HBME1 and CK19 in Non-Invasive Follicular Thyroid Neoplasm with Papillary-like Nuclear Features (NIFTP) vs Other Follicular Patterned Thyroid Lesions.

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Abstract

Background: Thyroid neoplasms with follicular architecture can have overlapping morphologic features and pose diagnostic confusion amongst pathologists. Various immunohistochemical stains have been investigated as potential diagnostic markers for PTC; amongst which HBME1 and CK19 have gained popularity. Non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) poses similar diagnostic challenges with interobserver variability and is often misdiagnosed as adenomatoid nodule or follicular adenoma. This study aims to evaluate expression of HBME1 and CK19 in NIFTPs in comparison to other well differentiated thyroid neoplasms and benign mimickers.

Method: 73 thyroid cases diagnosed over a period of 3 years at Methodist University Hospital, Memphis, TN were included in this study: 9 NIFTP, 18 papillary thyroid carcinoma (PTC), 11 follicular variant of papillary thyroid carcinoma, invasive (I-FVPTC), 24 follicular adenomas (FA), and 11 multinodular goiters/adenomatoid nodules (MNG). A tissue microarray (TMA) was constructed and HBME1 and CK19 IHC was performed.

Results: HBME1 was expressed in 77.8% NIFTPs, 88.9% PTC, 81.8% I-FVPTC, 16.7% FA, and 18.2% MNGs. CK19 expression was seen in 66.7% NIFTPs, 83.3% PTC, 81.8% I-FVPTC, 33.3% FA and 45.4% MNGs. Difference in expression of HBME1 and CK19 was statistically significant for NIFTP vs FA (qualitative; p<0.05) and NIFTP vs MNG (p<0.05). No statistically significant difference was found for HBME1 in NIFTP vs PTC (conventional and FVPTC), p>/= 0.2. Sensitivity of HBME1 and CK19 for NIFTP were 78% and 67%, ~88% each for PTC, and 89% and 100% for FVPTC respectively, while specificity of HBME1 and CK19 for NIFTP were 53% each, ~62% each for PTC and ~55% each for FVPTC.

Conclusion: Our study indicated that HBME1 and CK19 are valuable markers in differentiating NIFTPs from morphologic mimics like follicular adenoma and adenomatoid nodules/multinodular goiter. While HBME1 and CK19 are both sensitive in diagnosing lesions with PTC like nuclear features, CK19 stains a higher number of benign lesions in comparison to HBME1. No increase in sensitivity or specificity in diagnosis of NIFTP, PTC or FVPTC was noted on combining the two antibodies.

1. Introduction

The rate of thyroid cancer in the United States is escalating rapidly with a reported increase in incidence of 3.6% per annum and the number of newly diagnosed cases going up to 56,430 yearly [1-3]. Papillary thyroid carcinoma (PTC) makes up most of the thyroid malignancies with follicular variant being the most common subtype [4]. The follicular variant of papillary thyroid carcinoma (FVPTC) has become the most common architectural pattern with the percentage rising exponentially from 18 to 57% in the last few decades [5]. FVPTC had two known subtypes – infiltrative and encapsulated, with the latter demonstrating an indolent behavior. The encapsulated form could be invasive or non-invasive [6-12]. Several studies reiterated that non-invasive form of encapsulated FVPTC (NI-EFVPTC) exhibited a behavior comparable to that of benign nodules and was being over treated [13]. This led to the proposal...
of the new terminology “Noninvasive follicular thyroid neoplasm with papillary like nuclear features (NIFTP)” by the Endocrine Pathology Society working group (ESPWG) for these tumors with indolent behavior [13-14].

