Galectin-3 and HBME-1 Expression on Agarose Cell Blocks from Fine-needle aspirates of Follicular Cell-derived Thyroid Tumors

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Abstract

Aim: To test the expression of galectin-3 (gal-3) and Hector Battifora mesothelial antigen-1 (HBME-1) on agarose cell blocks (CBs) of fine-needle aspirates aiming to determine their diagnostic value in thyroid follicle cell-derived tumors. Materials and Methods: Forty patients with thyroid nodule were enrolled. Fine-needle aspiration biopsy was done and processed to produce smears and CBs. Immune staining was done on CBs using antibodies to gal-3 and HBME-1. Diagnostic value of tests was determined in comparison to pathology. Results: Current study included 17 papillary thyroid carcinoma (PTC), 15 follicular adenoma, and 8 follicular thyroid carcinoma (FTC). In PTC diagnosis, co-expression of gal-3/HBME-1 had a sensitivity of 94.1%, specificity of 73.3%, positive predictive value (PPV) of 80%, negative predictive value (NPV) 91.7%, and accuracy of 84.4%. In FTC diagnosis, combined gal-3/HBME-1 expression had a sensitivity of 75%, specificity of 78.6%, PPV of 50%, NPV of 91.7%, and accuracy of 77.8%. Co-expression of gal-3/HBME-1 raised the sensitivity of detection of differentiated thyroid carcinomas from 40% with cytomorphology to 92% and accuracy from 62.5% to 85%. Conclusion: The combined immunocytochemical expression of gal-3 and HBME-1 utilizing fine-needle aspirates can improve the sensitivity of detection and diagnostic accuracy of differentiated follicular cell-derived thyroid carcinomas.

Keywords: Agarose cell block, fine-needle aspirate, galectin-3, HBME-1, thyroid tumors

Introduction

Thyroid nodules represent a very frequent clinical problem in adult population with a prevalence rate of 5% for palpable thyroid nodules and 30–50% for nonpalpable nodules.1 In neoplastic nodules, adenomas are diagnosed in over 90% of instances, while carcinomas represent less than 10% of cases.2 Thyroid cancer is the most common endocrine malignancy and it showed an increasing incidence worldwide over the last three decades.3

Thyroid fine-needle aspiration cytology (FNAC) is the gold standard method for screening of thyroid nodules.4 However, differential diagnosis of thyroid lesions/tumors with follicular growth pattern, e.g., hypercellular microfollicular adenoma (FA), hyperplastic nodular lesions, and well-differentiated follicular carcinoma is challenging.5 Galectin-3 (gal-3) is a lectin family protein of 30 kD involved in cell signaling with specific binding affinity for beta-galactosidase residues on cell surface glycoproteins.6 This protein has been shown to play a role in physiologic processes such as cell adhesion, cell activation, and chemo-atraction, and has also been involved in pathologic conditions such as cancer progression and metastasis.7 Research studies on thyroid carcinoma demonstrated that gal-3 is the most sensitive and accurate diagnostic marker for such tumors.8,9 Hector Battifora mesothelial antigen-1 (HBME-1) is a monoclonal antibody developed against cultured mesothelial cells. It reacts with an unclear antigen in the microvilli of mesothelial cells, tracheal epithelium, and in various adenocarcinomas and sarcomas.10 Studies showed lack of HBME-1 expression in normal thyroid tissue. On the contrary, HBME-1 was shown to be overexpressed in most papillary thyroid carcinomas (PTCs), and also in a fraction of follicular carcinomas.11

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Several investigators recommended the use of a panel of immunomarkers against gal-3, HBME-1, and cytokeratin 19 proteins for differentiating benign from malignant thyroid lesions. Most studies evaluated HBME-1 and gal-3 expression on tissue sections for diagnosis of thyroid tumors. Only few studies have assessed these two markers on cell blocks (CBs) or thin layers prepared from fine-needle aspiration (FNA) samples.

