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Review

Diagnostic Utility of Galectin-3 in Thyroid Cancer

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Galectin-3 (Gal-3), which has received significant recent attention for its utility as a diagnostic marker for thyroid cancer, represents the most well-studied molecular candidate for thyroid cancer diagnosis. Gal-3 is a protein that binds to β-galactosidase residues on cell surface glycoproteins and has also been identified in the cytoplasmic and nuclear compartment. This marker has been implicated in regulation of normal cellular proliferation and apoptosis, as well as malignant transformation and the metastasis of cancer cells. We here present a mechanistic review of Gal-3 and its role in cancer development and progression. Gal-3 expression studies in thyroid tissue and cytologic tumor specimens and their methodological considerations are also discussed in this article. Despite great variance in their methodology, the majority of immunohistochemical studies found that Gal-3 was differentially expressed in thyroid carcinoma compared with benign and normal thyroid specimens, suggesting that Gal-3 is a good diagnostic marker for thyroid cancer. Recent studies have also demonstrated improved methodological reliability. On the other hand, Gal-3 genomic expression studies have shown inconsistent results for diagnostic utility and are not recommended. Overall, the development of Gal-3 as a diagnostic marker for thyroid cancer represents a promising avenue for future study, and its clinical application could significantly reduce the number of diagnostic thyroid operations performed for cases of indeterminant fine needle aspiration biopsy cytology, and thus positively impact the current management of thyroid nodular disease. ***(Am J Pathol 2010, 176:2067–2081; DOI: 10.2353/ajpath.2010.090353)***

Thyroid cancer represents one of the few cancer types that remains a diagnostic dilemma for the clinician. Thyroid nodules are extremely common in the general population, being identified in 5% of patients by palpation and 50% by ultrasound examination.[1] Fine needle aspiration biopsy (FNAB) represents the critical initial diagnostic test used for evaluation of thyroid nodules. However, diagnosis of thyroid cancer still remains uncertain in a large number of cases. In a review of more than 18,000 thyroid FNABs performed at the Mayo Clinic, FNAB had a reported sensitivity of 83%, specificity of 92%, and accuracy of 95%.[2] Furthermore, in up to 15% of cases, the diagnosis of cancer cannot be definitively determined by FNAB. This occurs in certain histological types of thyroid tumors in which benign and malignant thyroid lesions have overlapping cytomorphologic characteristics. There are also currently no patient or tumor characteristics that can reliably predict the presence of cancer in individuals diagnosed with a thyroid neoplasm of Hurthle cell or follicular subtype.[3,4]

Thus, when a thyroid tumor with this indeterminate cytology is identified by FNAB, the current recommended approach is a diagnostic operation for removal of either a portion of or the entire thyroid gland.[3–5] In addition to the emotional distress experienced by patients who undergo surgery for a possible cancer diagnosis, thyroid resection carries a low but significant risk of permanent injury to associated parathyroid glands and nerves that may lead to the need for life-long calcium supplementation or voice dysfunction, and extremely uncommonly the need for a long term tracheostomy. Indeed, only approximately one

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in five patients undergoing thyroid resection for indeterminant FNAB cytology will eventually be diagnosed with a thyroid cancer by histopathological evaluation.[3]

Study of the molecular characteristics of thyroid cancer has allowed for the development of potential molecular diagnostic tools. In the largest thyroid cancer diagnostic marker panel study reported to date, we recently found Galectin-3 (Gal-3) to be the most accurate standalone marker for differentiated thyroid cancer diagnosis (DTC) when compared with a panel of 56 other molecular markers.[6] The study used a tissue microarray containing 100 benign and 105 malignant thyroid tumors that was stained for expression of 57 markers. The most useful markers for DTC diagnosis were Gal-3, Cytokeratin 19, vascular endothelial growth factor, androgen receptor, p16, Aurora-A, and Hector Battifora mesothelial antigen-1 (HBME-1). Furthermore, the classification performance of Gal-3 alone (accuracy of 86.9%) was almost as good as the best multimarker panel (accuracy of 91.0%) determined by a Random Forests algorithm using marker combinations from the entire molecular marker panel.

Gal-3 is one of the best studied molecular markers for thyroid cancer diagnosis. Numerous studies have elucidated the mechanistic role of Gal-3 in normal physiology and cancer. More than 60 protein expression studies, evaluating more than 6000 thyroid specimens, have been reported in the current literature that investigate the utilization of Gal-3 as a thyroid cancer diagnostic marker. The aim of this review is to present: (1) a mechanistic overview of Gal-3 in thyroid cancer, (2) an evaluation of Gal-3 expression studies with focus on a Gal-3 immunohistochemical (IHC) testing methodologies, and (3) an overview of studies reporting utilization of Gal-3 expression for thyroid cancer diagnosis.

Gal-3 in Biology and Cancer

Galectins are a large family of proteins that recognize and bind β-galactosides on cell glycoproteins and glycolipids.[7] Gal-3 is a structurally unique 31-kDa member of the galectin family. Although other members exist as oligomers, Gal-3 is the only member that exhibits a pentameric structure and thus is capable of crosslinking glycoproteins at the cell surface to form new lattices that are involved in cellular signaling and receptor endocytosis. The C-terminal domain of Gal-3 contains a carbohydrate recognition domain responsible for the binding of the lectin to its specific carbohydrate.[8,9] The N-terminal domain is rich in proline, tyrosine, and glycine residues[7,10–13] and enables the formation of pentamers, and thereby plasma membrane galectin lattice microdomains[12,14] involved in cellular signaling and receptor stabilization[15,16] (Figure 1). On proteolytic cleavage of the N-terminal domain, the extracellular functions of Gal-3 are lost, presumably because of its inability to form pentamers.[14] Furthermore, Gal-3 phosphorylation can occur at the N-terminal domain on the Serine-6 and Serine-12 residues.[17]

Gal-3 has been identified in the nucleus, cytoplasm, and extracellular space. It has a role in the regulation of apoptosis, cell motility, and T-cell growth[11] and has also been implicated in thyroid cancer tumor progression.[18] An understanding of the role of Gal-3 in cellular physiology may provide insight into its significance in thyroid cancer.