Follicular lesions of the thyroid often pose diagnostic dilemmas due to the morphologic resemblance and architectural similarities in benign and malignant lesions. There are studies citing the prevalent interobserver variability in the diagnosis of thyroid lesions. Saxen et al reported a 58% agreement among pathologists for thyroid tumors [16]. Similar findings have been noted in further studies, especially pertaining to the follicular lesions of thyroid [17-20]. With increasing diagnostic perplexity, the focus shifted to use of immunohistochemical markers to delineate benign from malignant lesions and distinguish the various follicular neoplasms [7, 21,22,23]. Various immunohistochemical (IHC) stains have been investigated as potential diagnostic markers for PTC, which include CK19, HBME1 (Hector Battifora Mesothelial-1), FN1 (fibronectin1), CITED1 (Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain, 1, also known as melanocyte-specific gene 1) and GAL3 (galectin3) [4,17]. Amongst these, HBME1 and CK19 have gained popularity. HBME1 is a monoclonal antibody which is known to act against the microvillous surface of mesothelial cells and has shown to be expressed in thyroid malignancies, while negative in benign lesions [4, 22, 24-26]. CK19 has also proved useful in this regard, exhibiting strong and diffuse expression in thyroid malignancies and focal weak staining in benign nodules [22, 27-28].

This study aims at investigating expression of these two biomarkers (HBME1 and CK19) in the commonly encountered benign and malignant thyroid nodules in a random and blinded manner. Furthermore, the purpose was to study effectiveness of these markers in differentiating challenging cases of NIFTP from benign entities like follicular adenoma (FA) and adenomatoid nodules. No molecular studies were performed as part of this study.

2. Material And Methods

2.1 Case selection

After obtaining approval of this retrospective study by International Review Board (IRB) of University of Tennessee Health Sciences Center (UTHSC), the Methodist University hospital database was queried for thyroid cases belonging to the following categories: multinodular goiter (MNG)/ adenomatoid nodules, follicular adenoma (FA), papillary thyroid cancer (PTC), Invasive form of follicular variant of papillary thyroid carcinoma (I-FVPTC) and non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP). NIFTP cases were selected based on the revised, universally accepted, specific inclusion/exclusion criteria published in 2019 [6]:

A total of 73 cases over a period of 3 years from 2016-2019 were identified. Clinical data including age, gender, site and size of tumor was recorded for each case. Detailed review of H & E slides was conducted by Head and Neck Pathologist (SA) to characterize the tumors in each category. A change in the primary diagnosis in three cases was rendered as follows: Two cases of FVPTC were reclassified to NIFTP and
classical PTC respectively while one case previously diagnosed as NIFTP was altered to I-FVPTC making the total number as follows: MNG (n=11) including hyperplastic and adenomatoid nodules; FA (n=24) {Hürthle cell/oncocytic types in 4 cases, clear cell and macrofollicular type in 1 each}; PTC (n=18) {usual=13, hobnail=2, focal tall cell features=2, diffuse sclerosing=1}, FVPTC (n=11) and NIFTP (n=9).

2.2 Tissue microarray (TMA)

H&E slides were reviewed by the pathologist to select the best possible area representative of the diagnosis for the TMA. Formalin fixed paraffin embedded tissue blocks were then utilized to construct 1-mm single cores (n=73) using a semi-automated tissue microarrayer (Advanced Tissue Arrayer from Veridiam) to evaluate the immunohistochemical expression.

2.3 Immunohistochemistry (IHC)

IHC for HBME1 (Cell-Marque pre-dilute Clone HBME1) and CK-19 (Roche Pre-dilute Dispenser Clone = A53-B/A2.26) was performed on the TMA created from paraffin embedded tissue blocks of 73 selected cases at PathGroup, TN. IHC staining of all the cores was then analyzed in a random and blinded fashion. HBME1 and CK19 were considered positive if staining was membranous/luminal and membranous/cytoplasmic respectively, and moderate to strong in >10% lesional cells.

2.4 Statistical analysis

Positive/Negative IHC results (HBME1/CK19) were taken as categorical variables and analyzed by Chi-square analysis; result was expressed as a percentage (qualitative). Categorical variables were summarized as count and percentage and compared between disease types using the $X^2$ test. The association between HBME1 and CK19 expression and disease as a binary outcome (NIFTP, PTC, FA, etc) was conducted using the logistic regression analysis adjusting for demographic variables.

