CLINICAL STUDY

Follicular thyroid neoplasms can be classified as low- and high-risk according to HBME-1 and Galectin-3 expression on liquid-based fine-needle cytology

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Abstract

Design: Fine-needle aspiration biopsy (FNAB) is the most reliable diagnostic tool in the diagnosis of thyroid nodules. A cytologic diagnosis of follicular neoplasm with atypical cells of undetermined significance (FN/AUS) implies that the selection of patients between surgery and follow-up is difficult. In this setting immunocytochemical stainings might be helpful. The efficacy of a panel made up of HBME-1 and Galectin-3 antibodies is evaluated in cases processed by liquid-based cytology (LBC).

Methods: Out of 7091 thyroid FNAB processed by LBC method, 120 cases undergoing surgery successively were selected. These cases were classified as benign lesion (BL, eight cases), FN, including the ACUS category of the Bethesda classification (FN/AUS, 50 cases), suspicious for malignancy (SM, 59 cases), and malignant neoplasm (MN, three cases). Immunostains for HBME-1 and Galectin-3 were carried out on the LBC slides.

Results: All MN and BL were histologically confirmed. FN/AUS and SM showed a malignancy risk of 24 and 72.9% respectively. The complete immunocytochemical panel was positive in 83.3% of the cases resulting in malignancy and negative in 87.5% of cases resulting in benign histology. Among the FN/AUS, the complete positive immunocytochemical panel was detected in 76.9% of cases resulting as malignant and the complete negative immunocytochemical panel was observed in 96.8% of cases resulting as benign at histology.

Conclusions: The expression of HBME-1 and Galectin-3 in cases classified as FN/AUS on LBC-processed FNABs can effectively distinguish lesions, which need immediate surgery (high risk or FNH or Thy 3h) from those which can be followed-up (low risk or FNL or Thy 3l).

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Introduction

Fine-needle aspiration biopsy (FNAB) represents a valid diagnostic choice combined with clinical and ultrasound evaluation, in the characterization of thyroid nodules. It may achieve a precise interpretation of the nature of thyroid lesions in more than 70% of cases and it leads to a correct clinical strategy (mostly related to the surgical indication) in more than 90% of cases (1, 2). It is simple, safe and extremely cost-effective especially when performed under sonographic guidance (3, 4). Some problems may arise in the cytologic diagnosis of lesions exhibiting a predominant micro-follicular pattern. The real nature of these lesions can be assessed only by the detection of capsular and vascular invasion whereas the needle biopsy may sample only the center of the nodule. Several studies (5–9) have investigated different morphologic features (especially the nuclear 'atypia' of the follicular cells) that may

enable a distinction between the most important neoplasms, which are follicular adenoma (FA), follicular carcinoma (FC), and the follicular variant of papillary carcinoma (FVPC). However, this possibility is still speculative and the majority of authors feel that the diagnosis of indeterminate cytology is still unavoidable.

In thyroid pathology, immunohistochemistry has been used for the differential diagnosis of follicular and C cell-derived neoplasms. Recently, the expression of markers of malignancy has also been used as an additional tool for diagnosing malignant tumors, regardless of the presence of capsular or vascular invasion (10).

Among these markers of malignancy HBME-1 and Galectin-3 have reached high values of specificity and sensitivity in discriminating benign lesions (BL) from malignancies.

Galectin-3 is a component of the β -galactoside-binding lectins whose function is still unclear. It appears

to be involved in the cell–cell and cell–matrix modulation and, according to some authors (11), in cell growth and differentiation. Therefore, this antibody could play a role in the malignant transformation of thyroid cells and it is expressed in a high proportion of carcinomas, especially of the papillary histotype (11–13).

HBME-1 is an MAB directed against a still unknown membrane antigen of the microvillar surface of the mesothelioma cells. It yields positive in neoplastic follicular cells, mostly from papillary carcinoma, and negative in BL (14, 15).

However, none of them has shown a diagnostic accuracy sufficient for using as single antibody in the diagnosis of malignant thyroid neoplasms (10, 16).