The use of multiple ancillary techniques on FNA biopsy is often hampered by lack of sufficient number of preparations. One good solution for this limitation is CB preparation. With adequate cellularity, CB can yield up to 20–50 sections per aspirate, hence multiple adjuvant tests, e.g. special stains, immunohistochemistry, and molecular diagnostics can be applied on the same sample. The use of formalin-fixed paraffin embedded CB is preferred over cytologic preparations, e.g., thin Prep as it is comparable to surgical biopsies with good morphological interpretation and no background staining.

**Materials and Methods**

The research was approved by the Institutional Ethical Committee. This prospective study included 40 patients with clinically or radiologically detectable thyroid nodules that were referred to Cytology Unit between February 2015 and February 2016. Patients either presented with single thyroid nodule or with suspicious nodule among multiple nodules. All relevant clinical data were obtained from patient's files.

FNAC from thyroid nodules was performed with or without ultrasonography image guidance. At least two aspirations were performed for cytological examination and another one or two aspirations for preparing CB. The aspirations were done using 22 or 23 gauge needles. At least four slides were made for each case. One slide was left to be air dried and stained by Diff Quick stain for rapid on site evaluation (ROSE) of the adequacy of the aspirated material. Rest of smears were fixed immediately in 95% ethanol at room temperature for a minimum of 15 min prior to staining. Slides were then stained using modified Papanicolaou (Pap) stain. Aspirate obtained for CB was ejected into a tube containing 10% neutral buffered formalin to fix the cells for 8–12 h. Smears were then screened and cytologic diagnosis was done using the Bethesda system for reporting thyroid cytopathology. Cases cytologically diagnosed as follicular neoplasm, PTC, and follicular lesion of undetermined significance (FLUS) were included in the study. Cytological diagnoses were correlated with the histopathological ones.

To prepare CBs we developed a processing technique from thyroid fine-needle aspirates based on the method proposed by Kerstens et al. (2000) with minor modifications. A gel 2% was prepared using UltraPure low-melting agarose (Thermo Fisher Scientific Cat. No.: 16520100). An aliquot of gel was melted at 95°C in a water bath. The fixed cells were pelleted by centrifugation at 2000 rpm for 10 min in a flat bottom or centrifuge tube. The supernatant was discarded and 1 ml of liquefied gel at 55°C was added to the tube and quickly mixed with sedimented cells. Cells/gel mixture was immersed in another wider tube containing water warmed to 60°C. Tubes were centrifuged immediately at 3000 rpm for 8 min to sediment cells in the liquefied gel. The gel/cells pellet was allowed to solidify at 4°C for a minimum of 1 h. The gel/cells button was incubated in 70% ethanol overnight, routinely processed using standard laboratory protocol for biopsy tissue, and finally embedded in paraffin. CBs were sectioned at 4 µm thickness and stained with hematoxylin and cosin (H&E). A comparison was performed between paired FNAC smears (Pap stained) and CB (H&E stained) samples using five objective parameters [Table 1]. A cumulative score ranging between 0 and 10 points was calculated for each case and then categorized into the following three categories: (i) Category 1 (scores 0–2): unsuitable for diagnosis. (ii) Category 2 (scores 3–6): adequate for cytological diagnosis. (iii) Category 3 (scores 7–10): diagnostically superior.

For immunohistochemical staining two sections were cut from each CB on electrostatically charged glass slides, deparaffinized and pretreated for antigen retrieval in a microwave. Sections were stained with anti-galectin-3 (9C4) (mouse monoclonal antibody, ready to use, Cell Marque, USA, CMC25521021) and anti-HBME-1 (mouse monoclonal antibody, ready to use, Cell Marque, USA, CMC28321030) using avidin biotin peroxidase technique. The reaction was detected using diaminobenzidine (DAB) with hydrogen peroxide (H₂O₂). Appropriate positive (small bowel for gal-3 and mesothelioma for HBME-1) and negative controls (by substituting phosphate buffer saline for the primary antibody) were used. All slides were counter-stained with hematoxylin. Immuno-stained cytoblocks were assessed by two independent observers. Gal-3 protein expression was detected in cytoplasm ± nuclei of follicular cells while HBME-1 immunoreactivity was membranous. Gal-3 and HBME-1 expression was graded as negative if ≤10% cells