The following analyses were performed:

1. Expression of HBME1 and CK19 in NIFTP was compared to that observed in other well differentiated thyroid neoplasms and p value calculated.
2. HBME1 and CK19 were cross classified and their expression (cumulative) was compared between two diagnoses (NIFTP vs PTC; NIFTP vs FA; NIFTP vs MNG and NIFTP vs FVPTC) and p value calculated.
3. HBME1 and CK19 were cross classified and their expression (cumulative) was compared between all diagnoses and p value calculated.

A p-value ≤ 0.05 is interpreted as statistically significant.

A p-value >0.05 is interpreted as not statistically significant.
In addition, sensitivity, specificity, and predictive values for HBME1 and CK19 in diagnosing NIFTP were calculated. The association between HBME1 and CK19 expression and disease types as a binary outcome (NIFTP, PTC, FA, etc.) was conducted using the logistic regression analysis adjusting for demographic variables. The receiver operating characteristics (ROC) curves were produced for the prediction of the outcomes by the HBME1 and CK19 adjusting for covariates. All the statistical analysis was conducted using R version 3.5.3 (2019-03-11).

3. Results

3.1 Demographic data and Immunohistochemical expression

Table 1 summarizes the demographic data along with tumor size for the cases included in this study (n=73). HBME1 and CK19 were considered positive if staining was membranous and cytoplasmic/membranous respectively, and moderate to strong in >10% lesional cells. Results for HBME1 and CK19 immunohistochemical expression (number and percent positive cases) in the different diagnostic categories are summarized in Table 2.

Table 1: Demographic data and tumor size of thyroid neoplasms included in the study

|                     | NIFTP (n=9) | PTC (n=18) | FVPTC (n=11) | FA (n=24) | MNG (n=11) |
|---------------------|-------------|------------|--------------|-----------|------------|
| F/M                 | 3.5:1       | 2:1        | 4.5:1        | 3:1       | 4.5:1      |
| Median Age (y)      | 41          | 47         | 41           | 53        | 52         |
| Tumor Size (cm)     | 2.0         | 2.0*       | 2.0*         | 2.7       | 5.2        |

*Largest tumor nodule considered in multifocal cases.

Table 2: Immunohistochemical expression (qualitative) of HBME1 and CK19 in various well differentiated lesions.

| Diagnostic Category | Number of cases (n) | HBME1+ (X) | HBME1+ (%) | CK19+ (Y) | CK19+ (%) |
|---------------------|---------------------|------------|------------|-----------|-----------|
| NIFTP               | 9                   | 7          | 77.8       | 6         | 66.7      |
| PTC                 | 18                  | 16         | 88.9       | 15        | 83.3      |
| FVPTC               | 11                  | 9          | 81.8       | 9         | 81.8      |
| FA                  | 24                  | 4          | 16.7       | 8         | 33.3      |
| MNG                 | 11                  | 2          | 18.2       | 5         | 45.4      |
| Total               | 73                  | 38         | N/A        | 43        | N/A       |

X= Number of cases showing HBME1 positivity.
Y= Number of cases showing CK-19 positivity.

3.2 Statistical Analysis

3.2.1 Comparing NIFTP with each separate diagnoses.

1. FA: 16.7% expressed HBME1 while 33.3% showed positive expression for CK19 vs 77.8% and 66.7% for NIFTPs, respectively. The difference was found to be statistically significant for both antibodies ($p=0.002$ for HBME1 and $p=0.02$ for CK19).

2. MNG: 18.2% expressed HBME1 while 45.4% showed positive staining for CK19. The difference was statistically significant in both percentage ($p=0.008$, HBME1; $p=0.009$, CK19).

3. PTC: No statistically significant difference was found for either HBME1 or CK19 expression between PTC and NIFTP.

4. FVPTC: No statistically significant difference was observed.

3.2.2 Cross Classification of HBME1 and CK19 and Two-Way Diagnosis

This method of data analysis showed statistically significant results for NIFTP vs FA ($p=0.002$) and NIFTP vs MNG ($p=0.005$) while no significance was found for NIFTP vs PTC and/ or NIFTP vs FVPTC.