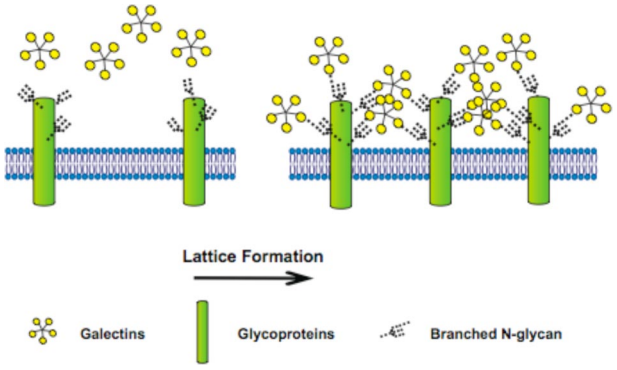

Figure 1. Formation of plasma membrane galectin lattic microdomains.

Regulation of Apoptosis

Upregulation of Gal-3, and its translocation into the nucleus, occurs in proliferating cells, suggesting a function in normal cell growth.[19] In disease, Gal-3 has been found to be significantly overexpressed in the nuclear compartment of rapidly proliferating human thyroid papillary carcinoma cells. In the nucleus, Gal-3 acts as an upregulator of thyroid-specific transcription factor 1 transcriptional activity, and thus Gal-3 expression contributes to the highly proliferative state of these cells.[20] Moreover, reduction of Gal-3 expression by siRNA silencing was found to induce cellular apoptosis in human papillary thyroid cancer cell lines[21] and human colorectal cancer cells.[22]

A functional domain at the COOH-terminal region of Gal-3 has been shown to have significant sequence homology with that of the highly conserved BH1 domain of the *bcl-2 gene* family that contains an apoptosis-inducing NWGR (Asp-Trp-Gly-Arg) amino acid motif.[23] This anti-death motif is responsible for the inhibition of cytochrome *c* release from mitochondria.[24] Furthermore, Gal-3 has recently been shown to belong to the p53/HIPK2 apoptotic pathway.[25] p53 is a sequence-specific transcription factor shown to transcriptionally suppress Gal-3 expression, and p53-induced apoptosis is dependent on its regulatory effects on Gal-3.[25] Indeed, studies using IHC expression show a positive correlation between *p53* mutations and Gal-3 expression in human thyroid carcinoma cells. Concordant expression of p53 and Gal-3 was found in 11/21 (52%) of poorly differentiated thyroid cancers and 5/7 (71%) of anaplastic thyroid cancers, and Gal-3 protein expression was comparatively higher in human thyroid cancer cell lines expressing p53 mutations compared with those with wild-type p53.[26] Furthermore, using MDM-2 (also a transcriptional target of p53) as a surrogate of p53 activity, positive Gal-3 expression was shown in 7/10 (70%) of tumors in a cohort of nonfunctioning p53 tumors characterized as having positive p53 expression and negative MDM-2 expression. In the same study,

mutant p53 overexpression in null or wild-type p53 expressing cells resulted in increased Gal-3 expression, and Gal-3 expression was reduced when mutant p-53 expression was disrupted by transient RNA transfection. Thus, these findings suggest that Gal-3 and p53 may have coordinate activity in thyroid cancer, and study of their expression pattern in tumors may have clinical utility. Furthermore, other proteins involved in the regulation of apoptosis, such as CD95[27] and nuclin,[28] have also been shown to interact with cytoplasmic Gal-3.

Cellular Transformation and Metastasis

Overexpression of Gal-3 by stable transfection of thyroid follicular cells have been found to lead to changes in cellular phenotype, including the development of anchorage-independent growth, increased cellular proliferation, and loss of contact inhibition when compared with nontransfected cells.[29] Upregulation of Gal-3 in human colon cancer cells has been reported to result in increased liver metastasis, whereas the opposite was seen with Gal-3 down-regulation.[30] Reduction of Gal-3 expression by siRNA knock-down in cancer cell lines was also found to result in the suppression of downstream signaling and induction of cellular apoptosis,[22] and the suppression of cellular transformation in diverse cancer types.[31] One example of this can be seen when infection of T-cells with human T-cell leukemia virus type 1 leads to a significantly increased level of Gal-3 expression in comparison with noninfected T-cells.[19] Thus, these studies suggest that Gal-3 is a regulator of normal cell proliferation and that overexpression of Gal-3 results in malignant transformation and metastasis.

Cellular Distribution of Gal-3

Gal-3 has a complex biology, and the relative contributions of the cytoplasmic and nuclear fractions of Gal-3 in tumorigenesis and metastasis are currently unknown. Gal-3 is predominantly identified in the nucleus and can be transported to the perinuclear and nuclear compartments.[32] In mouse 3T3 fibroblast cells, phosphorylated Gal-3 has been identified in both the nucleus and cytoplasm, whereas the nonphosphorylated form remains exclusively within the nucleus. Cell proliferation in these studies has been associated with an increased fraction of the phosphorylated, and thus cytoplasmic, form of Gal-3.[17]

Similarly, inhibition of the Ser-6 phosphorylation site (Ser-6) of Gal-3 by substitution mutation resulted in loss of antiapoptotic activity.[33] Transfection of Gal-3–deficient human breast carcinoma cell lines with wild-type Gal-3 resulted in phosphorylation and translocation of Gal-3 from the nucleus to the cytoplasm and resistance to apoptosis when treated with apoptotic inducing chemotherapeutic drugs. On the other hand, transfection with Ser-6 mutant Gal-3 led to persistence of Gal-3 in the nucleus and susceptibility to apoptosis on drug treatment.[34] Furthermore, in an experiment evaluating Gal-3 localization and function, transfection of a Gal-3–deficient prostate cell line with cytoplasmic-localized Gal-3 led to increased anchorage-independent cell growth, increased invasion on Matrigel, and enhanced tumor growth, whereas transfection with nuclear localized Gal-3 failed to demonstrate these effects.[35] In the clinical setting, these studies suggest that it is the cytoplasmic expression of Gal-3 in thyroid tumors, rather than its nuclear expression, that would be of critical importance to evaluate with a Gal-3 testing methodology.

