Immunohistochemical and Molecular Markers Associated with Differentiated Thyroid Carcinoma

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In the last decade, conventional diagnosis of thyroid nodules largely depended on fine-needle aspiration (FNA) and ultrasound. However, FNA has a limited ability to distinguish between benign and malignant lesions, especially in cases with indeterminate cytology. Although the clinical course of differentiated thyroid carcinoma is believed to be favorable, delayed diagnosis can make its clinical management difficult. Many immunohistochemical (IHC) or molecular adjunctive markers have been tested to improve the diagnostic accuracy for thyroid nodules. The common IHC markers galectin-3, Hector Battifora mesothelial-1, and cytokeratin-19 are used alone or as part of panels for both FNA and analysis of surgical specimens. A novel IHC marker, podoplanin, was recently introduced as an adjunctive marker for thyroid cancer diagnosis and prognosis and is associated with the progression of papillary thyroid carcinoma (PTC). Several researchers have identified molecular markers to increase the diagnostic accuracy of thyroid lesions of undetermined significance. Four promising molecular markers have been proposed and thoroughly investigated: B-type Raf kinase (BRAF) and RAS, rearranged in transformation/PTC (RET/PTC), paired box gene 8 (Pax8)/peroxisome proliferator-activated receptor gamma (PPARγ). BRAF mutations can be measured by immunohistochemistry using an antibody specific to the mutated protein. In this review, we focused on the limitations of current diagnostic tools and on determining the application of the above-mentioned markers to thyroid nodule diagnosis.

Key Words: Differentiated thyroid carcinoma, Immunohistochemistry, Molecular markers

Introduction

Thyroid cancers are rapidly increasing endocrine malignancies.1) Thyroid cancers account for 31.1% of women’s cancers and 6.4% of men’s cancers. It is estimated that 40,568 individuals (33,562 women and 7,006 men) were diagnosed with thyroid cancer in 2011. Of all thyroid cancers, differentiated thyroid carcinoma (DTC) is the most common type of follicular epithelial cell derived tumors.2,3) It accounts for about 90% of all thyroid cancers.4) Though DTC is famous for its propensity of hematic and lymphatic spread, its prognosis is excellent with the proper diagnosis and treatment. Basically, DTC diagnosis largely depends on microscopic examination by a pathologist, which is very important to initiate the appropriate treatment. Due to its good cost-effectiveness, fine-needle aspiration (FNA) is one of the first diagnostic procedure choices in the clinical management of thyroid nodules.5) Ultrasound (US) associated with FNA cytology is quite important to distinguish benign thyroid nodules from malignant nodules.6) The introduction of FNA in the diagnostic procedure increased the selection of adequate
patients for surgical treatment and allowed the prevention of unnecessary surgery, because the procedure allows about 65–80% of accurate diagnoses.

For the last decade, many studies were designed to improve FNA diagnostic accuracy. Many immunohistochemical (IHC) markers have been proposed, including galectin–3, Hector Battifora mesothelial–1 (HBME–1), cytokeratin–19 (CK–19), and novel molecular markers (i.e. B–type Raf kinase [BRAF]), rearranged in transformation/papillary thyroid carcinoma (RET/PTC), RAS, and paired box gene 8 (Pax8)/peroxisome proliferator–activated receptor gamma (PPARγ) to diagnose DTC. Data suggest that IHC markers improve the performance of FNA cytology in the diagnosis of patients with indeterminate cytology. Additionally, podoplanin, which is a proven prognosis factor in other cancers such as colorectal and breast cancer, has been studied in the prediction of lymph node metastasis in patients with DTC.

This review aimed at not only explaining the general characteristics of these numerous IHC and molecular markers in DTC diagnosis and prognosis but also discussing the individual efficacy of these markers for diagnosis.

**Immunohistochemical Markers**

Most thyroid cancers are easily diagnosed by using conventional histopathologic criteria, which include characteristic nuclear features such as irregular and enlarged nucleus, optical clearing, fine chromatin, elongation, overlapping, micronucleoli, nuclear grooves and pseudoinclusion. However, these cytology criteria are sometimes not enough to distinguish thyroid gland malignancies from benign lesions. Thus, IHC and molecular markers have been employed to improve the diagnostic accuracy of FNA cytology. A recent systematic review, analyzing cumulative data, reported that the combination of IHC positivity for galectin–3, HBME–1 and CK–19 as well as galectin–3 preoperative expression proved to be very sensitive tests to distinguish benign lesions from differentiated thyroid carcinoma. Another review of the literature also supported the use of ancillary techniques, involving a panel of antibodies for IHC as well as molecular analysis in the assessment of thyroid nodules combined with standard morphologic criteria.

