Differentiated thyroid carcinoma : diagnostic and therapeutic studies

Liu, Y.Y.

Chapter 2

Combined Immunostaining with Galectin-3, Fibronectin-1, CITED-1, HBME-1, Cytokeratin-19, PPAR-gamma and NIS Antibodies Increases the Diagnostic Accuracy in the Differential Diagnosis of Thyroid Neoplasms

Y.Y. Liu [1], H. Morreau [2], J. Kievit [3,4], T. van Wezel [2] , G vd Pluijm [1], M. Karperien [1], J.A. Romijn [1], J.W.A. Smit [1]

Department of
1) Endocrinology 2) Pathology 3) Medical Decision Making 4) Surgery
Leiden University Medical Center, The Netherlands

Submitted

Abstract

Background: The microscopic distinction between benign and malignant thyroid lesions is often difficult because in particular follicular lesions share many histological features.

Aim: This study was performed to evaluate the diagnostic value of Galectin-3 (Gal-3), HBME-1, cytokeratin (CK)-19, CITED-1, Fibronectin (FN)-1, PPAR-gamma (PPARγ) and cytoplasmic NIS (cNIS) staining in a large panel of thyroid neoplasms. Our study differed from earlier ones with regard to the identification of optimal semi-quantitative cut-off levels using Receiver Operator Curve (ROC) analysis and the use of hierarchical cluster analysis.

Methods: We used tissue arrays consisting of normal thyroid tissue (64), Graves disease (10), multinodular goiter (MNG, 14), follicular adenoma (FA, 12), papillary thyroid carcinoma (PTC, 53), follicular thyroid carcinoma (FTC, 13) and follicular variant of PTC (FVPTC, 11). Antibody staining was scored semi-quantitatively and differential expression was analysed in 2x2 tables and with hierarchical cluster analysis.

Results: In general, we found overexpression of FN-1, CITED-1, Gal-3, CK-19, HBME-1 and cNIS in malignant thyroid lesions. Gal-3, FN-1and cNIS had the highest accuracy in the differential diagnosis of follicular lesions. A panel of Gal-3, FN-1 and cNIS, identified by hierarchical cluster analysis had a 98% accuracy to differentiate between FA and malignant thyroid lesions. HBME-1 was found to be useful in the differentiation between FA and FVPTC (accuracy 88%).

Conclusion: A combination of antibodies increases the diagnostic value in the differential diagnosis of thyroid neoplasms. The combination of FN-1, Gal-3 and cNIS had the best accuracy (98%) whereas HBME-1 may be useful in the differentiation of FVPTC from FA.

Introduction

Although thyroid nodules are common, few are malignant and require surgical treatment. In particular, the microscopic distinction between follicular adenoma (FA), follicular thyroid carcinoma (FTC) and follicular variant papillary thyroid carcinoma (FVPTC)) is difficult because these follicular lesions share overlapping histological features. This is underscored by substantial inter-observer variability in the pathological and cytological assessment of thyroid nodules (1;2). As a result, up to 85% of patients with suspicious cytology who subsequently undergo surgery have benign lesions (3). Therefore, the identification of markers to distinguish benign from malignant tumours is important to avoid unnecessary surgery. In recent years, several immunohistochemical markers have been studied to improve the differential diagnosis of thyroid lesions.

The galectins are carbohydrate binding proteins involved in cell adhesion, cell growth and cell death. Galectin-3 (Gal-3) has been considered a marker with a high diagnostic potential to identify FTC (4-8), but in recent publications Gal-3 staining was also reported in benign lesions (9;10). HBME-1 (Hector Battifora mesothelial), is a monoclonal antibody developed against an unknown epitope of the microvillous surface of mesothelial cells and has been reported to be useful in the diagnosis of malignant thyroid tumours (11-15). Cytokeratins are intermediate filament proteins that are specific for epithelial cells. CK-19 has been found to be strongly and diffusely expressed in PTC, whereas it is heterogeneously expressed in FTC and absent or focally expressed in FA. However, CK-19 expression has also been reported in normal thyroid epithelium, Hashimoto's thyroiditis, and benign thyroid tumors (16-21).

Recent studies based on cDNA expression arrays have identified immunohistochemical markers for the differentiation between thyroid neoplasms. One study confirmed the differential expression of Gal-3 and also identified the extracellular matrix component Fibronectin-1 (FN-1) as a specific marker for PTC (22). In another study, Gal-3, FN-1 and the nuclear protein CITED-1 (*C*BP/p300-*I*nteracting *T*ransactivators with glutamic acid *[E]* and aspartic acid *[D]*-rich C-terminal domain) were found to be overexpressed in PTC (23). A combined approach using a panel of HBME-1, Gal-3 and CK-19 was followed by Casey et al (18), de Matos et al (19) and Prasad et al (24). In the study of de Matos et al, this combination had limited value. In contrast, in the study of Prasad et al (24) this panel had a high sensitivity and specificity for carcinomas.

In most of the before mentioned studies, fixed cut-off levels for positive staining are used. Therefore, we decided to evaluate the diagnostic value of Gal-3, HBME-1, CK-19, CITED-1, FN-1, the sodium iodide symporter (NIS) and peroxisome proliferator activated receptor gamma (PPARγ) in a large panel of thyroid neoplasms. We calculated cut-off levels for positive staining for each antibody using

receiver operator curve (ROC) analyses. We not only analyzed the diagnostic value of each individual antibody, but also the diagnostic accuracy of panels identified by hierarchical cluster analysis. We decided to include NIS and PPARγ because intracellular overexpression of NIS has been reported in a considerable percentage of malignant thyroid tumors (25). Apart from the pathophysiological implications, this expression pattern, if confirmed, may be helpful in the distinction between benign and malignant lesions. In the pathogenesis of thyroid tumors, decreased expression of PPAR-γ has been reported (26-28). Apart from the pathogenetic significance, PPAR γ may therefore also be used as a diagnostic marker.