Immunocytochemistry may find new application fields in fine-needle aspiration cytology, especially when a diagnosis of follicular neoplasm (FN/atypical cells of undetermined significance (AUS) – Thy 3) is made (4, 5). The latter category accounts for 10–25% of all cases and represents a therapeutic problem because of the low, though not irrelevant, risk of malignancy. Therefore, the cases diagnosed as such are often addressed to surgery that results in a large amount of inappropriate thyroidectomies. In fact, about 80% of all thyroid operations for FN/AUS yield a histologic diagnosis of benign nodule (5–8).

Immunocytochemical techniques can be applied on liquid-based cytology (LBC) (17–19). This technique has been introduced to obtain thin-layer smears in cervical cytology. The excellent results in this field prompted a wider application to virtually all cytological branches, including thyroid FNAB (20–27). The current investigation has been carried out to evaluate the effectiveness of a panel made up by HBME-1 and Galectin-3 on LBC in identifying two subsets of FN/AUS with low and high malignant risk that might be managed differently to achieve a reduction of inappropriate surgery.

Materials and methods

During the period August 1, 2006–December 31, 2008, 7091 thyroid FNAB were carried out in the Divisions of Endocrine Surgery, Endocrinology and Anatomic Pathology and Histology of the Catholic University, ‘Agostino Gemelli’ School of Medicine and Hospital of Rome. The diagnoses of FN/AUS were 953 (13.4%) and those of suspicious for malignancy (SM) 227 (3.2%). The series under investigation included 120 cases, 50 diagnosed as FN (FN/AUS), corresponding to Thy 3 of the BTA classification (28), which also includes oxyphilic neoplasms of the NCI Bethesda classification (9) and 59 lesions classified as SM (Thy 4 of BTA). The remaining 11 cases included eight cases diagnosed as non-neoplastic lesion (Thy 2 of the BTA classification) and three papillary carcinomas, which were used as

controls. The following inclusion criteria were applied: i) all cytologic diagnoses reviewed by an experienced pathologist (G F); ii) presence of abundant material so that immunocytochemical stainings could be carried out; and iii) availability of the definitive histology specimen. The morphologic criteria for the inclusion of each case processed by LBC in the corresponding cytologic category were presented in previous papers of our group (26, 27). The series included 35 male and 85 female patients with a median age of 32.5 years (range 13–78 years). All aspirations were performed with 25–27G needles under sonographic guidance with at least two until four consecutive passes for each lesion (4).

After the aspiration, the needle was rinsed in Cytolite solution and then the sample was processed by the ThinPrep 2000 method (Hologic Cytoc Co., Marlborough, MA, USA), fixed with 95% ethyl alcohol and stained with Papanicolaou.

Those cases showing a difficult cytologic picture were discussed by three cytopathologists (E D R, G F, and G F Z) until a final agreement was achieved.

Immunocytochemical staining was carried out with the avidin–biotin–peroxidase complex on LBC slides using the following antibodies: HBME-1 (Dako, Glostrup, Denmark, 1:100 dilution) and Galectin-3 (Ventana, Tucson, AZ, USA, 1:100 dilution). The slides were washed three times in PBS and then preincubated in normal serum with PBS (1:50) for 20 min before incubating at 4 °C with the primary antibody. Then the slides were washed three times with PBS and incubated with the biotinylated secondary antibody conjugated with the avidin–biotin–peroxidase complex. The reaction was developed using 3,3'-diamino-benzidine. All slides were counterstained with hematoxylin for 5 s, rinsed in water three times then mounted for the microscopic examination. The positivity was assessed, for each cytological case, when at least 50% of follicular cells showed a convincing cytoplasmic positivity. The cytodiagnostic groups of SM (Thy 4) and malignant neoplasm (MN) (Thy 5) were referred to surgery according with the BTA and ATA guidelines (29) whereas the BL (Thy 2) cases were operated on when esthetic or functional problems occurred.

For the FN (Thy 3) category, which shows a relevant risk of carcinomatous occurrence, the same guidelines

Table 1 Cytohistological comparison (120 cases).

Cytological diagnosis	Histological diagnosis			
	Benign	FA	Malignant	Overall
BL: Thy 2	8 (100%)	0	0	8
FN/AUS: Thy 3	20 (40%)	19 (38%)	11 (22%)	50
SM: Thy 4	6 (10.2%)	10 (16.9%)	43 (72.9%)	59
MN: Thy 5	0	0	3 (100%)	3

BL, benign lesion; FA, follicular adenomas; FN, follicular neoplasm; SM, suspicious of malignancy; MN, malignant neoplasms.