**Galectin–3**

Galectin–3 is a subtype of β–galactoside–binding lectins identified in the cytoplasm and nucleus. It plays an important role in cell–cell and cell–matrix interaction, cell growth, malignant transformation, and apoptosis. It is expressed by human macrophages and neutrophils, mast cells, and Langerhans cells. It is also known to be associated with inflammation, cell damage repair, and metastasis.

Many studies reported galectin–3 overexpression in...
malignant tumors. In a recent study, galectin–3 was depicted as the most accurate marker for DTC diagnosis when compared with other molecular markers. The reported frequencies of galectin–3 expression in DTC are presented in Table 1.

Galectin–3 staining shows a strong diffuse cytoplasmic staining in most cases of PTC, including the conventional and follicular variant types (Fig. 1A). Galectin–3 is observed in 64–100% of patients with conventional PTC and 44–100% of patients with follicular thyroid carcinoma (FTC) (Fig. 1B). Galectin–3 is less frequently detected in follicular adenomas (0–37.5%; mean, 21.84%, Fig. 1C). Hyperplastic nodules, nodular goiters, and normal follicular epithelium usually do not express galectin–3. In a large multicenter study comprising 226 preoperative FNA specimens of thyroid nodules (188 benign lesions and 34 carcinomas), galectin–3 immunodetection sensitivity, specificity, positive predictive value and diagnostic accuracy were 100%, 98%, 92%, and 99%, respectively. In contrast, a relatively lower specificity was estimated in smaller series due to the considerable expression of galectin–3 in follicular adenoma (11 out of 44 cases, 25%).

These studies imply that galectin–3 is a useful tool for DTC diagnosis. However, it should not be used as a single diagnostic marker. Clinicians have to be cautious when interpreting positive results in the absence of DTC definite morphologic features, due to the probability of false positive. Similarly, caution should be taken when evaluating unconventional forms of PTC.

**HBME–1**

HBME–1 is a monoclonal antibody reacting against unknown antigens existing on mesothelial cell sur-

![Fig. 1. Galectin–3 immunostaining. (A) Galectin–3 is diffusely expressed in papillary thyroid carcinoma, it is localized in both the cytoplasm and nuclei (×400). (B) Focal expression of galectin–3 is detected in follicular thyroid carcinoma (×200). (C) Galectin–3 is not detected in follicular adenoma (×200).](image-url)
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Originally, HBME-1 was commonly used in differential diagnosis of mesothelioma and adenocarcinoma. Several investigators reported HBME-1 as a useful diagnostic marker to differentiate thyroid cancers, especially DTC from other benign thyroid lesions like nodular hyperplasia and follicular adenoma. HBME-1 is highly expressed in both PTC and FTC, but weakly expressed in MTC and anaplastic thyroid carcinoma (ATC). It is involved in cancer cell proliferation and migration.

Table 2. HBME-1 immunohistochemical detection in thyroid surgical specimens*

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>NH</th>
<th>FA</th>
<th>PTC</th>
<th>FTC</th>
<th>PDTC</th>
<th>ATC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prasad et al.</td>
<td>0/59 (0)</td>
<td>1/29 (3)</td>
<td>2/21 (10)</td>
<td>57/67 (85)</td>
<td>3/6 (50)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cheung et al.</td>
<td>0/35 (0)</td>
<td>0/35 (0)</td>
<td>76/138 (55)</td>
<td>2/4 (50)</td>
<td>4/6 (67)</td>
<td>1/2 (50)</td>
<td>-</td>
</tr>
<tr>
<td>Mase et al.</td>
<td>8/62 (12)</td>
<td>17/62 (27)</td>
<td>35/36 (97.2)</td>
<td>33/39 (84.6)</td>
<td>-</td>
<td>0/2 (0)</td>
<td>-</td>
</tr>
<tr>
<td>Choi et al.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>65/67 (97)</td>
<td>30/30 (100)</td>
<td>11/12 (91.6)</td>
<td>2/10 (20)</td>
</tr>
<tr>
<td>Papotti et al.</td>
<td>-</td>
<td>-</td>
<td>1/15 (6.6)</td>
<td>14/14 (100)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saggiorato et al.</td>
<td>-</td>
<td>-</td>
<td>2/50 (4)</td>
<td>37/39 (94.8)</td>
<td>17/19 (89.5)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nikiforova et al.</td>
<td>-</td>
<td>-</td>
<td>3/23 (13)</td>
<td>-</td>
<td>11/33 (34)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Park et al.</td>
<td>0/54 (20.4)</td>
<td>17/35 (49)</td>
<td>166/181 (92)</td>
<td>22/25 (88)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Values are number/total number (percent). Hyphen indicate not determined in this study.