Material and methods

Patients

One hundred and seventy seven histological samples from surgically removed thyroid lesions, representing 7 different histological thyroid disorders and adjacent normal thyroid tissue were obtained from the pathological archive of the Leiden University Medical Center, the Netherlands. We selected normal thyroid tissue (64), Graves disease (10), MNG (14), FA (12), PTC (53), FTC (13, minimally invasive 5) and FVPTC (11).

Tissue microarrays

Ten percent formalin-fixed, paraffin-embedded blocks routinely prepared from surgical specimens of thyroid tumours were selected for this study. Representative areas containing tumor or adjacent normal tissues were identified by a pathologist (HM). Triplicate tissue cores with a diameter of 0.6 mm were taken from each specimen (Beecher Instruments, Silver Springs, MD, USA) and arrayed on a recipient paraffin block, using standard procedures (29).

Immunohistochemistry

Four μm consecutive tissue sections were cut from each arrayed paraffin block and prepared on pathological slides. The sections were deparaffinised in xylene followed by 0.3% hydrogen peroxide methanol at room temperature for 20 minutes for blocking endogenous peroxidase. After rehydration, except for Gal-3, antigen retrieval treatment was done for CK-19, HBME-1, FN-1, CITED-1, NIS and PPAR-γ immunostaining by microwave treatment in 0.01 M citrate buffer at pH 6.0. After 2 hours cooling down, endogenous avidin activity blocking was performed for NIS immunostaining by incubation with egg-white for 5 minutes followed by biotin for 15 minutes. The sections were incubated with primary antibodies (specified in Table 1) in PBS with 1% bovine serum albumin overnight at room temperature. The negative controls were stained with the primary antibody omitted. Next, sections

Table 1. Specification of Antibodies

Primary antibody	Dilution	Manufacturer	Type	Secondary Antibody	Antigen Retrieval	Extra procedure
NIS	1:200	Donated by Nancy Carrasco, NY, USA	Polyclonal	1	Na-Citrate heating	Endogenous Avidin activity block
PPAR γ	1:30	Santacruz, California, USA (sc-7273)	Monoclonal	2	Na-Citrate heating	No
Galectin-3	1:200	Novocastra, Newcastle, UK (NCL-GAL3	Monoclonal	2	No	No
HBME-1	1:50	DacoCytomation Glostrup, Denmark (M3505)	Monoclonal	2	Na-Citrate heating	No
Fibronectin	1:2000	DacoCytomation, Glostrup, Denmark (A0245)	Polyclonal	1	Na-Citrate heating	No
CITED-1	1:100	Abcam, Cambridge, UK (ab15096)	Polyclonal	1	Na-Citrate heating	No
CK-19	1:100	DacoCytomation, Glostrup, Denmark	Polyclonal	1	No	No
Secondary Antibodies						
(1) Swine-anti-Rabbit	1:400	DacoCytomation, Glostrup, Denmark				
(2) Rabbit-anti-Mouse	1:200	DacoCytomation, Glostrup, Denmark				

were incubated for 30 minutes with either the biotinylated rabbit-anti-mouse conjugate (Dako, Glostrup, Denmark, 1:200) or swine-anti-rabbit (1:400), followed by incubation for 30 minutes with the streptavidin-biotin-peroxidase conjugate (Dako, Glostrup, Denmark 1:100). This step was by a 10-minute incubation with 3,3'-diaminobenzidinetetrachloride substrate in a buffered 0.05 M Tris/HCl (pH 7.6) solution containing 0.002% hydrogen peroxide. The sections were counterstained with haematoxylin.

Scoring

A semi-quantitative assessment of immunohistochemical scoring was performed according to both the intensity of staining and the percentage of positive cells. The criteria are summarized in Table 2. Score results for triplicate samples were summarized in one total score. The resulting score ranged from 1 – 6.

Table 2. Immunohistochemistry staining score levels according to proportion of positive cells and staining intensity

Cells with positive staining (%)	0	10	30	50	100
Intensity			*Score*		
Faint	0	1	2	3	4
Moderate	0	2	3	4	5
Intense	0	3	4	5	6

Statistical analyses

Statistical analyses were performed using SPSS 12.0. Staining scores were summarized and expressed as median and ranges and proportion of samples with scores above the cut-off level. Analyses of significant differences in staining scores were analyzed on a 2x2 base using the Kruskall-Wallis test. Optimal cut-off values for each antibody were identified using Receiver Operator Curve (ROC) analysis for each individual marker. Diagnostic validity was expressed using Bayesian statistics as sensitivity, specificity and accuracy.

In addition to individual protein markers, analysis of the diagnostic accuracy of panels of antibodies was performed using hierarchal clustering analysis of tissue microarray data using Cluster and TreeView (Cluster and TreeView 2.11, Eisen Lab, University of California at Berkely, California). A p value of <0.05 was considered significant.

Results

Protein expression in thyroid lesions

Because a distinct intracellular distribution was observed for some antibodies, their staining scores were categorized according to these patterns: NIS staining was differentially categorized as membranous (mNIS) or cytoplasmic (cNIS). Accordingly, FN-1 was also categorized as mFN-1 and cFN-1. Gal-3 was categorized as cGal-3 or nuclear Gal-3 (nGal-3).

The median values, ranges of expression of the proteins and the proportion of samples with staining scores above the cut-off levels are given in Table 2. Statistically significant differences in protein expression between all categories of thyroid tissues were investigated in 2x2 tables, the results of which are given in Table 3. Examples of staining patterns are given in Figure 1 (see color image at page 150).

