (**c**)

Thyroid Tumor Classification

The diagnosis of thyroid tumors of follicular cell derivation is complex and subject to interobserver variability. It would be ideal to have biomarkers that can address borderline features and allow distinction of different entities. In this review, we will provide evidence that biomarkers can offer assistance in the distinction of (a) benign and malignant follicular-patterned tumors, (b) classical and follicular papillary thyroid carcinomas (PTCs), solid and poorly differentiated thyroid carcinomas, and (d) anaplastic carcinoma distinction from sarcoma and squamous carcinomas.

Follicular Lesions

For many years, follicular tumors of the thyroid have been classified within the spectrum of benign follicular nodular disease, follicular adenomas, follicular variant PTCs, and

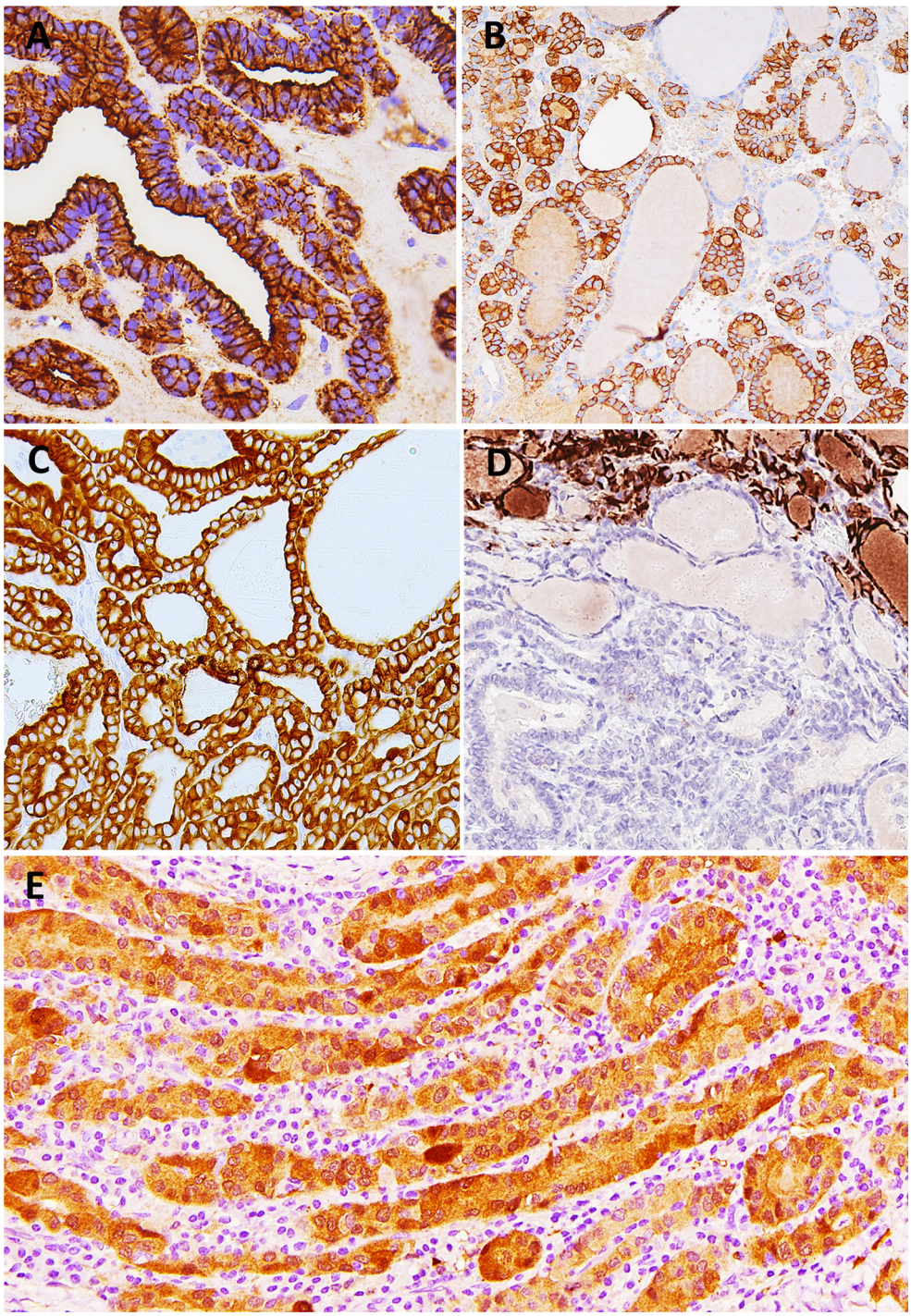

Fig. 8 Universal biomarkers used to support thyroid follicular malignancies. HBME-1 is the most commonly used immunohistochemical biomarker. Membranous and apical-luminal staining is traditionally considered to support malignancy (**a**, **b**). However, functioning thyroid nodules and NIFT-P (formerly known as noninvasive encapsulated follicular variant papillary carcinoma) can also be positive for this biomarker. While most BRAF-like papillary carcinomas show reactivity for HBME-1 (**a**), RAS-like papillary carcinomas tend to be either negative or variably positive (**b**). Diffuse membranous and cytoplasmic cytokeratin 19 (**c**) and loss of CD56 expression (**d**) can also be identified in follicular epithelial-derived thyroid malignancy. Nuclear and cytoplasmic galectin-3 reactivity also expands the spectrum of biomarkers of malignancy (**e**)