The Golgi enzyme, β1,6 N-acetylglucosaminyltransferase V (Mgat-V), is upregulated in multiple cancer types.[36–39] Its expression initiates the production of poly N-acetyllactosamine antennae on N-glycans, the high affinity ligand of Gal-3[15] (Figure 1). The presence of increasing amounts of Mgat-V produced N-glycans is commonly associated with the malignant transformation of both murine and human cells while also showing a correlation with disease progression.[39–41] Numerous glycoproteins, such as epidermal growth factor receptor (EGFR) and transforming growth factor β receptor, have multiple N-glycan binding sites.[15] The number of N-glycan chains is distinct for each glycoprotein, and by determining receptor affinity for the galectin lattice, can impact on receptor responsiveness to metabolic flux and their role in cellular growth and differentiation.[42] The galectin lattice has been shown to compete with caveolin-1 (Cav-1) cell surface microdomains by impeding diffusion of EGFR and limiting its down-regulation by endocytosis, thereby enhancing EGFR signaling capabilities and inducing cell survival and growth.[15,16] In addition, fibronectin polymerization and tumor cell migration are regulated by the degree of Gal-3 binding.[43] Expression of the galectin lattice, in concert with the presence of phosphorylated Cav-1 (pY14Cav-1), plays a role in tumor cell migration by stabilizing focal adhesion kinase and causing increased focal adhesion turnover.[44] Expression of Mgat-V and Gal-3 and recruitment of receptors to galectin lattice domains therefore stimulate local receptor-mediated signaling events that promote tumor cell proliferation and migration.[45,46]

Methodological Considerations in the Evaluation of Gal-3 Expression

A comprehensive evaluation of the English literature reporting Gal-3 protein expression in thyroid cancer was performed utilizing studies that were identified through the 'Pubmed' and 'Medline' databases with the search terms 'galectin' and 'thyroid.' All IHC studies reporting Gal-3 expression, utilizing tissue or cytological specimens, were included in this review. Only studies that report the corresponding pathological diagnoses were included. Molecular panel studies that included Gal-3 have been recently reviewed by our group[47] and receive limited discussion in the current review.

Numerous different methodologies have been utilized for the immunohistochemical study of Gal-3 expression in thyroid specimens. These different experimental techniques have included variations in biotin handling and antigen retrieval protocols, antibody characteristics, antibody dilution, marker localization, and criteria for positive

Table 1. Description of Methodology for Immunohistochemical Studies of Galectin-3 Expression in Thyroid Cytologic Specimens

Study	Year	Specimen type/FNAB needle	Cytologic diagnosis	Antibody	Source	Antigen retrieval	Biotin handling	Criterion pattern of staining	Cut-off for positivity (negative versus positive)*
Orlandi[48]	1998	Cell block/22G	Various	Rat monoclonal, M3/38	Boehringer	n.s.	Direct strep-ABC detection	Cytoplasmic	Intensity, distribution
Gasbarri[49]	1999	n.s./n.s	n.s.	Rat monoclonal, M3/38	Boehringer	Microwave heating	Indirect ABC complex	n.s.	<50% vs. ≥50%
Inohara[50]	1999	n.s./n.s.	Various	Rat monoclonal, M3/38 (‡)	ATCC	n.s.	Direct ABC detection	n.s.	n.s.
Bartolazzi[51]	2001	Cell block, smear/n.s.	n.s.	Monoclonal	Novocastra	Microwave heating	Indirect ABC complex	n.s.	<10% vs. 11% to 49%, >50%
Saggiorato[52]	2001	Cell block/22G	Follicular	Clone 9C4	Novocastra	Microwave heating	Direct strep-ABC detection	Cytoplasmic	Intensity, distribution
Aratake[53]	2002	Smear/n.s.	Various	Clone 9C4	Medac diagnostika	n.s.	n.s.	n.s.	Neg, weak vs. mod, strong
Papotti[54]	2002	Cell block/22G	Various	Clone 9C4	Novocastra	Microwave heating	Direct strep-ABC detection	Cyoplasmic	Nuclear vs. cytoplasm ± nuclear
Maruta[55]	2004	n.s./n.s. (§)	Follicular	Monoclonal	Medac diagnostika	n.s.	Block (Bio Genex)	Membranous	<50% vs >50%
Saggiorato[56]	2004	Cell block/22G	Follicular	Mouse monoclonal, Clone 9C4	Novocastra	Microwave heating	Biotin-free detection (EnVision System))	Cytoplasmic or cytoplasmic and nuclear	Intensity, distribution
Collet[57]	2005	Smear/26G, 27G	Indeterminte	Mouse monoclonal, NCL-GAL3	Novocastra	n.s.	Block (30% AB serum), n.s.	Cytoplasmic and/or membranous	<20% vs. 20% to 50%, >50%
Mills[58]	2005	Cell block/21G, 23 G	Various	Clone 9C4	Novocastra	Heat	n.s.	Cytoplasmic or membranous or nuclear	n.s.
Saggiorato[59]	2005	Cell block/22G	Follicular	Clone 9C4	Novocastra	Microwave heating	Biotin-free detection, (EnVision System)	Cytoplasmic	<10% vs. >10%
Aron[60]	2006	Smear/23 G	n.s.	Monoclonal	Novocastra	Microwave heating	Direct strep-ABC detection	Cytoplasmic and nuclear	Neg vs. weak, mod, strong
Carpi[61]	2006	Cell block/18G, 20 G	Follicular	Rat monoclonal	Mabtech	Microwave heating	Biotin-free detection	n.s.	Distribution
Kim[62]	2006	Cell block/n.s.	Follicular	Monoclonal	Novocastra	n.s.	n.s.	n.s.	Intensity, distribution
Torres-Cabala[63]	2006	Cell block/n.s.	n.s.	Mouse monoclonal	Novocastra	Microwave heating	Blocking solution (Dako)	n.s.	0% vs. <10%, 11% to 50%, >50%
Sapio[64]	2007	Cell block/n.s.	Indeterminant or suspicious PTC	Rat monoclonal	Mabtech	Pressure cooking	Biotin-free detection (Vector)	n.s.	<10% vs. ≥10%
Hooft[65]	2008	n.s./n.s.	Follicular	n.s.	n.s.	n.s.	Direct ABC detection	Cytoplasmic	Neg versus weak, mod, strong
Ersoz[66]	2008	n.s./n.s.	n.s.	Clone 9C4	Neomarkers	Microwave heating	Indirect ABC complex	Cytoplasmic	n.s.
Bartolazzi[67]	2008	Cell block/n.s.	Follicular†	Rat monoclonal	Mabtech	Microwave heating	Biotin-free detection (EnVision System)	Cytoplasmic	<5% vs. ≥5%
Franco[68]	2009	Cell block/n.s.	Indeterminant or suspicious	n.s.	Novocastra	n.s.	Direct strep-ABC detection	Membranous and cytoplasmic	<10% vs. ≥10%

*By percent of cell stained or degree of staining; †follicular lesions or suspected follicular tumors; ‡western blot; §aspiration from excised tissue. G indicates needle gauge; n.s., not specified; neg, negative; mod, moderate.

expression. The methodology utilized for each study is presented in Table 1 for thyroid cytologic specimens[48–68] and Supplemental Table S1 at *http://ajp.amjpathol.org* for thyroid tissue specimens.[18,48–51,60,63,64,69–105] Only a few of the studies had clearly reported all aspects of their IHC methodology. Each of the above parameters represents a critically important step for the evaluation of Gal-3 expression and should be considered in detail when developing a reliable and reproducible Gal-3 testing methodology.