In general, malignant tumors showed overexpression of Gal-3 (predominantly PTC), cFN-1 (all carcinomas), CK-19 (mostly PTC), HBME-1 (mostly PTC and FTC) and cNIS (mostly PTC and FTC). In contrast, expression of PPAR-γ and membranous NIS (mNIS) were low or absent in thyroid carcinomas.

In Graves disease, expression of mNIS was abundant as expected. PPAR-γ was also higher in adjacent normal tissues and benign thyroid lesions.

In general the most prominent differences were observed in PTC in comparison with benign lesions and adjacent normal thyroid tissues: PTC showed high expression levels of cFN-1 (median level 5, 96% of tumors), cGal-3 (median level 5, 92% of tumors), cNIS (median level 4, 83% of tumors), HBME-1 (median level 3, 74% of tumors), CITED-1 (median level 5, 98% of tumors) and CK-19 (median level 3, 78% of tumors) and absence of PPARγ and mNIS.

CK-19, Gal-3 and HBME-1 were differentially expressed between PTC and FTC.

FN-1, CK-19, Gal-3, HBME-1 and cNIS were differentially expressed between PTC and FVPTC (Table 4).

FTC had high expression levels of cFN-1 (median level 5, 86% of tumors), CITED-1 (median level 5, 86% of tumors) and cNIS (median level 4, 67% of tumors). In the comparison between FTC and FA, proteins differentially expressed were cFN-1 and cNIS (Table 4). No significant differences were observed in staining patterns between minimally invasive FTC and widely invasive FTC. In the comparison between FTC and FVPTC, the only differentially expressed protein was HBME-1 (Table 4).

FVPTC had a high expression of FN-1 (median level 4, 89% of tumors), CITED-1 (median level 5, 100% of tumors) and HBME-1 (median level 5, 89% of tumors)

Table 3. Protein expression in thyroid lesions

Protein	**Fibronectin 1**		**CITED-1**	**CK-19**	**Gal-3**		**HBME-1**	**NIS**		**PPAR-γ**
Cut-off level	*Cytoplasmic (1.5)*	*Membranous (0.5)*	*(3.0)*	*(1.5)*	*Cytoplasmic (2.5)*	*Nuclear (2.5)*	*(0.5)*	*Membranous (0.5)*	*Cytoplasmic (1.0)*	*(1.0)*
Normal Thyroid (64)	0(0-5) 14%	0(0-2) 2%	3(0-4) 51%	0(0-2) 4%	0(0-4) 10%	0(0-3) 2%	0(0-2) 2%	1(0-4) 59%	0(0-3) 12%	2(0-5) 56%
Benign lesions										
Graves(10)	0(0-4) 22%	0(0-0) 0%	4(2-5) 89%	0(0-0) 0%	1(0-2) 0%	0(0-3) 10%	0(0-0) 0%	6(2-6) 100%	0(0-0) 0%	3(2-4) 100%
MNG (14)	0(0-4) 15%	0(0-1) 8%	3(0-4) 79%	0(0-1) 0%	0(0-2) 0%	0(0-2) 0%	0(0-0) 0%	0(0-5) 45%	0(0-0) 0%	2(0-4) 64%
Benign tumors										
FA (12)	0(0-5) 40%	0(0-0) 0%	4(2-6) 80%	0(0-0) 0%	0(0-0) 0%	0(0-2) 0%	0(0-4) 11%	0(0-0) 0%	0(0-4) 22%	1(0-3) 50%
Malignancy										
PTC (53)	5(0-6) 96%	0(0-6) 40%	5(1-6) 98%	3(0-4) 78%	5(0-6) 92%	4(0-6) 80%	3(0-6) 74%	0(0-5) 11%	4(0-6) 83%	0(0-3) 10%
FTC (13)	5(0-6) 86%	0(0-3) 15%	5(0-6) 86%	0(0-1) 0%	0(0-5) 33%	0(0-5) 29%	0(0-6) 17%	0(0-0) 0%	4(0-5) 67%	0(0-6) 15%
FVPTC (11)	4(0-5) 89%	0(0-0) 0%	5(4-5) 100%	0(0-2) 22%	0(0-6) 33%	0(0-4) 33%	5(0-6) 89%	0(0-2) 11%	0(0-4) 33%	0(0-0) 0%

Classification of disorder (n)
MNG: Multinodular Goiter; FA: Follicular Adenoma; PTC: Papillary Thyroid Carcinoma; FTC: Follicular Thyroid Carcinoma; FVPTC: Follicular Variant PTC
Expressed as Median (Range) and proportion of samples with scores above the cut-off level for positivity.