follicular carcinomas [2, 92]. Frankly invasive lesions are readily identified and the distinction between follicular variant papillary carcinoma and follicular carcinoma is academic and likely unwarranted [99]. Similarly, the distinction of follicular nodular disease from adenoma is also academic [2, 100]. However, the distinction of a benign from a malignant lesion is of critical importance in patient management. Unfortunately, it is one of the areas of most intense disagreement [101–103]. This lack of consensus is largely because of disagreement of the value of nuclear membrane irregularity in the categorization of malignancy based on cytologic features. While it is easy to recognize florid nuclear atypia with clearing and inclusions, more subtle changes are harder to classify. In an elegant study of the three-dimensional features of papillary carcinoma nuclei [104], Papotti et al. defined the criteria based on previous morphometric analyses [105], and subsequently, Asioli et al. provided a biomarker, emerin, that could be used to distinguish round from irregular nuclei [106, 107]. This biomarker has not been widely adopted in clinical practice.

To address the discrepant diagnoses, a working group of the Endocrine Pathology Society attempted to define the nuclear features that correlate with malignant behavior in noninvasive encapsulated and well-delineated follicular neoplasms [108]. The results of this initiative, that recommended the terminology "Noninvasive follicular thyroid neoplasm with papillary-like nuclei" or "NIFT-P," provide guidelines for the distinction of RAS-like follicular neoplasms from the BRAF-like classical papillary thyroid carcinomas as identified in the TCGA study of papillary carcinoma [109] based on nuclear morphology. However, there are architectural requirements for this distinction as well, and subsequent studies pointed to the importance of papillae as an exclusion criterion for the diagnosis of NIFT-P; while initially it was proposed that there are < 1% papillae, it soon became evident that the presence of even a single papilla could portend a tumor with *BRAFV600E* mutation [110, 111], thus opening the door for classical variant papillary carcinomas with predominant follicular architecture to be included in the NIFT-P category. Since any classical PTC has a higher risk of local nodal metastasis than a true RAS-like follicular neoplasm, this led to a recommendation that additional studies, including the use of the VE1 antibody against the mutant *BRAFV600E* that characterizes approximately 70% of classical PTCs, be applied to ensure correct classification of a RAS-like lesion [110, 111]. Therefore, immunohistochemistry using VE1 currently plays a major ancillary role in the distinction of classical variant PTCs with predominant follicular growth as well as any *BRAFV600E*-mutant thyroid carcinoma; it remains to be seen whether *RAS* mutation-specific antibodies [112] will prove valuable in this regard (Fig. 7a, b). The application of *RET* immunohistochemistry for the diagnosis of PTC [42, 113, 114] has fallen by the wayside with the loss of reliable antisera, but this remains a valuable tool for potential molecular classification of differentiated thyroid carcinomas.

A number of other biomarkers have been proposed to assist in the diagnosis of malignancy in follicular-patterned lesions of the thyroid (Fig. 8). The most widely recognized as helpful is HBME-1, a monoclonal antibody that recognizes an unknown epitope [115–124]. Even though this is a mystery protein, the target of HBME-1 is reliably expressed in a proportion of thyroid carcinomas including papillary and follicular carcinomas as well as in NIFT-Ps [124–126]. The value of this biomarker is highest in follicular lesions where it helps to classify such a tumor as malignant and may have prognostic value [125].

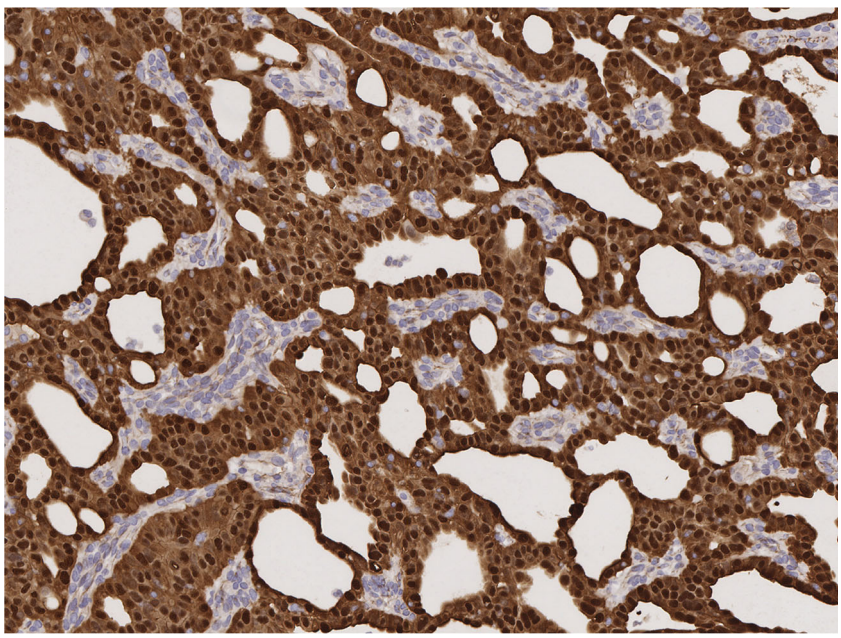

Fig. 9 Cribriform-morular variant papillary thyroid carcinoma. This rare variant of papillary thyroid carcinoma is characterized by diffuse nuclear and cytoplasmic beta-catenin staining. The identification of this variant may be a harbinger of FAP syndrome