Table 2 Cytohistologic comparison for the immunocytochemical results (all 120 cases).

Immunocytochemistry	Histological diagnosis		
	Benign ^a	Malignant	Overall
Concordant negative	42 (87.5%)	6 (12.5%)	48
HBME-1 +, Galectin-3 –	10 (45.4%)	12 (54.6%)	22
HBME-1 –, Galectin-3 +	2 (100%)	0	2
Concordant positive	8 (16.7%)	40 (83.3%)	48

^aIncluding follicular adenomas; $P < 0.001$.

suggest that surgery is routinely advised after a multidisciplinary discussion.

The surgical specimens were fixed in 10% buffered formaldehyde, embedded in paraffin and the 5 µm thick sections were stained with hematoxylin–eosin.

Immunostains for the above-mentioned antibodies, particularly in those cases that did not show a complete positivity of the panel in the preoperative cytology, were carried out also on the histological sections. It was performed by the ABC method with the same antibodies used for the immunostains on LBC slides.

Statistical analysis

Sensitivity, specificity, and overall accuracy of FNAB were calculated as follows: the BL category (Thy 2) were defined as benign, the categories FN/AUS and SM (Thy 3 and 4), were included in the malignant lesions. Inadequate specimens and microscopic carcinomas incidentally found in the final pathologic examination were excluded and the statistical analysis was performed considering only the histological diagnosis of the sampled lesion.

Benign and malignant lesions were compared for cytological and immunocytochemical pattern. Statistical analysis was performed by a commercially available statistic software package (SPSS 10.0 for Windows – SPSS, Inc., Chicago, IL, USA). The χ^2 test was used for categorical variables up to 6 degrees of freedom. A P value < 0.05 was considered as significant.

All patients had been appropriately informed regarding the use of LBC method for processing their aspiration samples and a written informed consent was signed by each of them.

Results

The 120 cytological cases were diagnosed as follows: 8 BL (Thy 2), 50 FN/AUS, which did not include any oxyphilic neoplasm (Thy 3); 59 SM (Thy 4), 3 papillary carcinomas (PC) (Thy 5). All cases underwent surgery and the histologic examination confirmed all three PC (one showing an insular pattern) and all BL (three revealed FAs at histology). Of the 50 FN/AUS, 11 (22%) resulted as carcinoma at histology (ten papillary

carcinomas and one FC, Table 1). Forty-three out of 59 cases of SM (72.9%) were malignant; all but one (which was an insular carcinoma arising from a papillary carcinoma) was diagnosed as PC.

The application of the immunocytochemical stainings to the ThinPrep 2000 processed material showed that 96 cases (80%) exhibited a concordant panel (Table 2). In the group of PC, the complete panel was positive in all cases and, accordingly, within the benign group all cases were negative for HBME-1 and Galectin-3. In the FN/AUS category (Thy 3, Table 3) the concordant panel resulted positive in 13 cases (resulting three benign and ten, 76.9%, MN) and negative in 31 cases (30 resulted benign neoplasms, including 14 adenomas, and one, 3.2%, was a PC). The results of IHC for the subgroup of SM showed a concordant positivity for HBME-1 and Galectin-3 in 32 cases (yielding five BL and 27, 84.4%, malignancies) and a concordant negativity in ten (five benign and five malignant, Table 4 and Fig. 1). In the latter category, the low efficacy of the immunocytochemistry compared with morphology explains the relatively low values of specificity and sensitivity for the complete series (Table 5).

Regarding the cases with discordant panel, the figures in Table 2 show that the best correlation between cytoplasmic positivity and occurrence of malignancy can be observed with HBME-1 instead of Galectin-3. In fact, out of 22 cases of HBME-1 positivity and Galectin-3 negativity 13 resulted in malignancy (59.1%) whereas in the opposite instance both cases were benign.

The correlation of the presence of the complete panel with the histological results for the category of FN/AUS (Table 3) shows that the simultaneous negativity of both antibodies is almost always associated with a benign diagnosis whereas complete positivity is associated with an MN in 10 out of 13 cases (76.9%).

The descriptive statistical analysis, depicted in Table 5, shows the differences in sensitivity, specificity, diagnostic accuracy, positive, and negative predictive values of the cytologic diagnosis alone, of the immunocytochemistry, of the category of FN/AUS (Thy 3 according to BTA guidelines) and of HBME-1 and Galectin-3 alone.