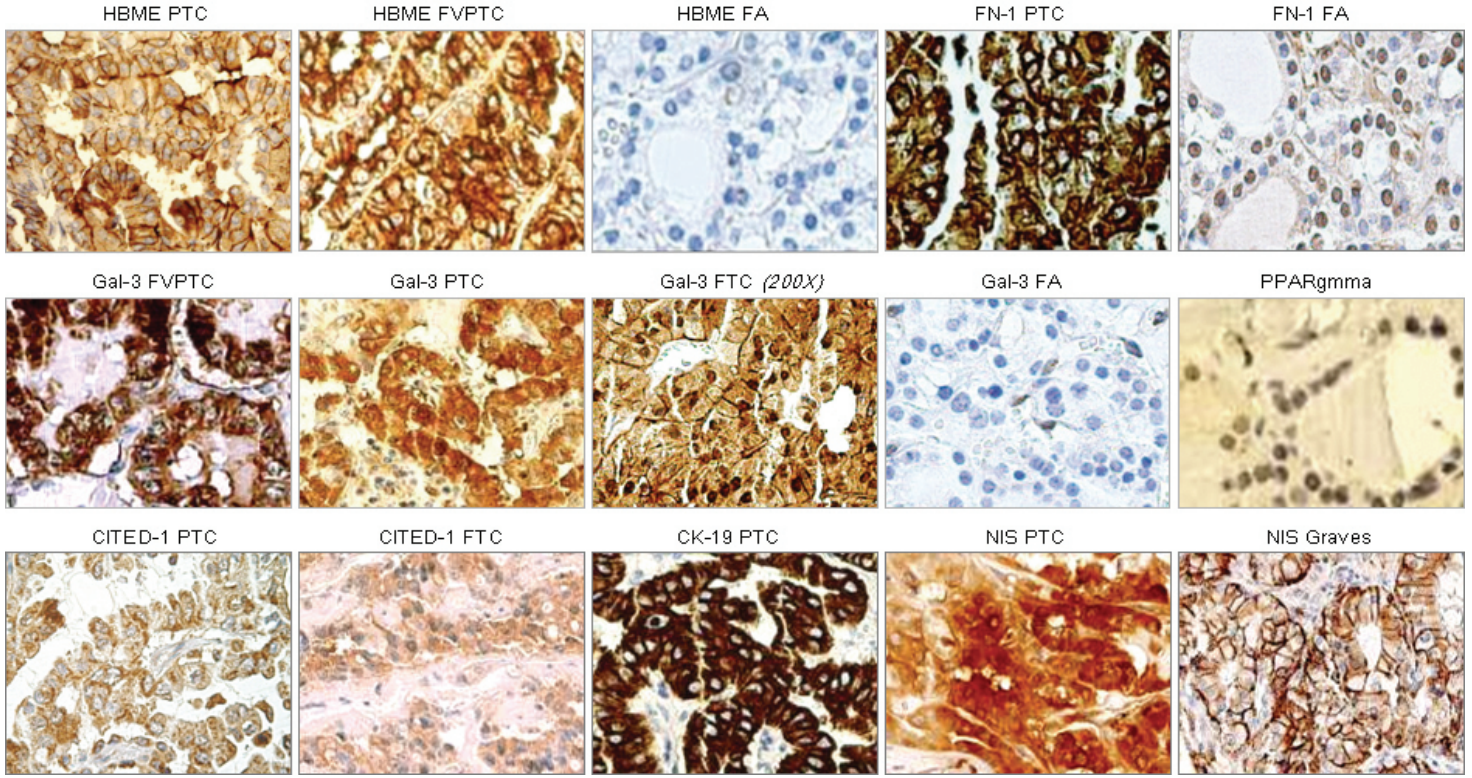

Figure 1. Immunostaining of thyroid tissues with HBME-1, Fibronectin-1 (FN-1), Galectin-3 (Gal-3), PPARγ, CITED-1, cytokeratin-19 (CK-19) and Sodium Iodide Symporter (NIS). Magnification was 200x. For immunohistochemical staining procedures, see Materials and Methods. HBME-1 gave membranous staining in Papillary Thyroid Carcinoma (PTC) and Follicular Variant PTC (FVPTC), and was absent in Follicular Adenoma (FA). FN-1 gave cytoplasmic staining in PTC. Gal-3 gave cytoplasmic or nuclear staining in thyroid carcinomas. PPARγ staining nuclear staining was observed in benign thyroid lesions. CITED-1 gave cytoplasmic staining in benign and malignant thyroid lesions. CK-19 was overexpressed in PTC. Cytoplasmic NIS was observed in FTC and PTC. Typical membranous staining was observed in Graves disease (see color image on page 150).

(Table 3). FVPTC differed from FA for cFN-1, PPAR-γ, HBME-1 and CK-19, whereas protein expression was different for FN-1, Gal-3, cNIS, HBME-1 and CK-19 in the comparison between FVPTC and PTC (Table 4).

Cytoplasmic NIS was mainly observed in PTC (median level 4, 83% of tumors) and FTC (median level 4, 67% of tumors) (Table 3). Remarkably, differences in CITED-1 were not prominent between benign thyroid tissues (median levels 3-4 in normal or benign lesions, with 51-89% of tissues positive) and malignant lesions (86-100% positive cases) in 2x2 comparisons.

Protein expression in follicular lesions

Because the clinical distinction between follicular lesions proves to be the most difficult, we focused our analyses on the diagnostic value of proteins found to be differentially expressed in follicular lesions (Tables 4 and 5). In 2x2 comparisons of the different follicular lesions, we first identified the optimal cut-off levels using ROC-analyses, aiming at the highest combination of sensitivity and specificity for each comparison. The cut-off values are given in Table 5. We subsequently calculated the percentages of correct diagnoses of both lesions in a 2x2 comparison as well as the accuracy, using these cut-off levels. The accuracy (total percentage of correct diagnoses) is the best indicator of the diagnostic or discriminating value of the antibody.

The highest accuracies were found in the discrimination between PTC and FVPTC, with the highest accuracy for cGal-3 (88%), cFN-1 (81%), CK-19 (78%) and cNIS (77%). In the comparison between FA and FTC, moderate accuracies were found for FN-1 (accuracy 71%) and cNIS (accuracy 65%). The distinction between FA and FVPTC had a high accuracy for HBME-1 (89%), PPAR-γ (74%) and FN-1 (74%). HBME-1 also gave a good discrimination between FVPTC and FTC (accuracy 84%).

Clustered expression pattern of Gal-3, FN-1 and cNIS distinguish benign thyroid tumors from thyroid carcinomas

To identify optimal combinations of antibodies, we performed an unsupervised hierarchical cluster analysis including all tissues and all antibodies.