Similarly, galectin-3 has value in supporting the diagnosis of malignancy [116–120, 125, 127–137]. Loss of CD56 expression is also a feature of progressive thyroid neoplasia [115]. An interesting biomarker is cytokeratin 19 (CK19); this protein is expressed in nontumorous thyroid, underexpressed or negative in follicular neoplasms, and overexpressed with intense membranous positivity in PTCs, including some with follicular architecture [124, 138–141]. The qualitative interpretation required for this stain differs from the positive versus the negative approach used for other biomarkers [142].

Papillary Lesions

The identification of a papillary lesion in the thyroid usually indicates the diagnosis of papillary carcinoma. The vast majority of tumors with papillae fall into the category of classical

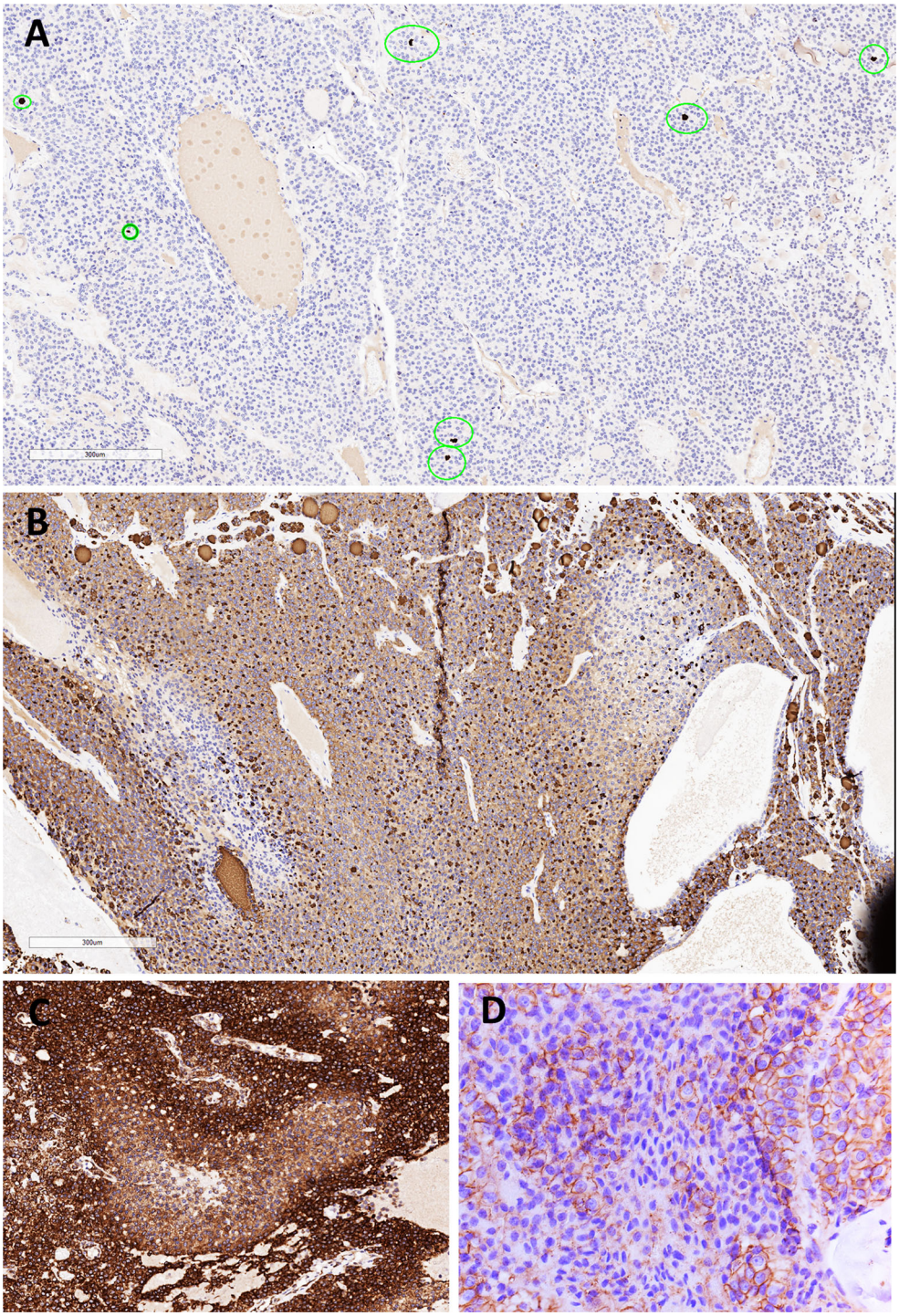

Fig. 10 Immunohistochemical biomarkers of poorly differentiated thyroid carcinoma. It is not uncommon to encounter a differentiated thyroid carcinoma with areas of poorly differentiated thyroid carcinoma. While most cases can be classified using morphological evaluation as defined in the Turin criteria, the application of biomarkers can facilitate the diagnostic workup of challenging cases. Demonstration of increased mitotic activity using phosphoHistone-H3 (**a**; mitotic figures are circled), MIB-1 (often > 10%), reduced expression of thyroglobulin (**b**), bcl-2 (**c**), and E-cadherin (**d**), as well as increased p53 expression and nuclear beta-catenin expression (in the absence of cribriform morular variant papillary thyroid carcinoma) can assist diagnosticians

variant papillary carcinomas, with variable amounts of a follicular component. Molecular studies have shown that the majority of these lesions harbor *BRAFV600E* mutations, and while this can be proven using immunohistochemistry with the VE1 mutation-specific antibody [143, 144], it is clinically and diagnostically unnecessary [145]. The tumors that lack this mutation may have *RET* rearrangements that also can be detected by immunohistochemistry [109], but again there is no clinical relevance.

Papillary architecture can be found in rare benign tumors, follicular adenomas, that are associated with activating mutations of *GNAS* or *TSHR*, resulting in autonomous hyperactivity and tumors that can be associated with clinical or subclinical hyperthyroidism [2, 146]. These lesions are usually recognized on routine histology because of the organized architecture of the papillae within intact follicles and the benign nuclear morphology; however, in difficult cases, application of immunohistochemistry can be used to show lack of HBME-1, CK19, galectin-3, and VE1 staining.