Endogenous Biotin

Thyrocytes have unique challenges for IHC study. The high affinity binding of avidin (and also streptavidin) for biotin is a key tool in IHC localization using the avidin-biotin-peroxidase complex (ABC) system (or streptavidin-biotin-peroxidase complex system). Biotin-labeled marker antigens are identified by an avidin-containing probe. However, thyrocytes have a high level of endogenous biotin,[106] which may lead to false-positive results for marker antigen expression. Thus, studies utilizing an avidin-based detection system without biotin blockade should be interpreted with caution. Furthermore, the reactivity of endogenous biotin is enhanced with heat-induced antigen retrieval procedures from formalin-fixed paraffin-embedded specimens, particularly with pressure cooking, and to a lesser extent with microwave heating.[107,108] In particular, Bussolati et al found positive biotin staining in 8/12 thyroid tumors after antigen retrieval even without application of marker antibody.[108] Use of a biotin treatment blockade prevented any staining in these tumors. Thus, biotin-free detection systems, or an avidin-biotin treatment blockade, are critically important for the accurate detection of Gal-3 marker antigen in thyroid tissues.

Heterogeneity of Gal-3 Antibody

Variations in the reactivity of different Gal-3 antibody types and concentrations may also affect study results.

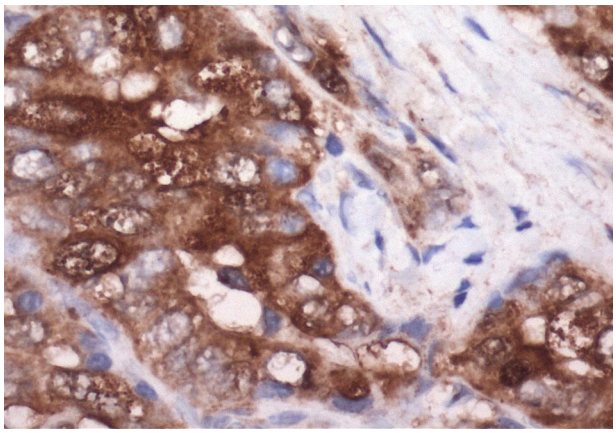

Figure 2. Sample tissue immunohistochemistry exhibiting increased Galectin-3 expression in thyroid papillary carcinoma.

Each antibody may recognize different isotypes or components of Gal-3. A wide variety of Gal-3 antibodies have been utilized in studies of Gal-3 expression in thyroid tumors. However, we are unable to determine whether a single antibody exists that has superior sensitivity or specificity for detection of thyroid cancer. To date, there have been no reports that have simultaneously evaluated and compared the utility of different Gal-3 antibodies for thyroid cancer diagnosis. The performance characteristics of each antibody are also effected by other methodological considerations such as the antibody dilution level, antigen retrieval process, and as already discussed biotin handling.

Scoring Criterion

Although IHC interpretation has often utilized a multipathologist consensus scoring strategy, with the exception of the study by Bartolazzi et al,[67] reports have not described the inter- and intraobserver reliability of Gal-3 IHC interpretation. A clearly interpretable definition for positive marker expression, such as the percentage of cells showing staining or a dichotomization of cases into negative or positive staining groups (irregardless of staining intensity), will be important for the successful future application of studies of Gal-3 expression to assist with thyroid cancer diagnosis (Figure 2).

Because of the complex and varied cellular distribution of Gal-3, the criterion for Gal-3 positivity by IHC interpretation warrants special consideration. Some studies have proposed the need for nuclear staining, in addition to cytoplasmic reactivity, for a reliable designation of Gal-3 positive expression. As already discussed, biological studies have implicated a more prominent role for cytoplasmic localized Gal-3 in tumorigenesis and metastasis, compared with nuclear localized Gal-3. In IHC studies utilizing paraffin-embedded thyroid tumor specimens, two reports have compared the evaluation of Gal-3 nuclear staining and cytoplasmic staining for their ability to diagnose thyroid cancer.[100,101] In a study reported by Weinberger et al on a cohort of papillary thyroid carcinoma (PTC), the use of cytoplasmic staining in a papillary thyroid carcinoma (PTC) cohort identified a higher number of Gal-3–positive cases when compared with the use of nuclear staining, 82% (28/34) and 62% (21/34), respectively. All cases of benign adenomas ($n = 48$) were negative for Gal-3 expression by both criteria.[100] Similarly, Liu et al also reported that cytoplasmic Gal-3 staining identified a higher proportion of PTC cases when compared with nuclear Gal-3 staining, 92% and 80% respectively ($n = 53$ PTC cases).[101] However, both studies reported a lower proportion of Gal-3 expression in PTC specimens when compared with other reports. Similarly, studies using either scoring methodology have not any consistently demonstrated any single criterion to be superior to another, although many other methodological factors influence the performance characteristics of the Gal-3 testing methodology.

Histological and Pathological Diagnoses

Evaluation of the performance characteristics of the Gal-3 testing methodology relies on an accurate histological diagnosis of the thyroid tumor specimen. Thus, any study reporting on Gal-3 expression first requires an accurate and reliable pathological diagnosis of the thyroid tumor. Furthermore, the cytomorphology of PTC may also represent a diagnostic challenge for the cytopathologist. By FNAB, nuclear features of PTC include: an irregular and enlarged nucleus, eccentric micronucleoli, fine chromatin, longitudinal nuclear grooves, and intranuclear pseudoinclusions.[109] However, even despite established cytomorphologic definitions, interpretation of the required threshold of nuclear change for a cancer diagnosis can often be difficult.[110]

Although independent reviewers were used in some studies for evaluation of Gal-3 expression, previous studies have generally not reported the utilization of independent reviewers for the evaluation of the histological diagnosis. The recent study reported by Bartolazzi et al is the first to use independent review of both histological and IHC specimens.[67] This group was thus able to report a more reliable determination of Gal-3 expression in thyroid cancer.