The results of this analysis are given in Figure 2. We found that 3 antibodies, cGal-3, cNIS and cFN-1 had the highest discriminating power to cluster benign and malignant thyroid lesions: all malignant lesions had positive staining for at least 2 of the 3 antibodies cGal-3, cNIS and cFN-1 (Cluster 1), whereas lesions with absent staining for all 3 antibodies were all benign (Cluster 5). All malignancies were combined because the subgroups were too small to allow separate cluster analysis. Initially, there was only one case of FA in cluster 1. Strikingly, after rechecking whole section histological H&E slides, this adenoma was identified harbouring classic focal papillary carcinoma and re-categorized.

Table 4. Proteins differently expressed between thyroid lesions

Diagnosis	**Normal**	**MNG**	**Graves**	**FA**	**PTC**	**FTC**
MNG	nGal-3**					
Graves	cCITED-1* cGal-3* nGal-3** mNIS** PPARγ*	mNIS** PPARγ*				
FA	cFN-1* CITED-1** mNIS**	mNIS* cGal-3*	cGal-3* mNIS* PPARγ*			
PTC	cFN-1** mFN-1** cCITED-1** nCITED-1** CK-19** cGal-3** nGal-3** HBME-1** mNIS** cNIS** PPARγ**	cFN-1** mFN-1* cCITED1** CK-19** cGal-3** nGal-3** HBME-1** cNIS** mNIS** PPARγ**	cFN-1** mFN-1* cCITED-1* CK-19** cGal-3** nGal-3** HBME-1** mNIS** cNIS** PPARγ**	cFN-1** mFN-1* CK-19** cGal-3** nGal-3** HBME-1* cNIS** PPARγ**		
FTC	cFN-1** mFN1* cCITED-1** nGal-3* HBME-1* mNIS** cNIS** PPARγ*	cFN-1** CITED-1* cNIS** mNIS* PPARγ*	cFN-1* mNIS** cNIS* PPARγ**	cFN-1* cNIS*	CK-19** cGal-3** nGal-3** HBME-1**	
FVPTC	cFN1 ** cGAL-3* nGAL-3** HBME1 ** mNIS* CK-19* CITED1* PPARγ*	cFN1** cCITED1** HBME1** cNIS* PPARγ*	cFN-1** cCITED-1** HBME-1** mNIS** PPARγ**	cFN1* CK-19* nGAL3* HBME1** PPARγ*	cFN-1* mFN-1* CK-19** cGal-3* nGal-3* HBME-1* cNIS*	HBME-1*

MNG: Multinodular Goiter; FA: Follicular Adenoma; PTC: Papillary Thyroid Carcinoma;
FTC: Follicular Thyroid Carcinoma; FVPTC: Follicular Variant PTC
Gal-3: Galectin 3 (c=intracellular, n=nuclear); NIS: sodium iodide symporter (m=membranous);
FN-1: Fibronectin1; CK-19: cytokeratin 19

* $p<0.05$ ** $p<0.001$

Table 5. Diagnostic value of proteins differentially expressed in follicular thyroid lesions

I / II	Protein	Cut-off value	FA			PTC			FTC		
			Correct (I)	Correct (II)	Accuracy	Correct (I)	Correct (II)	Accuracy	Correct (I)	Correct (II)	Accuracy
FVPTC	*PPAR γ*	0	100	50	74						
	HBME-1	1	89	90	89	73	11	63	80	89	84
	CK-19	1.5	100	22	63	78	78	78			
	cGal-3	2				94	56	88			
	nGal-3	2	80	44	63	88	56	82			
	cFN-1	2	60	89	74	95	11	81			
	mFN-1	1				40	100	50			
	cNIS	2				80	67	77			
FTC	*cFN-1*	2	60	83	71						
	cNIS	2	60	78	65						

FA: Follicular Adenoma; PTC: Papillary Thyroid Carcinoma; FTC: Follicular Thyroid Carcinoma; FVPTC: Follicular Variant PTC
Gal-3: Galectin 3 (c=intracellular, n=nuclear); NIS: sodium iodide symporter (m=membranous); FN-1: Fibronectin1; CK-19: cytokeratin 19

Table 6. Combined intracellular expression of Fibronectin (FN), Galectin 3 (Gal-3) and NIS in thyroid lesions

		All Malignant Thyroid Tumors						
		One-Antibody			Antibodies Combined			
	Antibody	Sensitivity of Malignancy (%)	Specificity of Malignancy (%)	Accuracy (%)	**co-expression**	Sensitivity of Malignancy (%)	Specificity of Malignancy (%)	Accuracy (%)
All Benign Thyroid Tissues	*cNIS*	72	90	82				
	cFN-1	92	82	86	*Two antibodies positive:*	97	100	99
	cGAL-3	75	94	85				
Follicular Adenoma	*cNIS*	69	88	71				
	cFN-1	92	56	88	*Two antibodies positive:*	97	100	98
	cGAL-3	78	100	81				

Gal-3: Galectin 3 (c=intracellular, n=nuclear); NIS: sodium iodide symporter (m=membranous);
FN-1: Fibronectin1; CK-19: cytokeratin 19