One variant of PTC with classical architecture, the diffuse sclerosing type, has been reported to harbor *ALK* rearrangements that can be detected by immunohistochemistry for ALK [147] (Fig. 7c).

Another variant PTC, the cribriform morular variant, typically manifests with a complex growth pattern that can also include papillary growth in addition to cribriform, follicular, and solid architecture. The recognition of this variant is of clinical significance as this histological variant of PTC can be associated with germline mutations of *APC* in the setting of the familial adenomatous polyposis (FAP) syndrome ([2], [148]). Staining for beta-catenin (Fig. 9) shows nuclear translocation due to activated WNT signaling and can confirm this diagnosis. Rare cases are sporadic and harbor somatic mutations of *CTNNB1* that encodes beta-catenin [149].

Solid Lesions

Thyroid tumors with solid architecture include solid variant PTC, MTC, and poorly differentiated, or "insular" thyroid carcinoma [2]. Immunohistochemistry is critical to diagnose MTC based on the expression of chromogranin, calcitonin, and CEA. The distinction of solid variant PTC from poorly differentiated carcinoma requires histologic features, including cell size and nuclear morphology [150] and the identification of mitoses and necrosis [1]. However, other biomarkers can play a role in this distinction. In our experience, poorly differentiated thyroid carcinomas show loss of bcl-2, membranous E-cadherin and beta-catenin, and thyroglobulin [2], as well as increased p53 nuclear reactivity (Fig. 10). The use of phosphoHistone-H3 staining can assist in mitotic counts and Ki67 labeling using the MIB-1 antibody help to identify more proliferative lesions (Fig. 10). Hyalinizing trabecular neoplasms can also be distinguished by a membranous pattern of MIB-1 staining [1] (Fig. 11).

Spindle and Giant Cell Lesions

The diagnosis of anaplastic thyroid carcinoma is easy when it is associated with a more differentiated thyroid carcinoma with clear progression and dedifferentiation. Immunohistochemistry confirms progression with loss of thyroglobulin, bcl-2, E-cadherin, and beta-catenin; increasing p53; and ultimately loss of TTF-1 followed by loss of PAX8 reactivity. As mentioned earlier, reactivity for PAX8 can be seen in a subset of anaplastic thyroid carcinoma as well as in