Reliability of Methodology: Toward a Clinical Gal-3 Test Method

A standardized protocol for widespread clinical application of Gal-3 expression as a diagnostic tool for discrimination of benign and malignant thyroid lesions has yet to emerge. Unfortunately, previous studies have been lacking in reporting of key methodological steps in the IHC evaluation of Gal-3 expression by thyroid tumors. Despite these limitations, results from these studies have been encouraging. Certainly, the recent study reported by Bartolazzi et al[67] represents an important step toward the universal application of a Gal-3 testing methodology. In this study the methodological steps for Gal-3 testing were

Table 2. Results of Immunohistochemical Studies of Galectin-3 Expression in Thyroid Cytological Specimens

	(%, Number of positive tumors)*											
Study	Papillary carcinoma		Papillary carcinoma, classic variant		Papillary carcinoma, follicular variant		Follicular carcinoma		Hurthle cell carcinoma		Poorly differentiated carcinoma		Medullary carcinoma	
Orlandi[48]			100	8/8	100	7/7	67	4/6	100	8/8				
Gasbarri[49]	100	2/2					100	3/3						
Inohara[50]	100	8/8					100	1/1						
Bartolazzi[51] (block)	98	45/46					84	16/19	92	12/13	25	1/4		
Bartolazzi[51] (smear)	100	12/12					100	11/11	100	5/5				
Saggiorato[52]							94	16/17						
Aratake[53]	100	37/37					83	5/6					33	1/3
Papotti[54]	89	25/28												
Maruta[55]							89	31/35						
Saggiorato[56]	100	26/26					87	34/39						
Collet[57]	96	24/25					33	1/3					0	0/1
Mills[58]	50	2/4												
Saggiorato[59]					98	41/42	85	28/33						
Aron[60]	80	8/10					75	3/4						
Carpi[61]							92	11/12						
Kim[62]							74	25/34						
Torres-Cabala[63]	100	3/3			25	1/4	50	1/2						
Sapio[64]	65	11/17					40	2/5						
Hooft[65]							79	19/24						
Ersoz[66]	83	5/6					60	3/5					0	0/1

(*table continues*)

very clearly reported. Currently this is the only study evaluating Gal-3 expression in thyroid tumors that utilized a multicenter consensus pathology review and reported on the inter- and intraobserver reliability in IHC Gal-3 interpretation.

The recent utilization of HER-2 testing of breast cancer may serve as a model for the successful wide-spread clinical application of a testing methodology for marker expression. In a consensus report by the National Comprehensive Cancer Network Task Force on HER-2 testing in breast cancer, laboratories that offer clinical HER-2 testing must undergo a defined process for validation of their testing methodology. The process involved: (1) use of 50 to 100 samples of the tumor type proposed for clinical testing, (2) application of the test method at least twice on the same samples for proof of its internal validity, (3) comparison of test results with an established laboratory or test method utilizing the same tumor samples with a 95% concordance rate.[111] This procedure has proven to be an effective approach in the transfer of a laboratory testing methodology to the clinical setting and could potentially be applied to Gal-3 testing of follicular thyroid tumors as an adjunctive clinical diagnostic tool.

Table 2. *Continued*

(%, Number of positive tumors)*												
Anaplastic carcinoma		Follicular adenoma		Adenoma, unspecifiec		Goiter		Hyperplasia		Thyroiditis, graves		Comments
		10	3/29									
		0	0/7			0	0/11	0	0/11	0	0/2	
		0	0/5									
100	1/1	9	3/35					0	0/32	0	0/4	Multicenter immunopanel with Gal-3 and CD44v6 for benign versus malignant lesions showed SN 81%, SP 94%.
		6	2/35					1	1/151	0	0/2	Multicenter immunopanel with Gal-3 and CD44v6 for benign versus malignant lesions showed SN 88%, SP 98%.
				8	4/52							
100	3/3	14	2/14			0	0/16					
						0	0/12					Gal-3 expression identified in 100% (4/4) of lymph node metastases of PTC.
		25	11/44									No significant association with capuslar invasion, vascular invasion, metastasis, age, or tumor size. Immunopanel with Gal-3 and CD44v6 in FTC versus FA showed SN 66%, SP 93%.
		11	12/105									
		7	1/14	13	1/8							Hurthle cell adenoma cases utilized—results indicated in Adenoma table heading.
		0	0/1									
		6	3/50									Immunopanel for benign versus malignant lesions: Gal-3 and HBME-1 showed SN 97%, SP 90%; Gal-3 and CK19 showed SN 99%, SP 84%; Gal-3, HBME-1 and CK19 showed SN 100%, SP 82%.
		100	1/1			0	0/5	0	0/2			Only cases with tissue diagnosis have been extracted and are presented here.
		16	3/19					0	0/54			
		5	2/42					0	0/5			
		0	0/2					0	0/2			Corresponding Gal-3 expression using tissue block from patient is reported. Only cases with tissue diagnosis have been extracted and are presented here.
												Only cases with tissue diagnosis have been extracted and are presented here. Gal-3 positivity expression identified in 15% (6/41) of benign lesions.
		25	3/12									Immunpanel for benign versus malignant lesions: Gal-3 and HKIII: showed SN 92% and SP 75%; Gal-3, HKIII, and CyclA showed SN 96%, SP 75%.
0	0/1	0	0/2			0	0/18			0	0/4	5/6 PTC showed Gal-3 expression. FNAB sample of remaining case was taken from dominant nodule without malignant features.

SN indicates sensitivity; SP, specificity.
*Compared to pathologic diagnosis.
Results for study by Bartolazzi et al[67] and Franco et al[68] are discussed in the text.

Cytologic Specimens

Ideally, new operating protocols, such as antibody dilutions and antigen retrieval processes, would require validation for the transition from tissue to cytologic specimens.[112] Needle aspiration biopsy specimens are utilized as direct smears or spun into cell blocks for marker analysis. Direct smears have the advantage of utilizing a small amount of material and a retrieval procedure that is familiar to the clinician. However, because of the limited material, testing of marker panels is unlikely and the appropriate number of negative controls may be difficult to obtain. Cell blocks are easily stored and handled. However, cytologic preparations may be performed in a variety of different fixatives that can affect IHC staining, and thus the methodology utilized must be carefully considered and results validated by comparison with Gal-3 expression in pathology samples.