Fig. 2. HBME-1 immunostaining. (A) Papillary thyroid carcinoma exhibits a strong membranous staining (×200). (B) Follicular thyroid carcinoma exhibits a diffuse cytoplasmic staining (×200). (C) Follicular adenoma shows focal expression of HBME-1 (×200).
In PTC, most cases show diffuse positive staining for HBME-1 (55–100%; mean, 88%). In FTCs, HBME-1 detection showed a high variability between studies (50–100%; mean, 75%). A few studies indicated HBME-1 staining in follicular patterned tumors of uncertain malignant potential that had either questionable vascular/capsular invasion or incomplete nuclear features of PTCs (29–66% of cases). Many studies reported the absence of HBME-1 in normal and hyperplastic thyroid. However, HBME-1 has been detected in cases of adenomatous goiter (3–12%) and follicular adenomas (0–27%). HBME-1 sensitivity, specificity, positive predictive value, and diagnostic accuracy in distinguishing benign from malignant lesions as a single marker are 80%, 96%, 96.7%, and 86.4%, respectively. The results of reported HBME-1 staining in thyroid nodules by IHC are presented in Table 2. Various HBME-1 staining patterns in either benign or malignant thyroid nodules are shown in Fig. 2.

A meta-analysis of IHC markers, with a special focus on HBME-1, comprising 3900 samples reported that the diagnostic odds ratio (dOR) of HBME-1 (40.97) was higher than that of galectin-3 (23.41) and CK-19 (14.73). dOR was estimated using sensitivity and specificity values directly. This measurement shows the overall diagnostic power of each individual marker (a high dOR implies that the test presents a good diagnostic power).

HBME-1 positivity has been considered as a useful adjunctive marker in the discrimination of thyroid lesions using FNA cytology, due to its high sensitivity and specificity. However, its prognostic value is still in doubt and further studies are needed.

Fig. 3. CK-19 immunostaining. (A) Papillary thyroid carcinoma exhibits a diffuse expression of CK-19 (X200). (B) Focal expression of CK19 is detected in follicular thyroid carcinoma (X200). (C) CK-19 is not detected in follicular adenoma (X200).
CK-19

CK-19 belongs to the keratin (CK) family (48–60 kDa). It is expressed in simple or glandular epithelium like the thyroid gland. Many studies have been designed using various antibodies against CKs to identify the most effective patterns in normal parenchyma, benign nodules, and malignant tumors. Among all CKs, CK-19 has become one of the most useful markers to diagnose thyroid nodule lesions. In fact, CK-19 detection in follicular adenomas and other benign nodules is less intense and more focal than in DTC (Fig. 3). Many authors stress the importance of the distribution pattern and intensity of CK-19 staining as the most crucial factors for an accurate interpretation. Its intense immunoreactivity might help diagnose DTC using IHC. CK-19 reported sensitivity and specificity when used as a single marker are as high as 92% and 97%, respectively. CK-19 can be a crucial marker along with a panel of other markers due to its high expression in DTC. However, CK-19 staining of cytologic specimens should be interpreted with great caution because of its well-known reactivity in atypia and chronic lymphocytic thyroiditis that can be observed in PTCs.

BRAF

The V600E mutation of the BRAF gene is a well-known genetic event in PTC and thought to be involved in the development and progression of malignancy. Various methods have been reported for BRAF mutation analysis with different accuracies, including gene sequencing (Sanger sequencing, pyrosequencing) and polymerase chain reaction (PCR). However, these methods are somewhat complex and expensive and often complicated by inadequate DNA preservation in formalin-fixed and paraffin-embedded tissues and by the presence of reactive thyroid parenchyma. In a recent study, in which 144 patients with PTC were enrolled, the expression of the mutated BRAF V600E protein was evaluated with a novel mutation specific antibody, called clone VE1, using IHC. They reported that 76 cases (52.8%) showed definite diffuse cytoplasmic expression of the mutated protein and these selected cases were confirmed by sequencing analysis. All patients presented the BRAF T1799A point mutation. The authors concluded that BRAF antibody may be a promising diagnostic marker alone or as part of a panel of markers. However, BRAF mutation–specific antibody positive staining did not present a high sensitivity in this study. Additionally, BRAF mutation–specific antibody positive staining can warrant further gene mutation study. The BRAF antibody staining patterns are presented in Fig. 4.