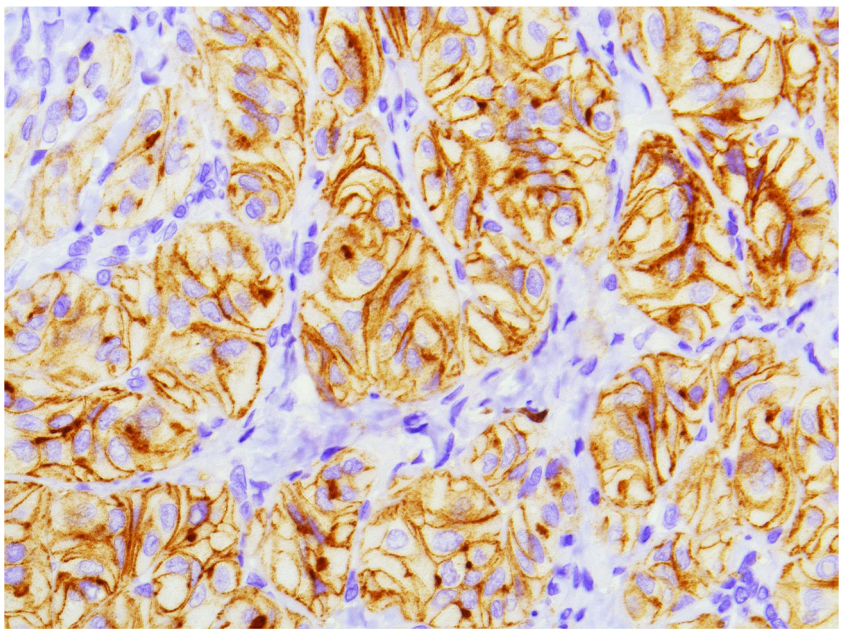

Fig. 11 Paradoxical membranous MIB-1 reactivity is the hallmark of hyalinizing trabecular thyroid neoplasms. This finding is typically identified when the test is performed at room temperature

primary squamous cell carcinomas of the thyroid gland. In cases with no differentiated tumor, the diagnosis of anaplastic carcinoma is one of exclusion and requires extensive immunohistochemistry to rule out other spindle and giant cell tumors.

Prognostic Features of Primary Thyroid Malignancies

Well-differentiated thyroid carcinomas tend to be indolent cancers that are amenable to targeted therapy with radioactive iodine. However, some develop locoregional lymph node metastases, others recur locally, and few develop distant metastatic spread with significant morbidity and mortality. The distinction of localized tumors that can be cured by surgery from those that require more aggressive management with total thyroidectomy to pave the way for radioactive iodine ablation is the biggest challenge in thyroid oncology.

A few immunohistochemical biomarkers have been suggested to predict behavior by well-differentiated thyroid carcinomas. In contrast to the driver genes that are known to be mutated in these cancers [109], most of the alterations implicated in behavior are due to epigenetic dysregulation. Upregulated genes include CITED1 [116, 118, 151],

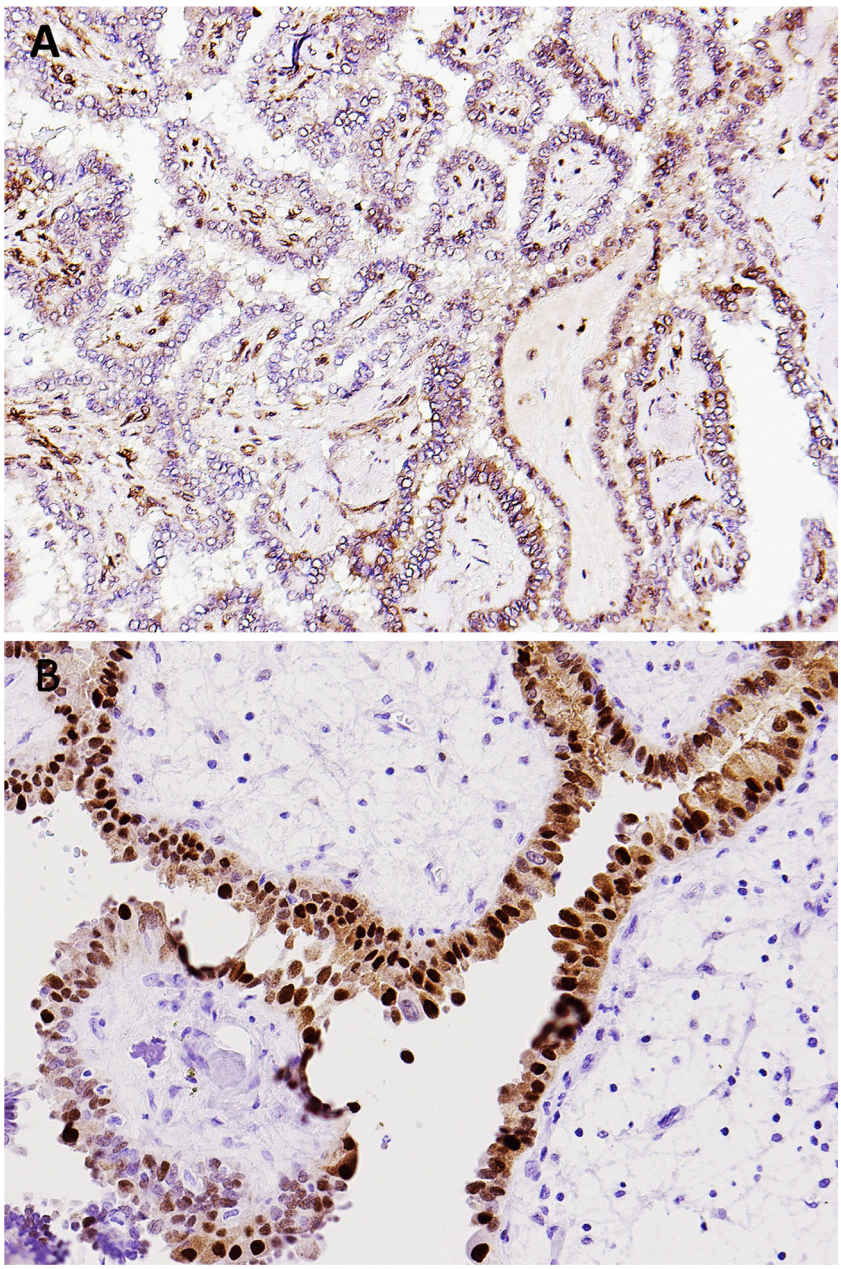

Fig. 12 p27 and cyclin D1 in risk stratification. Loss of p27 (**a**) and overexpression of cyclin D1 (**b**) can predict lymph node metastasis in papillary thyroid carcinoma

galectin-3 [116, 118, 135–137], CK19 and high molecular weight cytokeratins [116, 118, 124, 140], CD57 and CD44V6 [152], as well as the mysterious protein detected by HBME-1; downregulation in differentiated thyroid cancer is mainly seen with CD56 [115]. These biomarkers may have prognostic value [125].