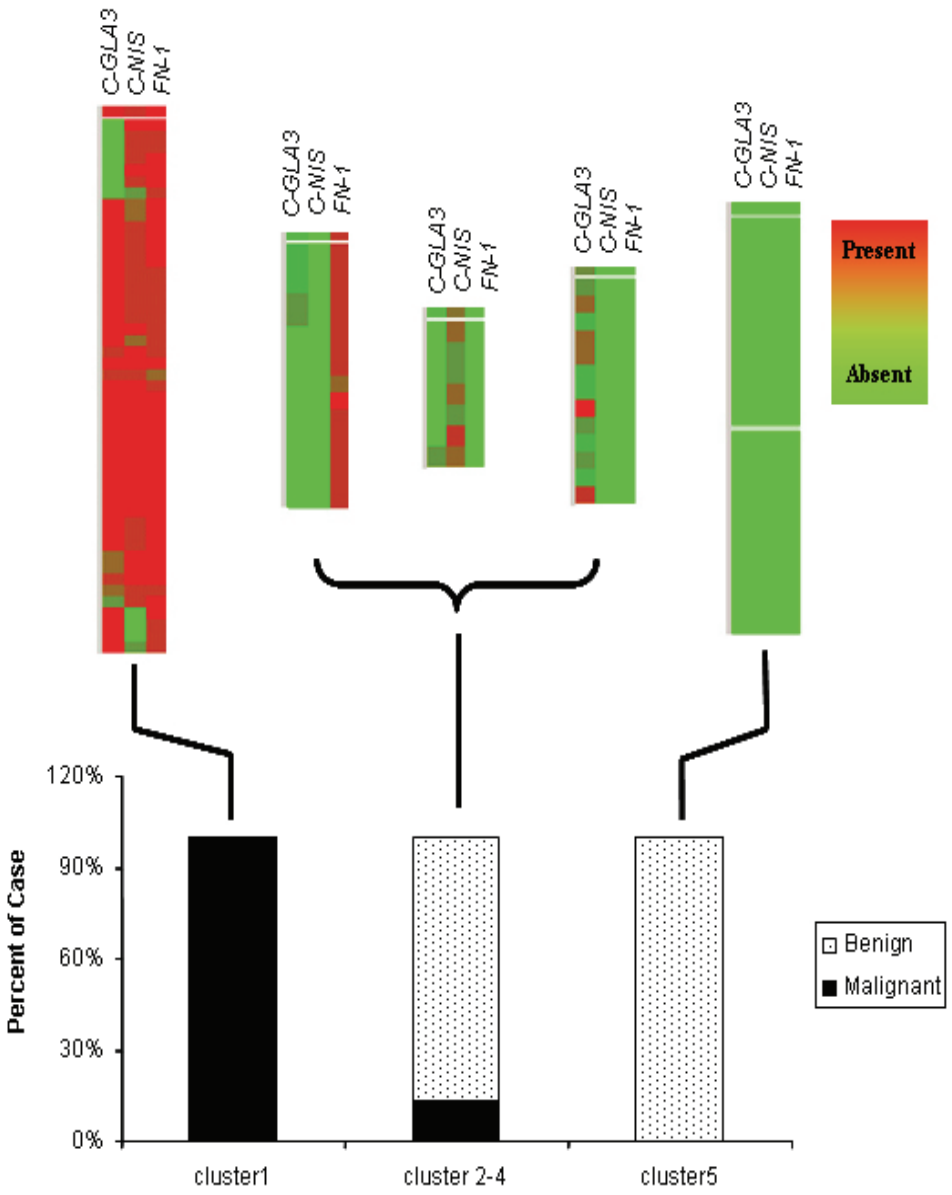

Figure 2. Hierarchical cluster analyses using 7 antibodies in all thyroid tissues. cNIS, FN-1 and Gal-3 were identified as the best predictors of benign or malignant thyroid lesions. Presence of 2 of these antibodies (Cluster 1) gave an almost 100% clustering of malignant thyroid lesions, whereas absence of these proteins (Cluster 5) was suggestive of benign thyroid lesions.

We therefore used the combined staining patterns of these antibodies to discriminate between benign and malignant thyroid lesions and FA and malignant thyroid lesions (Table 6). We found that positive staining for 2 of the 3 antibodies cFN-1, cGal-3 and cNIS had a high sensitivity (97-98%) and high specificity for thyroid carcinoma (100%).

Discussion

The present study was performed to evaluate the diagnostic value of Gal-3, HBME-1, CK-19, CITED-1, FN-1, PPAR-γ and NIS staining in a large panel of thyroid neoplasms, focussing on the differential diagnosis of follicular thyroid lesions.

Our study differed from earlier ones with regard to the identification of optimal semi-quantitative cut-off levels using ROC analysis and the use of hierarchical cluster analysis.

We initially analyzed differentially expressed antibodies comparing all thyroid tissues. In general, we found overexpression of FN-1, CITED-1, Gal-3, CK-19, HBME-1 and cNIS in thyroid carcinomas, whereas membranous NIS and PPARγ showed decreased expression in carcinomas in comparison with benign thyroid tissues.

The most challenging differential diagnosis is between FA and thyroid carcinoma. We found all proteins to be differentially expressed between FA and PTC. The differences between FA on the one hand and FTC and FVPTC on the other hand were less prominent, but we found a differential expression of PPARγ, HBME-1, Gal-3, cNIS and FN-1. We could not confirm the differential expression of CITED-1 and CK-19 between FA, FVPTC and FTC as reported by Prasad et al (24).

CK-19 is the most commonly used cytokeratin in investigating thyroid lesions. We and others found that CK-19 is relatively specific for PTC (16;18;19). However, in our analyses CK-19 has limited use in the differential diagnosis of follicular thyroid lesions. This has also been reported by Sahoo et al (17). In the study of Prasad et al (24), CK-19 had a sensitivity of 64% for thyroid carcinoma.

Several recent studies have reported that HBME-1 expression is a useful diagnostic marker for PTC (23;24). We found HBME-1 expression predominantly in PTC and FVPTC and in a limited number of FA with relatively high accuracy. Therefore, HBME-1 may indeed be useful in the differential diagnosis of FVPTC and FA (accuracy 88%).

We found CITED-1 expression both in benign and malignant thyroid lesions. Although the highest proportion of positive samples was found in PTC (as

previously reported (23;24)) and in FVPTC, the considerable proportion of positive samples in benign lesions makes CITED-1 in our opinion a less attractive marker for differential diagnosis.