**Table 1: Scoring point system for smears and cell blocks**

| Parameter             | Degree                          | Score point |
|-----------------------|---------------------------------|-------------|
| Background            | Marked (>50% of smear/section) | 0           |
|                       | Moderate (10-50% of smear/section) | 1           |
|                       | Minimal (<10% of smear/section)  | 2           |
| Cellularity           | Mild (<10% tumor cellularity)   | 0           |
|                       | Moderate (10-25% tumor cellularity) | 1           |
|                       | Abundant (>25% tumor cellularity) | 2           |
| Cell degeneration     | Marked                          | 0           |
|                       | Moderate                        | 1           |
|                       | Minimal                         | 2           |
| Cell trauma           | Marked                          | 0           |
|                       | Moderate                        | 1           |
|                       | Minimal                         | 2           |
| Architecture          | Minimal (diagnosis not possible) | 0           |
|                       | Moderate (some preservation)    | 1           |
|                       | Marked (architecture display)   | 2           |
show staining, weak if 11–50% of cells reveal stain, moderate if 51–75% showed expression or strong positive when 75–100% of cell exhibit protein expression. Divergent or unreliable immunostaining results were not considered for the diagnosis.

For statistical analysis, numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher’s exact test was used to examine the relation between qualitative variables. Evaluation of the two markers was done by calculating sensitivity, specificity, positive predictive values (PPV), negative predictive value (NPV), and accuracy of the cytomorphologic diagnosis by itself and when combined with immunocytochemistry (ICC) using gal-3 and HBME-1, against gold standard (pathology). Kappa test was used to evaluate agreement between two diagnostic methods and independent observers. All tests were two-tailed. A P value <0.05 was considered significant.

RESULTS

In the current study, the mean age of patients with thyroid lesions was 45.5 ± 14.7 years (range: 17–70). The mean age for adenoma patients was significantly lower (37.5 ± 13.2 years) than that for carcinoma patients (50.4 ± 13.6 years); P ≤ 0.05. Females represented the majority of cases with female to male ratio of 4:1.

According to Mair et al., point scoring system, 75% of smears and 90% of CBs [Figures 1b, 2b] in this study were considered diagnostically superior (Category 3). Twenty-five percent of the smears and 10% of CBs were diagnostically adequate (Category 2). None of the smears or CBs were unsuitable for diagnosis (Category 1) as adequacy of the aspirated material was assessed by ROSE.

Smears of FNAC of thyroid lesion (number = 40) revealed that 10 cases (25%) were PTC [Figure 1a], 19 (47.5%) were follicular neoplasms, and 11 cases (27.5%) were FLUS. Pathological examination of H&E stained sections from corresponding thyroidectomy specimens showed that 17 cases (42.5%) were PTC, 15 cases (37.5%) were FA, and 8 cases (20%) were follicular carcinoma. When cytomorphological and histopathological diagnoses were compared, pathology confirmed cytological diagnosis of all PTC with no false positive result. Follicular neoplasm group (19 cases) was classified as FA (number = 8) [Figure 2a], follicular carcinoma (number = 6), and PTC (number = 5). Tissue diagnosis of the indeterminate cases of FLUS (11) revealed that 7 were FA, 2 were follicular carcinoma, and 2 were PTC.

Two independent observers examined gal-3 and HBME-1 immune stained slides and recorded an interobserver agreement (kappa value or κ) of 0.95. Gal-3 displayed predominantly cytoplasmic staining [Figure 1c], while HBME-1 expression was mainly membranous [Figure 1d]. Table 2 shows that gal-3 protein was expressed in the majority (80%, 20/25) of thyroid carcinoma (PTC and FTC) and some (3/15, 20%) of FAs. The difference was statistically significant (P = 0.000). Figure 2c shows a case of FA displaying negative expression of gal-3 protein.