The cell cycle regulators p27 and cyclin D1 are dysregulated with loss of the tumor suppressor p27 and overexpression of the cyclin D1 in papillary carcinomas with the potential for lymph node metastasis [125, 153–155] (Fig. 12). Expression of p27 may be epigenetic through miRNAs [156], but posttranslational processing that is PTEN-mediated through Skp2 degradation can be inhibited by vitamin D [157, 158].

Fibronectin is also overexpressed in locally invasive tumors [(118], [159–161)] as is MAGE-A [160, 162], CEACAM1 [163, 164], and osteopontin, which mediates CD44v6 and CEACAM action [165, 166]. ERβ expression is also upregulated in more aggressive classical PTCs [125]. Rap1 (Ras-proximate-1 or Ras-related protein 1) is a GTPase that acts as a putative oncogene similar to RAS. It is regulated by the Rap1 GTPase-activating protein (GAP) Rap1GAP. This gene is downregulated in PTCs [167], but the methylation status of this gene has not been reported.

One of the best predictors of distant metastatic spread by a differentiated thyroid carcinoma is vascular invasion [168]. Indeed, in the thyroid gland, there is a markedly distinct difference in prognosis between tumors that exhibit lymphatic invasion, which spread to locoregional lymph nodes and can be cured with radioactive iodine, and those with angioinvasion that spread to the lungs, bone, brain, and liver. Assessment of lymphatic versus vascular invasion may require the use of biomarkers such as D240 (Fig. 13) that decorates lymphatic channels versus CD31, CD34, and ERG that highlight blood vessels. Among the three vascular markers, ERG is the most specific and offers clean staining. Since true vascular invasion in vivo induces a thrombotic reaction, special staining for fibrin and immunolocalization of fibrinogen can be helpful.

Other prognostic features include loss of biomarkers of differentiation, including reduced thyroglobulin and NIS in poorly differentiated thyroid carcinomas, and loss of almost all markers of differentiation in anaplastic carcinomas.

In MTC, loss of calcitonin with retained CEA positivity is associated with more aggressive behavior. The role of Ki67 as a proliferation marker in this type of neuroendocrine neoplasia and in other thyroid tumors [169, 170] remains to be validated.