In total, 19 cases (15.8%) were selected for the repetition of the immunostains for HBME-1 and

Table 3 Cytohistological comparison for immunocytochemical results (only FN: Thy 3; 50 cases).

Immunocytochemistry	Histological diagnosis	
	Benign ^a	Malignant
HBME-1 +, Galectin-3 +	3 (23.1%)	10 (76.9%)
HBME-1 +, Galectin-3 –	5 (100%)	0
HBME-1 –, Galectin-3 +	1 (100%)	0
HBME-1 –, Galectin-3 –	30 (96.8%)	1 (3.2%)
Overall	39 (78%)	11 (22%)

^aIncluding follicular adenomas; $P < 0.001$.

Table 4 Cytohistological comparison for immunocytochemical results (only suspicious of malignancy diagnoses, Thy 4, 59 cases).

Immunocytochemistry	Histological diagnosis	
	Benign ^a	Malignant
HBME-1 +, Galectin-3 +	5 (15.6%)	27 (84.4%)
HBME-1 +, Galectin-3 –	5 (31.3%)	11 (68.7%)
HBME-1 –, Galectin-3 +	1 (100%)	0
HBME-1 –, Galectin-3 –	5 (50%)	5 (50%)
Overall	16 (27.1%)	43 (72.9%)

^aIncluding follicular adenomas; $P < 0.001$.

Galectin-3 on the histological specimens and all were confirmed. The selection criteria included 12 cases exhibiting a discordant expression of the antibodies of the panel, five FN/AUS and two cytologically BL.

Discussion

Thyroid nodules are a common finding in everyday clinical practice, whereas only a small percentage of them is malignant. The efficacy of FNAB in making a correct diagnosis of both malignant and BL is definitely stated in literature (1, 9, 30). On the other hand, in those cases defined as FN, the cytologic examination is not always able to distinguish malignant or suspicious nodules requiring surgical treatment from those which can be followed-up safely.

The category of FN (Thy 3 in the BTA classification) represents an indeterminate category responsible for a high rate of unnecessary thyroidectomies (5–8). The problem may be due to the discrepancies between the cytologic and the corresponding histologic pictures because this category embraces BL showing scattered nuclear pleomorphism (e.g. hyperplastic nodules in Hashimoto's thyroiditis and toxic adenomas) and malignant lesions with moderate pleomorphism of the follicular cells (e.g. well-differentiated FC).

The cytologic criteria for diagnosing FN are not universally accepted and the category itself is debated. Though the cytologic picture of a follicular-patterned proliferation is well established, the diagnostic features which are helpful to assess the malignant nature of the nodule (e.g. nuclear atypia, nuclear clearing) are much less reproducible (31–33).

To address the problem of the management of these indeterminate diagnoses, immunohistochemical procedures using antibodies against antigens associated with malignancy were introduced. Among them Galectin-3 and HBME-1 have shown the highest reliability, particularly in differentiated carcinoma (10–13, 34).

The quality of the immunocytochemical reaction carried out on LBC slides is better than conventional smears in terms of morphologic details and purity of

background, as some studies have demonstrated, including some from our group (17–19, 34).

The combination of the antibody panel made up of HBME-1 and Galectin-3 results in a significant increase of the diagnostic accuracy of the technique (78.3% compared with 54.2% of the morphology alone) but this improvement turns out even more striking in FN as both the overall and the concordant panel results are considered (82 and 91.1% respectively), as is also depicted in Table 5. It is important to emphasize the relevance of an appropriate cellularity to assess the positivity or negativity of the immunocytochemical reaction. In case of scant cellularity, which is defined as not 6 groups of follicular cells, the immunocytochemical diagnosis should not be made.