HBME-1 was expressed significantly more (84%) in thyroid carcinomas than FAs (26.6%) (P = 0.000). Overall, large number of malignant tumors (FTC, PTC) showed strong and

Figure 1: Papillary thyroid carcinoma: (a) FNA smear showing enlarged pale nuclei with powdery chromatin, intranuclear pseudo-inclusions, nuclear groove, and eccentric micronucleoli (Papanicolaou stain, ×1000). (b) Cell blocks section displaying a papillary structure having fibrovascular core covered by malignant cells with clear nuclei and pseudo-inclusions (H&E, ×200). (c) Galectin-3 diffuse intense cytoplasmic brownish staining (ICC: Galectin, x100). (d) HBME-1 diffuse intense membranous brownish staining (ICC: HBME-1, x100)

Figure 2: Follicular adenoma: (a) FNA smear showing groups of follicular cells arranged in follicular pattern with no colloid in the background (Papanicolaou stain, ×400). (b) Cell block section showing large colloid-filled follicles with flattened uniform lining epithelium (H and E, 200). (c) Galectin-3 negative immunocytochemical reaction (ICC: Galectin, x200). (d) HBME-1 focal, weak positive membranous immunocytochemical reaction (ICC: HBME-1, x200)
diffuse reactivity for each marker, while small number of FA showed mainly focal and weak expression [Figure 2d].

Twenty-one samples (52.5%) reacted positively to both gal-3 and HBME-1 markers and 13 (32.5%) cases were negative to both. The agreement between the two markers was substantial ($\kappa = 0.688$, $P < 0.001$) [Table 3]. Twenty three out of 25 (92%) of malignant cases (PTC and FTC) showed positive ICC reaction to either gal-3 or HBME-1 or both, while 73.3% of FAs showed negative reaction to both gal-3 and HBME-1 ($P < 0.001$).

Addition of gal-3 marker staining results to cytomorphology elevated the sensitivity of diagnosis of thyroid carcinoma from 40 to 80%, and accuracy from 62.5 to 80% but lowered specificity from 100 to 80% [Table 4]. HBME-1 expression was more sensitive than gal-3 in differentiating follicular cell-derived thyroid carcinoma from adenoma but less specific (73.3%). Combined expression of both markers elevated the sensitivity of diagnosis of thyroid carcinoma to 92% and the accuracy to 85%.

Fifteen (88.2%) of PTC showed positive staining for gal-3 and 16 (94.1%) expressed HBME-1. In PTC, the sensitivity of detection was raised from 58.8% with cytomorphology alone to 88.2% with gal-3 immune stain and 94.1% with HBME [Table 5]. Combined use of both markers showed a sensitivity of 94.1%. On the contrary, the specificity of detection of PTC was lowered from 100% (with cytomorphology alone) to 80% for gal-3, 73.3% for HBME-1, and 73.3% for combined marker use. The diagnostic accuracy of FNAC was elevated from 78.2 to 83.4% and 84.4% when either galactin-3 or HBME-1 was used, respectively, and to 84.4% when both markers were studied.

Gal-3 was expressed in 5/8 of FTC with sensitivity of 62.5%, specificity of 80%, PPV of 62.5%, NPV of 80%, and 73.9% accuracy. Positive immunoreactivity to HBME-1 was evident in 5/8 FTC samples with a sensitivity, specificity, PPV, NPV, and accuracy values of 62.5, 73.3, 55.6, 78.6, and 69.6%, respectively. Combined gal-3 HBME-1 expression had a sensitivity of 75%, specificity of 78.6%, PPV of 50%, NPV of 91.7%, and accuracy of 77.8% in diagnosis of follicular carcinoma.

Among 11 cases diagnosed cytologically as FLUS, the two PTC showed positivity for both galectin-3 and HBME-1. Regarding FTC, one case reacted for gal-3, and the other for HBME-1. Two out of the seven adenomas were positive for both markers. The results, however, could not be evaluated

### Table 2: Expression of galectin-3 and HMABE-1 in thyroid benign and malignant tumors

| Marker     | *FA (Total 15) | †FTC (Total 8) | ‡PTC (Total 17) | Total     |
|------------|----------------|----------------|-----------------|-----------|
| Gal-3      | Positive 3     | 5              | 15              | 23/40 (57.5%) |
|            | Negative 12    | 3              | 2               |           |
| HBME-1     | Positive 4     | 5              | 16              | 25/40 (62.5%) |
|            | Negative 11    | 3              | 1               |           |
| Both       | Positive 3     | 3              | 15              | 21/40 (52.5%) |
|            | Negative 11    | 1              | 1               |           |