The ability to sample an adequate number of cells for analysis can be a challenge, particularly for small thyroid nodules. The application of ultrasound guidance and the utilization of large-needle aspiration biopsies (LNB) have

both been studied.[67,113] In a review of LNB in thyroid nodules, complications were few and resolved without intervention in the majority of cases. There have been no serious complications attributed to LNB reported in the literature.[113] Furthermore, the use of ultrasound guidance with LNB could theoretically reduce complications and also allow for sampling of small nodules. The use of LNB could also potentially be of benefit for molecular marker studies, and various techniques for cytological sampling have been reported (Table 1). In the Gal-3 expression study by Bartolazzi et al, 90% of cell blocks created from LNB-derived specimens had five or more sections available for further study. Compared with specimens obtained by FNAB, only 10% of cell blocks had one to two sections that remained for further study.[113]

Protein Expression of Gal-3 in Thyroid Cancer

Immunohistochemical Studies Evaluating Gal-3 Expression in Thyroid Tissue Specimens

Results of IHC studies evaluating Gal-3 expression in thyroid tissue specimens are summarized in Supplemental Table S2 at *http://ajp.amjpathol.org*.[18,48–51,60,63,64,69–105]

Follicular neoplasms represent one of the most troublesome thyroid cytologic diagnostic groups. Expression of Gal-3 has ranged from 20% to 100% in reported cases of follicular thyroid carcinoma (FTC). The largest series, reported by Bartolazzi et al, identified Gal-3 expression in 95% (54/57) of FTC cases.[51] Similarly, for the follicular variant of PTC (FVPTC), Gal-3 positivity ranged from 33% to 100% of cases. However, the majority of studies have also identified Gal-3 expression in greater than 75% of FVPTC cases.

In studies of PTC, Gal-3 expression has been reported in 58% to 100% of cases. However, the majority of studies reported Gal-3 positivity in 90% to 100% of PTC cases. Furthermore, few studies have reported Gal-3 expression in PTC by histological subtype. Gal-3 positivity was identified in 82% to 100% of the classic variant of PTC. In FVPTC, reported results for Gal-3 positively varied widely and ranged from 33% to 100%, with the majority of studies reporting positivity in more than 75% of cases. Comparing results within each study, Gal-3 expression tended to be moderately lower in FVPTC compared with the classic variant of PTC.

The clinical significance of papillary microcarcinomas (PMC) also warrants discussion. Most cases of PMC are detected incidentally and are not believed to have a significant impact on patient outcome.[114] However, a small proportion of PMC cases present with lymph node metastasis,[114] and a recent series by Noguchi et al reporting 2070 patients diagnosed with PMC showed that cancers measuring 6 mm to 10 mm had a significantly reduced recurrence-free survival compared with cancers measuring 5 mm or less.[115] In a study by Cvejic et al, Gal-3 expression was identified in 81% (51/63) of PMC cases, suggesting that alteration of Gal-3 expression is an early event in PTC progression and thus may be involved in PTC tumorigenesis.[116]

In contrast, Gal-3 positivity was found in a small number of cases of benign thyroid tumors and goiters, and not in normal thyroid specimens. Gal-3 was expressed in 0% to 45% of cases of follicular adenomas (FA), with the exception of a study reported by Mehrotra et al.[81] Similarly, studies evaluating adenomas (type non-specified) identified Gal-3 expression in 0% to 63% of cases. However, the majority of studies reported Gal-3 positivity in between 0% and 30% of adenomas. The high rate of expression of Gal-3 in 72% (23/32) of FA cases reported by Mehrotra et al may be a consequence of their use of a direct avidin-biotin peroxidase complex detection system without biotin blockade. Furthermore, Gal-3 expression was not identified in any thyroid goiter cases, with the exception of the reports from Beesely et al[73] and Prasad et al,[87] that found Gal-3 positivity in 38% and 55% of goiter specimens, respectively. The expression of Gal-3 by normal thyroid tissue was uniformly negative across all studies (533 tissue specimens).

Thyroiditis cases have been found to have variable Gal-3 expression that has been reported to range from 0% to 100% in the current literature. In particular, the association of Hashimoto thyroiditis (HT) with PTC warrants further study. Nuclear changes typical of PTC have been reported in HT.[117] In a molecular marker panel study of HT cases with nuclear atypia, Gal-3 expression was identified in 87% (20/23) of cases, including 2 cases with Gal-3 positivity that were reclassified as PTC by pathological review.[118] Thus, the expression of Gal-3 may allow for identification of early malignant changes in a subset of HT cases.

Of the other malignant histological types of thyroid cancer, variable Gal-3 expression was identified in studies of medullary thyroid cancer (MTC), Hurthle cell carcinoma, and poorly differentiated thyroid cancer. However, in cases of anaplastic thyroid cancer (ATC), Gal-3 expression was identified in the majority of cases (75% to 100% of reported cases). Increasing evidence suggests that DTC can progress, or undergo anaplastic transformation, into ATC.[119] In an IHC marker panel study of ATC and DTC, Gal-3 expression was found to be up-regulated in ATC compared with adjacent associated DTC foci, although the change was not statistically significant.[119]

The variability in reported study results highlights the need for the development of a standardized protocol and criteria for evaluation of Gal-3 expression. Several studies have shown encouraging results for ability of Gal-3 to discriminate thyroid cancer from benign thyroid nodules.[49,51,73] In a large multicenter trial, Bartolazzi et al identified Gal-3 expression in 95% (54/57) of FTC cases compared with 3% (4/125) of FA cases.[51] Similarly, Gasbarri et al reported Gal-3 positivity in 100% (14/14) of FTC cases compared with 3% (1/37) of FA cases,[49] and Beasley et al identified Gal-3 expression in 100% (12/12) of FTC cases compared with 10% (2/20) of FA cases.[73] Thus, the use of Gal-3 expression represents a promising adjunctive test method for aiding in thyroid cancer diagnosis.

Immunocytochemical Studies Evaluating Gal-3 Expression in Needle Aspiration Biopsy Specimens

Results of immocytochemical studies evaluating Gal-3 expression in needle aspiration biopsy specimens are summarized in Table 2.[48–68]

Similar to reports utilizing thyroid tissue specimens, the majority of studies evaluating Gal-3 expression in thyroid cytologic specimens have also identified a high proportion of Gal-3 expression cases of FTC compared with FA. With reference to studies evaluating more than 20 cases in each histological group, Gal-3 expression had a reported sensitivity ranging from 74% to 100% for FTC diagnosis. The calculated specificity for differentiation of FTC and FA ranged from 75% to 100%, and the accuracy ranged from 78% to 100%. The majority of these studies utilized FNAB cases with a follicular histological diagnosis (Table 1). However, the calculated specificity and accuracy reported are derived from studies using variable proportions of FTC and FA cases, and thus must be interpreted with caution when considering clinical application.

PTC has also been reported to exhibit Gal-3 expression in FNAB specimens, being identified in 80% to 100% of reported cases. Similar to results reported utilizing thyroid tissue specimens, Gal-3 was not expressed in cases of goiter (62 cases), hyperplasia (257 cases), or thyroiditis (12 cases).