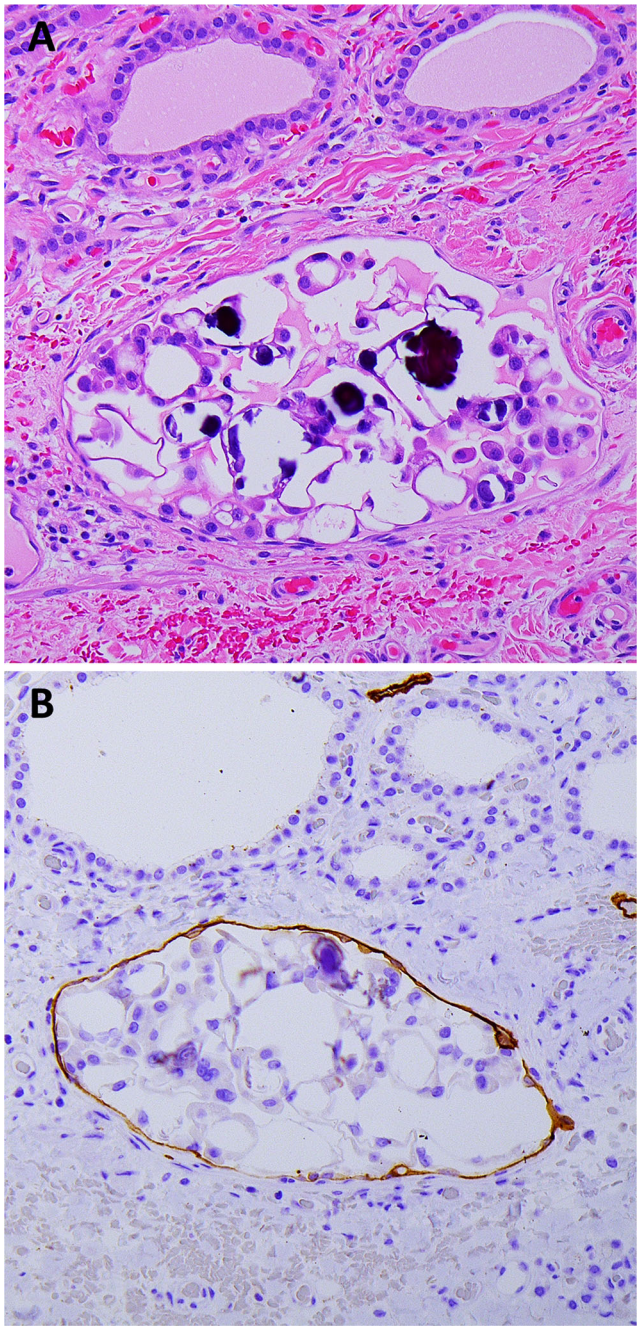

Fig. 13 Distinction of lymphatic invasion from vascular invasion. This distinction is one of the most important in differentiated thyroid carcinomas of follicular epithelial derivation. Vascular channels can be highlighted using ERG immunohistochemistry, whereas D240 can be used to distinguish lymphatic channels. This composite photomicrograph illustrates lymphatic invasion (**a**: H&E, **b**: D240)

Identification of Genetic Predisposition

Genetic predisposition to thyroid neoplasia is becoming increasingly recognized as a feature that the pathologist can predict. The first studies of this type of genetic analysis were initiated with the identification of C-cell hyperplasia in patients with multiple endocrine neoplasia type 2 [67], a diagnosis that relies entirely on immunohistochemical confirmation. While many patients with MEN2 exhibit florid C-cell hyperplasia (Fig. 14) with neoplasia illustrating the progression of this disorder, some cases are diagnosed by identifying

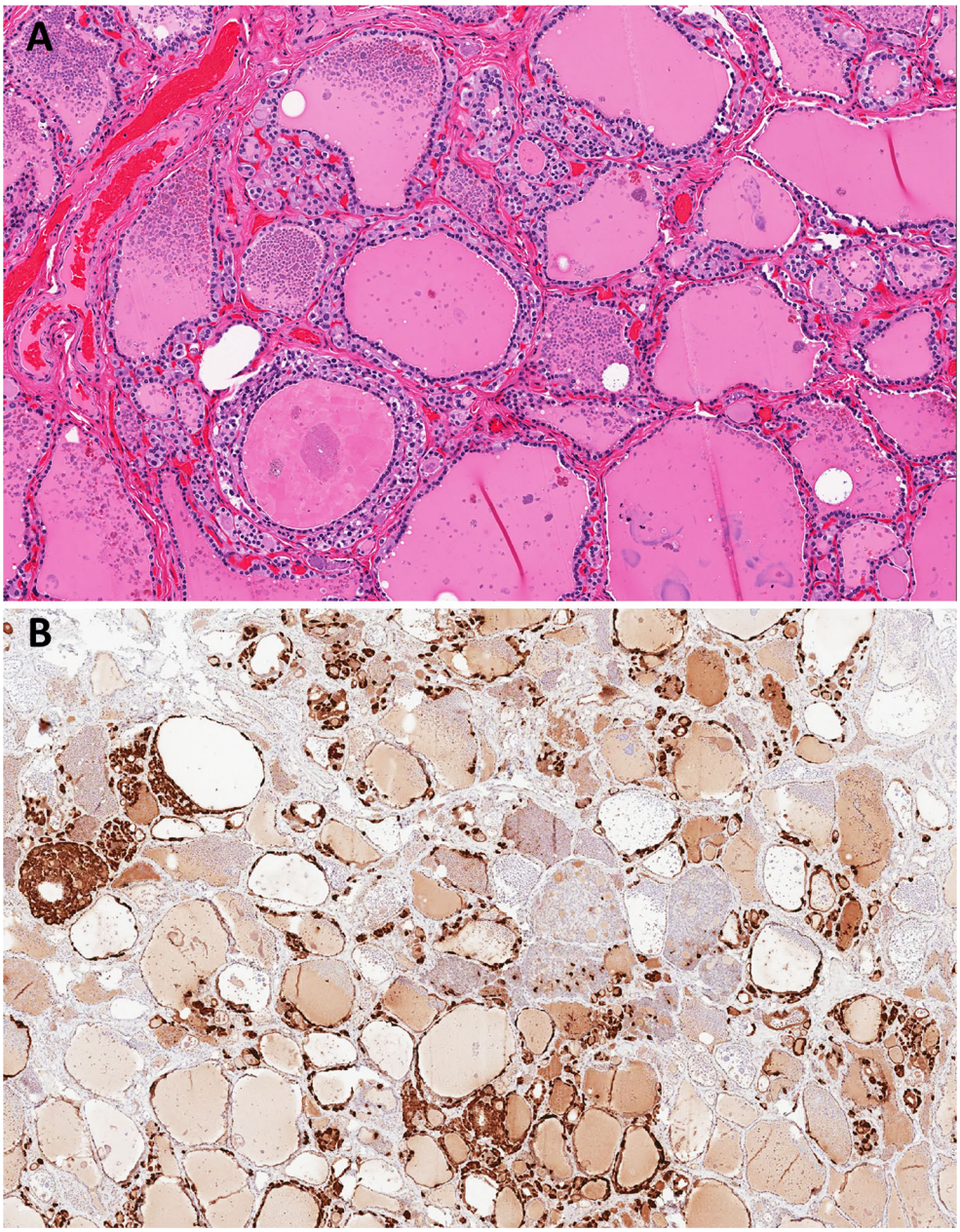

Fig. 14 Bilateral and multifocal C-cell hyperplasia is the hallmark of germline RET-disease (MEN2 syndrome). This composite photomicrograph illustrates an incidental finding in a thyroidectomy specimen. The identification of bilateral florid C-cell hyperplasia (**a**) led to positive germline *RET* testing. Linear and micronodular C-cell proliferations are highlighted using calcitonin immunohistochemistry (**b**)