*FA: Follicular adenoma, †FTC: Follicular thyroid carcinoma, ‡PTC: Papillary thyroid carcinoma

### Table 3: Agreement between galectin-3 and HBME-1 expression in current study

| Galectin-3 | HBME-1 | Total |
|------------|--------|-------|
|            | Positive | Negative |       |
| Positive   | 21      | 2      | 23    |
| Negative   | 4       | 13     | 17    |
| Total      | 25      | 15     | 40    |

### Table 4: Value of test in discrimination of thyroid carcinoma from adenoma

| For malignancy | Cytomorphology (%) | Galactin-3 (%) | HBME-1 (%) | Combined (%) |
|----------------|--------------------|---------------|------------|--------------|
| Sensitivity    | 40                 | 80            | 84         | 92           |
| Specificity    | 100                | 80            | 73.3       | 73.3         |
| *PPV           | 100                | 65.2          | 84         | 85.2         |
| *NPV           | 50                 | 70.6          | 73.3       | 84.6         |
| Accuracy       | 62.5               | 80            | 80         | 85           |

*PPV: Positive predictive value, *NPV: Negative predictive value

### Table 5: Diagnostic value of tests in identification of papillary thyroid carcinoma

| For malignancy | Cytomorphology (%) | Galactin-3 (%) | HBME-1 (%) | Combined (%) |
|----------------|--------------------|---------------|------------|--------------|
| Sensitivity    | 58.8               | 88.2          | 94.1       | 94.1         |
| Specificity    | 100                | 80            | 73.3       | 73.3         |
| *PPV           | 100                | 83.3          | 80         | 80           |
| †NPV           | 68.2               | 85.7          | 91.7       | 91.7         |
| Accuracy       | 78.2               | 83.4          | 84.4       | 84.4         |

*PPV: Positive predictive value, †NPV: Negative predictive value
statistically due to small number of cases within subgroups, thus a larger number of cases are needed to evaluate the value of galectin-3 and HBME-1 in cases of FLUS.

**Discussion**

Thyroid cytology is an easy affordable method for screening of thyroid nodules that can be relied upon, generally with no complications.[2] Verification of malignant nature of follicular neoplasms—which is a histopathologic matter—is a limitation for thyroid FNAC.[4] ICC when applied as complimentary to thyroid cytomorphology can raise its diagnostic accuracy.[6,9] Galectin-3 and HBME-1 have shown the highest reliability in distinguishing benign from malignant lesions, particularly in papillary and follicular carcinomas.[23]

In the current research, majority (90%) of CBs were considered diagnostically superior (Category 3) and 10% were diagnostically adequate (Category 2). This was attributed to CB preparation technique that concentrated and focused cells in a relatively sharp layer in the gel disc. Furthermore, formalin fixation and low-melting point agarose preserved cell morphology and protected the cells from degeneration or trauma during processing, respectively. When compared to smears, CBs contributed significantly to diagnosis in FNAC from thyroid hemorrhagic nodules or low-cell yield lesions.

Excellent interobserver agreement was scored (κ = 0.95) during assessment of gal-3 and HBME-1 immune stained slides cut from CBs. This was due to preservation of morphology that facilitated interpretation of immune staining results in terms of stain intensity and distribution.

In this research, gal-3 protein was expressed in the majority of included thyroid carcinoma cases (80%) and some of FAs (20%). The difference was statistically significant (P = 0.000). Addition of gal-3 marker expression results to cytomorphology elevated the sensitivity of diagnosis of thyroid carcinoma from 40 to 80%, and accuracy from 62.5 to 80%, but lowered specificity from 100 to 80%.