3.2.3 Cross Classification of HBME1 and CK19 and All Diagnosis

This method showed a $p$ value of <0.0001, which indicated highly significant results. Figure 3 shows the distribution of all diagnoses with cross classification of HBME1 and CK19. It is quite evident from the bar diagram that benign diagnoses like FA and MNG are clustered on the left-hand side of the graph with most cases staining negative for both antibodies (HBME1-CK19: Neg-Neg) while PTC and NIFTP have a higher distribution along the right-hand side of the graph (HBME1-CK19: Pos-Pos).

3.2.4 ROC curve analysis results

Table 3, 4 and 5 summarize the sensitivity, specificity, positive predictive value and negative predictive value of HBME1 and CK19 (as calculated by the ROC curve analysis and DeLong’s test) for NIFTP, PTC (classical), and I- FVPTC, respectively.

Table 3: Sensitivity, specificity and predictive values of HBME1 and CK19 in diagnosis of NIFTP

|          | Sensitivity | Specificity | Positive predictive value | Negative predictive value |
|----------|-------------|-------------|---------------------------|----------------------------|
| HBME1    | 0.78        | 0.53        | 0.19                      | 0.94                       |
| CK19     | 0.67        | 0.53        | 0.18                      | 0.91                       |
| HBME1 + CK19 | 0.56     | 0.57        | 0.17                      | 0.89                       |
Table 4: Sensitivity, specificity and predictive values of HBME1 and CK19 in diagnosis of PTC

|          | Sensitivity | Specificity | Positive predictive value | Negative predictive value |
|----------|-------------|-------------|---------------------------|---------------------------|
| HBME1    | 0.89        | 0.62        | 0.44                      | 0.94                      |
| CK19     | 0.88        | 0.63        | 0.44                      | 0.94                      |
| HBME1 + CK19 | 0.82        | 0.68        | 0.47                      | 0.92                      |

Table 5: Sensitivity, specificity and predictive values of HBME1 and CK19 in diagnosis of FVPTC

|          | Sensitivity | Specificity | Positive predictive value | Negative predictive value |
|----------|-------------|-------------|---------------------------|---------------------------|
| HBME1    | 0.89        | 0.55        | 0.24                      | 0.97                      |
| CK19     | 1.0         | 0.57        | 0.26                      | 1.0                       |
| HBME1 + CK19 | 0.89        | 0.62        | 0.27                      | 0.97                      |

Discussion

Papillary thyroid carcinoma is usually a morphologic diagnosis with characteristic nuclear features such as large, overlapping, ground glass nuclei, nuclear grooves, and pseudo inclusions and rarely requires immunohistochemistry to confirm the diagnosis. Histologically, classic PTC and follicular variant are the two major low risk sub-types of PTC with other high-risk variants like tall cell, diffuse sclerosing and hobnail variants reported in literature [29,30].

FVPTC encompasses a wide spectrum of morphology ranging from micro- to macro- follicular and diffuse growth pattern and could be encapsulated or infiltrative often creating diagnostic confusion with other follicular neoplasms. Tallini et al [31] in 2017 published a detailed historical review of the emergence of the term “Follicular Variant of Papillary Thyroid Carcinoma”. FVPTC was first officially defined by Chen and Rosai [32] in 1977 after Lindsay found papillary carcinoma like nuclear features in a subset of follicular carcinomas [29]. In 1980s, the encapsulated variant of FVPTC was recognized. This led to the classification of thyroid tumors showing predominant follicular growth pattern with nuclear characteristics of PTC into 3 main groups: 1. Encapsulated FVPTC without invasion (EFVPTC); 2. Encapsulated FVPTC with capsular and/or vascular invasion and infiltrative FVPTC without a tumor capsule [31]. In 2016, non-invasive EFVPTC was re-categorized as non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) by Nikiforov et al [13].
Since the new classification in 2016, several studies have evaluated biologic behavior of NIFTPs. Analysis of 94 NIFTP cases by Thompson et al [33] and 129 cases by Rosario et al [7] supported the low-risk behavior and conservative approach in treating the patients with NIFTP. Molecular studies on the encapsulated/well circumscribed FVPTCs have found primarily RAS mutations and thereby suggested their close relationship with other follicular neoplasms of the thyroid such as follicular adenoma and follicular carcinoma [34-37].