An immunocytochemical-based classification of indeterminate thyroid lesions has never been attempted because of the difficulty in performing immunocytochemistry on conventional smears. Nevertheless, the uncertainty regarding the most appropriate treatment of FNs (Thy 3 in the BTA classification, ACUS and FNs in the Bethesda classification) that often sent to surgery but result in being found as benign on the definitive histology, has prompted the development of different techniques to define the cancer risk preoperatively (e.g. immunocytochemical and molecular studies (35–40)). The results of these investigations are quite promising but there is still a relevant amount of lesions that undergo unnecessary surgery or, on the other hand, may harbor an undiagnosed cancer. The current investigation achieves results similar to the molecular studies with a significantly cost-effective method still based on the morphologic evaluation of the follicular cells. The results of Nikiforov *et al.* (39, 40) compared with the current data show a better positive predictivity for the molecular analysis compared with

Figure 1 Malignancy rates for different antibodies combinations in the cytological categories. Conc. Neg., concordant negative panel; Conc. Pos., concordant positive panel; HB + GAL –, HBME-1 positive/Galectin-3 negative; HB – GAL +, HBME-1 negative/Galectin-3 positive; Foll. Neopl., follicular neoplasm.

Table 5 Descriptive statistics for the morphologic and immunocytochemical yieldings in all cytologic categories and only in follicular neoplasm lesions. Data are presented as %.

	Cytology ^a	ICC only ^b	FN panels			
			All	Conc.	HBME-1	Galectin-3
Sensitivity	100	89.7	91.7	90.9	72.3	80
Specificity	12.7	67.7	78.9	91.2	88	72.3
Diagnostic accuracy	54.2	78.3	82	91.1	80	76.6
PPV	51	72.2	57.9	76.9	89.6	69
NPV	100	87.5	96.8	96.9	71	84

^aFollicular neoplasms; Thy 3, and suspicious of malignancy; Thy 4, are counted as malignant; ICC, immunocytochemistry.

^bAll combinations of antibodies where at least one of them was positive were accounted as malignant; All panels, all possible combinations of HBME-1 and Galectin-3 expressions (see Table 2); Conc. panels, HBME-1 and Galectin-3 are both positive or negative; FN, follicular neoplasm (Thy 3); PPV, positive predictive value; NPV, negative predictive value.

immunocytochemistry in the indeterminate (FN/AUS) lesions (100 vs 76.9%) but a lower negative predictive value (16.2 vs 3.2%). As a rule in our institution, lesions classified as FN (Thy 3) undergo immunocytochemical analysis for HBME-1 and Galectin-3 expression: those showing a simultaneous positivity for both antibodies are referred to surgery whereas those exhibiting a simultaneous negativity should be carefully followed-up, with the surgical option being held for potential future use.

Regarding the SM lesions, our results (Table 4) may look somewhat surprising but they can be easily explained. This category is usually cytologically dyshomogeneous being composed of a mixture of large atypical cells and smaller thyrocytes as is often observed in cases of FVPC. Thus, the chosen cutoff of 50% expression of HBME-1 and Galectin-3 may result in some false-negative cases that nonetheless do not represent a true diagnostic problem as the inclusion of a case in the SM category is mainly based on the cytologic features.

As a consequence of these results, HBME-1 has a far better diagnostic accuracy than Galectin-3 and, in case of availability of only one antibody, the use of the former as diagnostic aid in the classification of a thyroid nodule is strongly suggested.

Based on the results (Fig. 1) of the present investigation, the suggested management of a thyroid lesion undergoing FNAB includes: i) accurate examination of clinical history and serum analysis to rule out the possibility of a toxic adenoma or a Hashimoto's thyroiditis; ii) evaluation of the nuclear atypia of the follicular cells to define the inclusion of the case in the benign, FN/AUS, SM, and malignant categories; and iii) if the lesion is classified as FN/AUS (Thy 3) the assessment of the expression of HBME-1 and Galectin-3 in the follicular cells would identify two additional subtypes: low risk (Thy3l or FNL – resulting HBME-1 and Galectin-3 negative) and high risk (Thy 3h or FNH – HBME-1 and Galectin-3 positive). These subgroups are characterized by a significant difference in the risk that a MN is being detected at histology (76.9% when the

concordant panel is positive and 3.2% when the same panel is negative).

Therefore, the application of this antibody panel to any cytological classification that includes the category of FN/AUS may represent the first step in achieving a correct preoperative diagnosis of thyroid nodules. It improves the accuracy of the morphologic evaluation of these indeterminate lesions enabling a better selection of patients as candidates for surgery from those who should be treated pharmacologically. In this perspective, LBC is an improvement of conventional cytology as it represents an invaluable storage for the cellular material that can be successfully used for additional investigations.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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