Higher expression of gal-3 in malignant thyroid tumors compared to benign lesions was reported by several investigators.[11,12,24] Results of our research are in agreement with those reported by de Matos et al.[25] Authors conducted a systemic review and diagnostic meta-analysis for 66 published articles concerning value of marker expression (CK 19, galectin-3 and HBME-1) in differentiation of thyroid lesions. Globally gal-3 showed a sensitivity of 82% and specificity of 81%.

In the current research, HBME-1 was a more sensitive marker for thyroid carcinomas (papillary and follicular carcinomas together) than gal-3. It had a sensitivity of 84% compared to 80% for galactin-3. But it was less specific (73.3%) in differentiating carcinomas (follicular and papillary) from adenoma.

Several prior researches reported higher immune reactivity of HBME-1 in thyroid follicular cell-derived carcinoma compared to benign tumors (but in adenomas expression is focal).[11,13] In a systemic review conducted by de Matos et al.[25] HBME-1 demonstrated a sensitivity of 77% and specificity of 83% in the diagnosis of malignant thyroid lesions. Dunderovic et al.[26] reviewed 25 studies and reported that HBME-1 had an average sensitivity of 76% (range: 34–100%) and an average specificity of 87% (range: 54–100%) in diagnosis of follicular cell-derived thyroid carcinomas. Although HBME-1 does not show any immune reactivity in normal thyroid tissue, however, literature recorded a positive expression from 10 to 56% in thyroid FAs.[13]

In this research, co-expression of both markers (gal-3 and HBME-1) showed a sensitivity of 92%, specificity of 73.3%, PPV of 85.2%, NPV of 84.6%, and accuracy of 85% in discriminating thyroid carcinomas (PTC and FTC) from FA.

Dunderovic et al.[26] analyzed expression of a panel of markers (CD56, CK19, galectin-3, and HBME-1) in tissue microarrays containing 201 thyroid lesions (neoplastic and nonneoplastic). In their study, co-expression of gal-3 and HBME-1 markers had a sensitivity of 85.9% and a specificity of 100% in thyroid malignancy diagnosis.

The marked variability in diagnostic values of gal-3 and HBME-1 markers recorded by different studies could be attributed to variation in cut-off values for interpreting negative and positive stain results, tumor types (e.g., differentiated and undifferentiated thyroid carcinomas), and the interpretation of stain as membranous and/or cytoplasmic (e.g., HBME-1).[25,26] The sensitivity of detection of PTC increased from 58.8% with cytomorphology alone to 88.2% with gal-3 immune stain and to 94.1% with HBME. Combined use of both markers showed a sensitivity of 94.1%. On the contrary, the specificity of detection of PTC was lowered from 100% (with cytomorphology alone) to 80% for gal-3, 73.3% for HBME-1, and 73.3% for combined marker use.

Wu et al.[27] analyzed expression of CK 19, gal-3, HBME-1, and HER-2/neu markers with the aim to determine their diagnostic value in PTC (331 cases). Investigators recorded a sensitivity of 81.9% and specificity of 92.3% for PTC diagnosis using gal-3. While for HBME-1, sensitivity was 79.2% and specificity was 97.9%. Majority of studies have reported gal-3 positivity in 90–100% of papillary carcinoma cases.[28,29] In few studies who have reported gal-3 expression in PTC by histological subtype, positivity was identified in 82–100% cases of classic variant of papillary carcinoma.[7] These results are consistent with our findings.

In this study, combined expression of gal-3 and HBME-1 markers increased the detection of follicular carcinoma as they were positive in 85.5% (7/8) of included cases (2 positive for galectin-3, 2 for HBME-1, and 3 positive for both). While, they were both negative in 11 out of 15 (73.3%) FAs. Thus the positivity of one or both markers in a follicular lesion on thyroid FNA samples should raise suspicion of follicular carcinoma and should prompt surgical excision.[30]
**CONCLUSION**

The present study demonstrates that combined immunocytochemical expression of gal-3 and HBME-1 utilizing fine-needle aspirates can improve the sensitivity of detection and diagnostic accuracy of well-differentiated follicular cell thyroid carcinoma.

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**Conflicts of interest**

There are no conflicts of interest.

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