Gal-3 was predominantly expressed in PTC (92%) and to a lesser extent in FTC and FVPTC. Other investigators have used Gal-3 in differentiating FTC from FA in fine-needle aspirates (7), however Gal-3 was also reported in benign thyroid lesions (10). We found a reasonable accuracy (88%) in the differential diagnosis between FA and FVPTC for Gal-3. We also found Gal-3 to be a useful marker in a panel of antibodies.

FN-1 was first reported to be overexpressed in PTC (22;23). In a subsequent study, FN-1 appeared to be a valuable marker for the differentiation of FA and thyroid carcinomas (24). The percentage of FA (40%) positive for FN-1 in our study was higher than reported by Prasad et al (24). We found accuracies of 74% for the differentiation between FA and FVPTC and 71% for the differentiation between FA and FTC. Cluster analysis also identified FN-1 as a useful marker.

Although some studies report decreased NIS protein expression in thyroid carcinoma (30), Dohan et al reported cytoplasmic overexpression of NIS in a large series of human thyroid cancers (25). We confirmed cytoplasmic NIS overexpression in PTC (83% of tissues) and FTC (67% of tissues). As we used the same antibody as Dohan et al. it may well be that the differences with other studies are related to differences in antibody specificity. Nevertheless, the differential expression of cNIS between subtypes of thyroid neoplasms makes it a candidate for differentiating between these lesions. The accuracies of 68% (FA vs. FTC) and 77% (PTC vs. FVPTC) however are moderate. Cytoplasmic NIS was also identified by cluster analysis as a potential useful marker in the discrimination between FA and malignant carcinomas.

PPARγ has found to be downregulated in experimental models of thyroid carcinoma (26-28). The importance of the downregulation of PPARγ is also illustrated in the PPARγ/PAX8 rearrangement (31) which was initially observed in a series of FTC. Although the PPARγ/PAX8 rearrangement was therefore considered a specific marker for FTC, later studies also reported the rearrangement in benign thyroid lesions (32;33). We found decreased PPARγ nuclear staining in malignant tumors, whereas in non-malignant lesions, the percentage of positive cells varied from 50-100%. Although our results confirm the decreased expression of PPARγ in thyroid carcinoma, the diagnostic accuracies for the differentiation between follicular lesions were limited.

As no marker in itself has a superior diagnostic value, a combination of markers may be more accurate than any single marker. We performed a cluster analysis including all tissues and antibodies. To our knowledge, this has not been done before for immunohistochemistry in thyroid lesions. In this study, hierarchical clustering analysis on all valid samples confirmed that thyroid carcinomas, FA and benign lesions could

be categorized with high sensitivity, specificity and accuracy. Our study shows that a diagnostic immunohistochemical panel comprising Gal-3 and FN-1 was 97% sensitive for all thyroid carcinomas, whereas specificity was 100%. The diagnostic values of CK-19, CITED-1 and HBME-1 in our series were not sufficient to be included in the panel, which is in line with the results of de Matos et al (19). However, HBME-1 was found to be a useful marker for the differentiation between FA and FVPTC. Because the number of FVPTC was small, hierarchical clustering did not allow a separate analysis of this group of tumors. Prasad et al. also found a limited accuracy for HBME-1, CK-19 and CITED-1 (24).

In conclusion, Gal-3, FN-1 and cNIS is a useful diagnostic panel in the differential diagnosis of thyroid lesions. The absence of Gal-3, FN-1 and cNIS is highly suggestive for a benign lesion. HBME-1 may be useful in the specific differentiation of FVPTC from FA.

References

1. Hirokawa M, Carney JA, Goellner JR et al. Observer variation of encapsulated follicular lesions of the thyroid gland. Am J Surg Pathol 2002; 26(11):1508-1514.
2. Franc B, de la SP, Lange F et al. Interobserver and intraobserver reproducibility in the histopathology of follicular thyroid carcinoma. Hum Pathol 2003; 34(11):1092-1100.
3. Haugen BR, Woodmansee WW, McDermott MT. Towards improving the utility of fine-needle aspiration biopsy for the diagnosis of thyroid tumours. Clin Endocrinol (Oxf) 2002; 56(3):281-290.
4. Xu XC, el-Naggar AK, Lotan R. Differential expression of galectin-1 and galectin-3 in thyroid tumors. Potential diagnostic implications. Am J Pathol 1995; 147(3):815-822.
5. Orlandi F, Saggiorato E, Pivano G et al. Galectin-3 is a presurgical marker of human thyroid carcinoma. Cancer Res 1998; 58(14):3015-3020.
6. Gasbarri A, Martegani MP, Del Prete F, Lucante T, Natali PG, Bartolazzi A. Galectin-3 and CD44v6 isoforms in the preoperative evaluation of thyroid nodules. J Clin Oncol 1999; 17(11):3494-3502.
7. Bartolazzi A, Gasbarri A, Papotti M et al. Application of an immunodiagnostic method for improving preoperative diagnosis of nodular thyroid lesions. Lancet 2001; 357(9269):1644-1650.
8. Saggiorato E, Aversa S, Deandreis D et al. Galectin-3: presurgical marker of thyroid follicular epithelial cell-derived carcinomas. J Endocrinol Invest 2004; 27(4):311-317.
9. Martins L, Matsuo SE, Ebina KN, Kulcsar MA, Friguglietti CU, Kimura ET. Galectin-3 messenger ribonucleic acid and protein are expressed in benign thyroid tumors. J Clin Endocrinol Metab 2002; 87(10):4806-4810.
10. Kovacs RB, Foldes J, Winkler G, Bodo M, Sapi Z. The investigation of galectin-3 in diseases of the thyroid gland. Eur J Endocrinol 2003; 149(5):449-453.
11. Miettinen M, Karkkainen P. Differential reactivity of HBME-1 and CD15 antibodies in benign and malignant thyroid tumours. Preferential reactivity with malignant tumours. Virchows Arch 1996; 429(4-5):213-219.
12. Mase T, Funahashi H, Koshikawa T et al. HBME-1 immunostaining in thyroid tumors especially in follicular neoplasm. Endocr J 2003; 50(2):173-177.
13. Mai KT, Bokhary R, Yazdi HM, Thomas J, Commons AS. Reduced HBME-1 immunoreactivity of papillary thyroid carcinoma and papillary thyroid carcinoma-related neoplastic lesions with Hurthle cell and/or apocrine-like changes. Histopathology 2002; 40(2):133-142.
14. van Hoeven KH, Kovatich AJ, Miettinen M. Immunocytochemical evaluation of HBME-1,