A few studies have reported especially promising results when evaluating the diagnostic utility of Gal-3 in cytological specimens.[51,56,59] Saggiorato et al utilized FNAB cases diagnosed as follicular neoplasms by cytomorphology, and Gal-3 expression was identified in 85% (28/33) of FTC cases, 98% (41/42) of FVPTC, and 6% (3/50) of FA cases.[59] In an earlier study by Saggiorato et al, Gal-3 expression was reported in 87% (34/39) of FTC cases compared with 11% (12/105) of FA cases.[56] Furthermore, a study reported by Bartolazzi et al in 2001 identified Gal-3 expression to have a sensitivity of 100% (11/11) and a specificity of 94% (33/35) in differentiating FTC from FA.[51]

In a recent prospective multicenter trial performed by Bartolazzi et al, the clinical utility of Gal-3 for thyroid cancer diagnosis was evaluated using FNAB samples with a cytological diagnosis of either "follicular lesions" or "suspected follicular tumors."[67] Formalin-fixed and paraffin-embedded cell blocks were created from FNA-derived preparations, and a biotin-free immunoperoxidase technique was used with two different detection methods. Gal-3 positivity was determined by greater than 5% of thyroid cells exhibiting cytoplasmic staining and was reviewed by two pathologists at each center. An additional blinded central review was performed for unselected histological specimens, although cytologic samples were not assessed centrally. In the study cohort, 85% of cases did not have atypical features, whereas 15% of cases exhibited atypia by cytology. Gal-3 expression had an overall reported sensitivity of 78% (95% confidence interval 74–82), specificity of 93% (95% confidence interval 90–95), and accuracy of 88% for distinguishing benign and malignant thyroid tumors. Interestingly, some of the false-negative cases also exhibited Gal-3 expression in their corresponding tissue specimens, and some benign nodules were reclassified as being of uncertain malignant potential when re-examined. Notably, the study was performed at expert centers and thyroid nodules smaller than 1 cm in diameter were excluded.

After this, Franco et al reported a two center-based prospective study that evaluated 248 FNAB specimens with an indeterminant or suspicious diagnosis.[68] Formalin-fixed paraffin-embedded cell blocks were created from the FNAB specimens and stained for Gal-3 using an ABC-detection system. Cytoplasmic staining of more than 10% of thyroid cells was required for Gal-3 positivity, and FNAB and pathological specimens underwent independent pathologist review. The method of IHC assessment was not reported. Gal-3 expression was found to have a sensitivity of 83%, specificity of 81%, positive predictive value of 84%, and negative predictive value of 80%.

The recent studies reported by Franco et al and Bartolazzi et al highlight the diagnostic utility of a Gal-3 testing methodology and address many of the technical issues that must be reported in future studies before its widespread clinical application.

Clinicopathologic Correlates of Gal-3 Expression

Few studies have investigated the clinicopathologic correlates, and thus the prognostic utility, of Gal-3 expression by thyroid cancer (Supplemental Table S2 at *http://ajp.amjpathol.org*[18,48–51,60,63,64,69–105]). Among cases expressing Gal-3, correlations were determined by comparison of cases with weak and strong Gal-3 staining or comparison of the proportion of Gal-3–positive cells. Ito et al identified a significant correlation between Gal-3 positivity and the presence of vascular or capsular invasion in cases of PTC,[85] and Cvejic et al[116] and Faggiano et al[75] reported a significant correlation with Gal-3 expression and the presence of lymph node metastasis in MTC. Lymph node metastasis specimens were all uniformly found to express Gal-3 across various histological thyroid cancer types, including cases of PTC ($n = 56$) as reported by Torregoso et al,[98] and cases of PTC ($n = 20$), FTC ($n = 2$), and ATC ($n = 3$), as reported by Inohara et al.[50] However, other studies did not identify any significant correlations between Gal-3 positivity and various thyroid cancer clinicopathologic prognosticators such as capsular invasion, tumor size, tumor grade, nodal status, or disease stage, in PTC and FTC cases.[71,100,116] Thus, the prognostic utility of Gal-3 expression by IHC study of thyroid cancer tissue specimens warrants further investigation.

Immunopanel Evaluation with Gal-3

Several studies have evaluated the use of additional molecular markers to increase the diagnostic performance of Gal-3 for thyroid cancer. The Hector Battifora mesothelial antigen-1 (HBME-1) and Cytokeratin-19 (CK-19) have been the most commonly studied adjunctive markers to Gal-3. Their use has often enhanced the sensitivity and specificity for distinguishing between benign and malignant thyroid lesions, when compared with Gal-3 alone, although this difference was usually small[84,91,93,96] (Table 2). Furthermore, in a recent review of marker panel studies for thyroid cancer diagnosis reported by our group, Gal-3 was often identified as a top performer, and in several cases, the individual performance of Gal-3 was better than any other marker combination investigated.[47]

Genomic Expression of Gal-3 in Thyroid Cancer

Genomic Profiling Studies in Thyroid Cancer

A number of genome-wide mRNA expression profiling studies have reported the potential value of Gal-3 as a diagnostic tool for thyroid cancer. In a recent metaanalysis of 21 thyroid tumor gene expression profiling studies performed by our group, the gene encoding the Gal-3 protein (LGALS3) was found to be up-regulated in thyroid cancer compared with benign thyroid lesions.[120] Three independent studies, all using the Affymetrix Genechip microarray platform (HG-U95A or HG-U133A) and comprising 107 malignant and benign thyroid samples, reported Gal-3 to be significantly up-regulated in cancer compared with benign or normal samples, with an average fold-change of 3.7 (range 3.5 to 3.8).[121–123] Furthermore, since the publication of our metaanalysis, three additional gene expression profiling studies have been reported in the literature. Two of these studies identified Gal-3 as significantly up-regulated in PTC compared with benign thyroid nodules,[124,125] whereas the third study, evaluating FTC, did not identify Gal-3 as being differentially expressed.[126] Overall, despite the use of several different expression profiling technologies and analytic methodologies, most reports have identified Gal-3 as an important differentially expressed mRNA molecule in thyroid cancer.