Follicular patterned lesions of the thyroid have high level of interobserver as well as intraobserver disagreement [37, 38]. A considerable degree of discordance has been reported among pathologists in the diagnosis of FVPTC, the encapsulated type, in particular [20, 38]. The diagnostic criteria for NIFTP includes: Encapsulated/well demarcated tumor without any invasion, no papillary growth, no evidence of psammomatous calcifications or tumor necrosis, < 30% solid/trabecular or insular growth pattern and nuclear features of PTC with nuclear score of 2-3 [13, 34].

A high degree of interobserver variability has been observed, even among expert pathologists as the nuclear features of PTC could be only focal/subtle [13, 38]. Unfortunately, there are no established criteria like required percentage of the follicular neoplasm showing nuclear features of PTC and/or the more reliable nuclear features (overlapping vs irregular nuclear outlines) that can help diagnose this entity as EFVPTC vs FA [38, 39]. NIFTP is still an evolving diagnosis and the struggle in diagnosing this entity is real.

Immunohistochemistry, although seldom required, can be helpful in differentiating FVPTC from other follicular lesions [26,40,41]. Various IHC markers have been explored to characterize the immunohistochemical profile of thyroid tumors especially the follicular patterned lesion which causes significant diagnostic confusion with high rate of interobserver disagreement. Among these, most notable are HBME1, Cytokeratin 19 (CK19), galectin-3 (GAL3), CITED1, and Thyroid peroxidase (TPO). HBME1 (Hector Battifora Mesothelial-1) a monoclonal antibody directed against microvilli, and a marker of mesothelial and other epithelial cells, has shown significant expression in malignant thyroid with a sensitivity of 78.8% for thyroid malignancy, 87.3% for PTC, and 65.2% for follicular carcinomas and specificity of 82.1% [37, 42].

CK19 is a low molecular weight cytokeratin which is demonstrated in both simple as well as complex epithelium and has been widely utilized in thyroid neoplasms. [17,21, 43]. Baloch et al [44] employed a panel of cytokeratins including CK5/6/ and CK 18, 10/13, 14, 17, 18, 19 and 20 in FVPTC. The authors found that CK19 was useful in diagnosis of PTC (showed diffuse staining pattern), only focally expressed in follicular tumors but was expressed in normal thyroid tissue. It has also been proven a helpful marker in cytology specimens of unequivocal cases of PTC [28, 45]. Khurana et al [45] reported a sensitivity and specificity of 93% and 100% which was comparable to that reported by Nasser et al [28]. In our series, the sensitivity and specificity values for CK19 in diagnosis of PTC were 88% and 63% respectively. Although the staining was weak to moderate in intensity, we did see about 33% FAs and 45% MNG nodules showing CK19 expression. Our findings agree with those of Baloch et al and Haiyan Lu et
al [37,40] who also found CK19 expression in normal thyroid parenchyma. We did not find any difference in antigen localization amongst positive malignant vs positive benign cases. Casey et al [46], also reported weak to moderate positive expression of CK19 in 12/30 benign thyroid cases with papillary hyperplasia with a sensitivity and specificity of 100% and 60% for PTCs.

In our study, we found a significant difference in the expression of CK19 and HBME1 in NIFTP cases in comparison to other benign follicular lesions (p<0.02 for both markers). HBME1 was expressed in 77.8% cases of NIFTP, while only 16.7% and 18.2% cases of FA and MNG showed positive staining, respectively. Frequent expression was also noted in cPTC (88.9%) and FVPTC (81.8%) cases which agree with the percentage reported in literature [37,47]. Gucer et al [48] reported an expression score of 77% for HBME1 in noninvasive RAS like PTCS/ NIFTPs, corroborating with our findings. The sensitivity of HBME1 and CK19 was found to be 78% and 67% respectively, for diagnosis of NIFTP while specificity was 53% for both biomarkers.