C-cell hyperplasia during thyroid surgery for unrelated lesions. This requires the application of CEA and/or CT in all sections from the lateral thyroid lobes [1, 2]. The distinction of nodular C-cell hyperplasia from infiltrative medullary microcarcinoma can benefit from the application of immunohistochemistry for collagen type IV that delineates the basement membranes of follicles [171].

Other familial syndromes associated with thyroid neoplasia include PTEN hamartoma tumor syndromes (Cowden syndrome, Cowden-like syndrome, Bannayan–Riley–Ruvalcaba syndrome, Proteus syndrome, and Proteus-like syndrome), frequently due to *PTEN* inactivation mutations, and familial polyposis coli (FAP) syndrome due to germline *APC* mutations. Patients with Cowden syndrome or other PTEN-related disorders develop multifocal follicular neoplasms [172], and staining for PTEN can confirm global loss attributable to this disorder (Fig. 15). Some authors also recommend the use of SDHB immunohistochemistry as germline mutations or variants in *SDH* genes have also been identified in patients with *PTEN* wild-type Cowden and Cowden-like syndromes [1, 173].

Patients with FAP usually develop the cribriform morular variant of thyroid carcinoma with nuclear translocation of beta-catenin that is diagnostically helpful and usually indicates a germline disorder; however, sporadic cases have also been described [174].

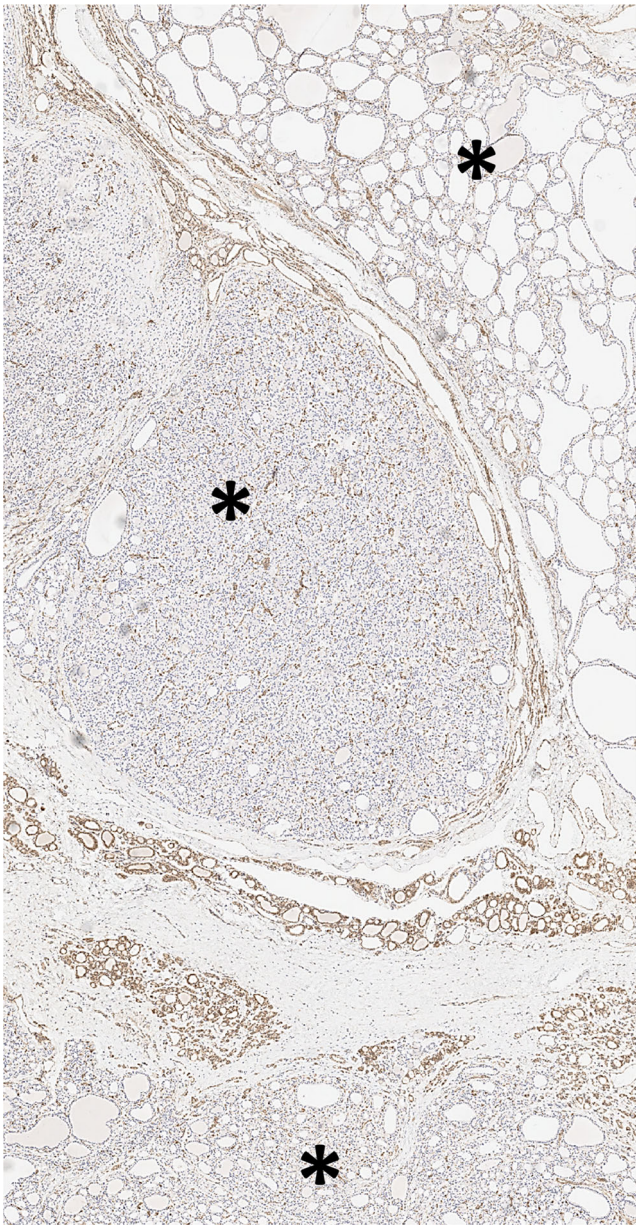

Fig. 15 PTEN immunohistochemistry in PTEN-hamartoma tumor syndrome. Global loss of PTEN in multiple nodules is the hallmark of PTEN-hamartoma tumor syndrome. Asterisks indicate nodular proliferations; please note that the nonproliferative thyroid tissue retains PTEN expression as do nontumorous stromal elements

Conclusions

While much of thyroid pathology is based on examination of slides stained with hematoxylin and eosin, there is a significant role for immunohistochemistry in ensuring the correct diagnosis of thyroid nodules, defining predictive biomarkers for behavior, and potentially identifying genetic predisposition to disease.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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