Reverse Transcriptase–Polymerase Chain Reaction Studies of Gal-3 Expression in Thyroid Cancer

A number of studies have investigated the potential value of Gal-3 nuclear transcription as a diagnostic marker of thyroid malignancy. Overall, these studies have reported varying levels of promise for Gal-3. Gasbarri et al examined Gal-3 mRNA levels by RT-PCR on a retrospective panel of 28 formalin-fixed paraffin embedded and 17 fresh surgically resected thyroid tissue specimens.[49] Gal-3 mRNA was not identified in normal or benign thyroid tissue but was consistently detected in all cases of thyroid malignancies. In another study, RT-PCR analysis demonstrated overexpression of Gal-3 in all six PTC and three FTC cases evaluated, whereas Gal-3 mRNA expression was either undetectable or identified at low levels in two of three FA cases and in all four normal thyroid tissue specimens.[53]

Despite this early promise, a number of less convincing studies have followed. Martins et al found that while Gal-3 mRNA expression was higher in PTC and FTC than in benign thyroid lesions, Gal-3 transcripts were also present in FA and multinodular goiter cases.[127] Utilizing qRT-PCR analysis of 37 snap-frozen thyroid tissue specimens (8 PTC; 9 FTC; 6 FA; 7 adenomatous nodule, AN; 7 normal), Bernet et al found that measurement of Gal-3 mRNA was useful for diagnosis of PTC but not for differentiating between FTC from FA.[128] Furthermore, Takano et al confirmed that while Gal-3 mRNA was significantly increased in PTC compared with normal thyroid tissue, benign (GT, FA), or other types of malignant thyroid tissues (MTC, ATC, FTC), there was no significant difference observed for FTC compared with FA,[129] and Gal-3 mRNA was identified to be ubiquitously expressed in both benign and malignant thyroid tumors. However, in a subsequent study from this group, the TFF3/GAL-3 mRNA ratio was found to distinguish FA from FTC with a sensitivity and specificity of 80.0% and 91.5%, respectively.[130]

A recent qPCR analysis of FNAB specimens also found no significant difference in Gal-3 mRNA levels when comparing benign and malignant thyroid tumors (5 GT; 7 FA; 4 PTC; 2 FTC).[131] In a 2004 review of five PCR-based Gal-3 expression studies, Bojunga et al concluded that detection of Gal-3 expression by RT-PCR was not a robust marker for thyroid cancer diagnosis.[132] Currently, with the possible exception of PTC, studies of Gal-3 expression by RT-PCR have continued to report inconsistent results for its utilization as a diagnostic marker for thyroid malignancy.

Gal-3 is constitutively expressed by foamy macrophages and endothelial cells.[69] Thus, study methodologies such as Western Blotting or RT-PCR, which use entire tumor tissues for measure of Gal-3 expression, would invariably be erroneous and are not recommended. Furthermore, the cellular localization of Gal-3 is a crucial factor for marker positivity, as discussed above. At this time, IHC appears to be a superior and more accurate testing methodology for Gal-3 expression in thyroid cancer.

Conclusions and Future Directions

Overall, Gal-3 protein expression evaluated utilizing IHC techniques is a sensitive, specific, and accurate marker for the diagnosis of thyroid cancer. Multiple studies have demonstrated differential expression of Gal-3 in cases of thyroid cancer when compared with benign thyroid tumors or normal thyroid tissues, particularly for PTC. Although results for follicular thyroid lesions, either FTC or FVPTC, were not as robust, many reports show very

promising results. Studies have also focused on the use of Gal-3 expression as a diagnostic tool for needle aspiration biopsy specimens with encouraging initial observations. Initial studies reported variable methodologies with wide-ranging results. Only recently, Bartolazzi et al reported on a multicenter prospective trial of a Gal-3 testing methodology for indeterminant FNA specimen that addresses many important methodological considerations discussed in this review.[67] Results from this study reported a sensitivity of 78%, specificity of 93%, and accuracy of 88% for Gal-3 expression when utilized for thyroid cancer diagnosis. Furthermore, options for cytological sampling, such as the use of large needle aspiration biopsy or core biopsy, are currently being studied.[113]

Interestingly, the measurement of serum levels of Gal-3 and its utility as an adjunctive tool for thyroid cancer diagnosis has been recently reported.[105,133] Serum Gal-3 levels were measured by an investigator-derived ELISA and compared with histological diagnosis after thyroidectomy.[105] Although results did not differ between individuals with benign thyroid nodules, with thyroid cancer, or healthy controls, further study of this potentially useful adjunctive diagnostic tool, that may compliment Gal-3 molecular testing methods and traditional cytomorphologic analysis, is needed.

Gal-3 is highly expressed in thyroid cancer, but not in normal thyroid tissue, and infrequently in benign thyroid lesions, and thus, Gal-3 may also represent an attractive target for therapy of thyroid cancer. In one study, disaccharide methyl β-lactosaminide analogues were developed and evaluated for their ability to selectively block binding of Gal-3 to Gal-3 binding glycoproteins. In particular, allyl lactoside was found to be a potent inhibitor of cellular apoptosis and tumor cell aggregation in a melanoma and nonsmall cell lung cancer cell line.[134] In a novel approach reported by Bartolazzi et al, radiolabeled antibodies to Gal-3 were utilized as a diagnostic tool for identification of thyroid tumors in a mouse model transplanted with human Gal-3 expressing tumors or Gal-3 knockout tumors.[135] Radioimmunological imaging accurately visualized Gal-3 expressing tumors in the mice, whereas murine models that received Gal-3 knockout tumors did not show any radioimmuno signal. These reports represent exciting avenues of future study of Gal-3 for improving diagnosis and treatment of thyroid cancer.

Clearly, a standardized protocol is needed for evaluation of Gal-3 expression in thyroid tissues. As an example, the widespread clinical use of IHC and fluorescent *in situ* hybridization testing of HER-2 expression in invasive breast cancer is possible with the use of extensively validated and clearly defined operating guidelines.[111] Standardized methodologies for IHC procedures and marker interpretation will impact on the accurate reporting of Gal-3 expression. Validation of a Gal-3 testing methodology in large multicenter prospective trials has now been reported in the literature, and further validation in diverse populations is required. The relative contribution of nuclear, cytoplasmic, and cell surface expression of Gal-3 in thyroid tumorigenesis and progression also requires further study. Similarly, other apoptotic markers, such as p53, as well as signaling pathways of various tumorigenic molecular markers such as the EGFR and the transforming growth factor β receptor, which have been directly linked with Gal-3 expression, signaling, and activity, also represent other exciting directions for future study.

As presented in this review, there is accumulating evidence in the current literature that suggests clinical utility of Gal-3 as a thyroid cancer diagnostic marker. Additionally, the use of Gal-3 as an adjunct to cytomorphologic evaluation of FNAB, and as a target for therapy, have become a growing area of clinical study. Thus, the further study and development of Gal-3 as a thyroid cancer diagnostic molecular marker could potentially lead to improved outcomes for individuals diagnosed with nodular thyroid disease.

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