Similarly, CK19 showed expression in NIFTP (66.7%), cPTC (83.3%) and FVPTC (81.8%) in comparison to FA (33.3%) and MNG (45.4%). Similar findings have been reported by Liu et al [49] who reported statistically significant expression of CK19 and HBME1 in PTC vs benign thyroid lesions. Sensitivity of CK19 and HBME1 in diagnosis of PTC were reported to be 96.30% and 85.3% respectively while the reported specificity was 40% and 62% respectively [49]. Our series reported a sensitivity and specificity of ~89% and ~63% respectively, for both antibodies in diagnosis of PTC (Table 4). For FVPTC, the sensitivity of HBME1 and CK19 were found to be 89% and 100% respectively, while specificity was ~55% for both antibodies (Table 5).

HBME1 was found to be the most sensitive marker of thyroid malignancy by Palo et al [50], followed by CK19, in differentiating FVPTC from FA and follicular carcinoma. Palo et al reported an increase in sensitivity with combined use of HBME1 and CK19 in differentiating benign from malignant thyroid lesions (94% with combined use vs 86% (HBME1) and 75% (CK19)). Saleh et al [47] did not report increase in sensitivity or specificity with combined use of CK19 and HBME1 vs isolated use of either biomarkers. Our series found no increase in the sensitivity when combining the two antibodies.

Huiyan Liu et al [37] published a review article in 2015 in which they analyzed various studies evaluating role of IHC in diagnosing thyroid lesions. The authors concluded that there is no single biomarker sufficient to differentiate between benign and malignant thyroid lesions. Their review found strong and diffuse HBME1 expression while CK19 had low sensitivity as well as specificity for papillary thyroid carcinomas. The authors suggested including TROP2 (trophoblastic cell surface antigen 2) in the panel along with HBME1, CK19 and Galectin-3 as an aid in diagnosis of PTCs.

Our study has some limitations, and the findings need further validation. First, our sample size is small with limited number of NIFTP cases (n=9). Second, we recognize that the study used TMA for IHC analysis, and the results might not be completely generalizable as some of these lesions can exhibit heterogeneity for antigen expression.
Conclusions

Thyroid lesions with follicular architecture have several overlapping histologic features with problems arising particularly in differentiating encapsulated FVPTC/ NIFTP from follicular adenomas or adenomatoid nodules in MNG. Our study revealed that HBME1 and CK19 are sensitive markers for diagnosis of NIFTPs, PTC and FVPTC and can help in rendering the correct diagnosis in challenging cases of EFVPTC without invasion/ NIFTP with focally developed PTC like nuclear features. Further, our statistical analysis did not find added significance of combining these two markers in aiding the diagnosis of NIFTP/ PTC or FVPTC. We acknowledge that the sample size of this study is small and further studies with larger number of cases (particularly NIFTP) is needed to further validate the findings. Nevertheless, the entire tumor capsule interface should be examined to rule out capsular and/or vascular invasion to avoid missing diagnosis of invasive carcinoma.

Declarations

- Ethics approval: The study was reviewed and approved by the International Review Board (IRB) at University of Tennessee Health Sciences Center (UTHSC).
- Consent for participation and publication: Not applicable.
- Availability of data and materials: All data generated or analyzed during this study are included in this published article.
- Competing interests: The authors declare that they have no competing interests
- Funding: The cost for immunohistochemistry (HBME1 and CK19) performed at PathGroup laboratories was paid with faculty development funds (SA).
- Authors’ contributions: SA designed the study, selected the blocks for TMA, read and interpreted the immunohistochemistry, supervised the work, and edited the final manuscript. QS helped collect the cases for study and helped in data collection and RS helped with TMA preparation. QS and RS contributed equally towards drafting the manuscript. DTD and QZ analyzed the raw data using statistical analysis.
- Acknowledgements: None

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