CA 19-9, and CD-15 (Leu-M1) in fine-needle aspirates of thyroid nodules. Diagn Cytopathol 1998; 18(2):93-97.
15. Sack MJ, Astengo-Osuna C, Lin BT, Battifora H, LiVolsi VA. HBME-1 immunostaining in thyroid fine-needle aspirations: a useful marker in the diagnosis of carcinoma. Mod Pathol 1997; 10(7):668-674.
16. Beesley MF, McLaren KM. Cytokeratin 19 and galectin-3 immunohistochemistry in the differential diagnosis of solitary thyroid nodules. Histopathology 2002; 41(3):236-243.
17. Sahoo S, Hoda SA, Rosai J, DeLellis RA. Cytokeratin 19 immunoreactivity in the diagnosis of papillary thyroid carcinoma: a note of caution. Am J Clin Pathol 2001; 116(5):696-702.
18. Casey MB, Lohse CM, Lloyd RV. Distinction between papillary thyroid hyperplasia and papillary thyroid carcinoma by immunohistochemical staining for cytokeratin 19, galectin-3, and HBME-1. Endocr Pathol 2003; 14(1):55-60.
19. de Matos PS, Ferreira AP, de Oliveira FF, Assumpcao LV, Metze K, Ward LS. Usefulness of HBME-1, cytokeratin 19 and galectin-3 immunostaining in the diagnosis of thyroid malignancy. Histopathology 2005; 47(4):391-401.
20. Miettinen M, Kovatich AJ, Karkkainen P. Keratin subsets in papillary and follicular thyroid lesions. A paraffin section analysis with diagnostic implications. Virchows Arch 1997; 431(6):407-413.
21. Hirokawa M, Inagaki A, Kobayashi H, Kanahara T, Manabe T, Sonoo H. Expression of cytokeratin 19 in cytologic specimens of thyroid. Diagn Cytopathol 2000; 22(3):197-198.
22. Wasenius VM, Hemmer S, Kettunen E, Knuutila S, Franssila K, Joensuu H. Hepatocyte growth factor receptor, matrix metalloproteinase-11, tissue inhibitor of metalloproteinase-1, and fibronectin are up-regulated in papillary thyroid carcinoma: a cDNA and tissue microarray study. Clin Cancer Res 2003; 9(1):68-75.
23. Huang Y, Prasad M, Lemon WJ et al. Gene expression in papillary thyroid carcinoma reveals highly consistent profiles. Proc Natl Acad Sci U S A 2001; 98(26):15044-15049.
24. Prasad ML, Pellegata NS, Huang Y, Nagaraja HN, de la CA, Kloos RT. Galectin-3, fibronectin-1, CITED-1, HBME1 and cytokeratin-19 immunohistochemistry is useful for the differential diagnosis of thyroid tumors. Mod Pathol 2005; 18(1):48-57.
25. Dohan O, Baloch Z, Banrevi Z, LiVolsi V, Carrasco N. Rapid communication: predominant intracellular overexpression of the Na(+)/I(-) symporter (NIS) in a large sampling of thyroid cancer cases. J Clin Endocrinol Metab 2001; 86(6):2697-2700.
26. Kato Y, Ying H, Willingham MC, Cheng SY. A tumor suppressor role for thyroid hormone beta receptor in a mouse model of thyroid carcinogenesis. Endocrinology 2004; 145(10):4430-4438.
27. Ying H, Suzuki H, Furumoto H et al. Alterations in genomic profiles during tumor progression in a mouse model of follicular thyroid carcinoma. Carcinogenesis 2003; 24(9):1467-1479.
28. Ying H, Suzuki H, Zhao L, Willingham MC, Meltzer P, Cheng SY. Mutant thyroid hormone receptor beta represses the expression and transcriptional activity of peroxisome proliferator-activated receptor gamma during thyroid carcinogenesis. Cancer Res 2003; 63(17):5274-5280.
29. Kononen J, Bubendorf L, Kallioniemi A et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998; 4(7):844-847.
30. Caillou B, Troalen F, Baudin E et al. Na+/I- symporter distribution in human thyroid tissues: an immunohistochemical study. J Clin Endocrinol Metab 1998; 83(11):4102-4106.
31. Kroll TG, Sarraf P, Pecciarini L et al. PAX8-PPARgamma1 fusion oncogene in human thyroid carcinoma [corrected] [published erratum appears in Science 2000 Sep 1;289(5484):1474]. science 2000; 289(5483):1357-1360.
32. Cheung L, Messina M, Gill A et al. Detection of the PAX8-PPAR gamma fusion oncogene in both follicular thyroid carcinomas and adenomas. J Clin Endocr Metab 2003; 88(1):354-357.
33. Castro P, Rebocho AP, Soares RJ et al. PAX8-PPARgamma rearrangement is frequently detected in the follicular variant of papillary thyroid carcinoma. J Clin Endocrinol Metab 2006; 91(1):213-220.