Podoplanin

Podoplanin, a mucin–type transmembrane glycoprotein specific to the lymphatic channel is expressed in various human cancers, including colorectal, breast, and pancreas cancer, and is regarded as a factor promoting tumor progression. Its expression by cancer–associated fibroblasts is known to be an indicator of poor prognosis in some types of cancer. Though podoplanin biological functions are not yet completely understood, the available data suggest that it can play an important role in tumor cell invasion, using IHC, RT–PCR, and western blotting in cancer cell lines, reported that podoplanin
Molecular Markers

It is well recognized that IHC assessment of thyroid nodules presents few limitations, including a large spectrum of sensitivity and specificity, inconsistency in the method, and various scoring systems. These limitations prompted us to investigate the diagnostic efficacy of molecular markers. To date, the three largest reported studies are: Nikiforov and colleagues’ study (513 indeterminate nodules), which used BRAF V600E (termed BRAF mutation in this review) and RAS mutation genetic tests as well as RET–PTC and PAX8/PPARγ rearrangements; Bartolazzi and colleagues’ study (432 indeterminate nodules), which used galectin–3 IHC; and Alexander and colleagues’ study (265 indeterminate nodules), which used the 142 gene expression classifier.

In this review, we try to focus on the recent reports on the above-mentioned molecular markers, BRAF and RAS mutations, as well as RET–PTC and PAX8/PPARγ rearrangements. Table 3 summarized DTC diagnosis efficacy of these four molecular markers in FNA specimens.

**BRAF Mutation**

BRAF is a member of the mitogen–activated protein kinase (MAPK) pathway that is associated with cell proliferation, cell differentiation, and apoptosis. Though many different BRAF mutations have been involved in the pathogenesis of several malignancies, a specific activating mutation resulting from the substitution of a glutamic acid for a valine at position 600 (V600E) has been observed in up to 50% of classic PTCs and 25% of its follicular variant (FVPTC).

However, it was reported to be positive in only 16% of thyroid cancers that presented an indeterminate or suspicious FNA cytology and PTC or FVPTC histology. The prevalence of the BRAF mutation in follicular thyroid carcinoma is just around 1%. Thus, diagnostic testing for this mutation cannot serve as an independent cancer diagnostic marker in evaluation of indeterminate nodules. Nevertheless, it is promising as part of a panel. Despite a low sensitivity, BRAF mutation specificity is quite high with a false positive rate of only 0.2%.

A more recent meta-analysis, summarizing the results of 6 studies and including 2800 malignant and benign lesions, reported a BRAF mutation specificity of 97.9% and a positive predictive value of 99.9%. Only seven false positive results were identified in three different studies.

In term of prognostic value, a multicenter study showed a strong association of BRAF mutation with lymph node metastasis, extrathyroidal extension, manifestation in advanced disease (stages III and IV), and recurrence. Additionally, BRAF mutation has previously been associated with the loss of radioiodine avidity of recurrent papillary thyroid cancer, making the disease refractory to radioiodine treatment. Many studies indicated an association between BRAF mutation and decreased or negative expression of thyroid iodide-handling genes, including SLC5A5 (also known as NIS), thyroid stimulating hormone receptor (TSHR), SLC26A4 (also known as pendrin), TPO, and TG. BRAF mutation has also been shown to lead to the overexpression of many tumor-promoting molecules such as vascular endothelial growth factor.

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**Table 3.** Molecular markers used in the preoperative evaluation of thyroid nodule FNA. From Rodrigues et al.

<table>
<thead>
<tr>
<th></th>
<th>Average SN</th>
<th>Average SP</th>
<th>Average PPV</th>
<th>Average NPV</th>
<th>Average AC</th>
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</thead>
<tbody>
<tr>
<td>BRAF mutations</td>
<td>52.35</td>
<td>97.92</td>
<td>99.85</td>
<td>51.62</td>
<td>70.54</td>
</tr>
<tr>
<td>RAS Mutations</td>
<td>23.00</td>
<td>97.20</td>
<td>82.20</td>
<td>63.20</td>
<td>65.00</td>
</tr>
<tr>
<td>RET rearrangements</td>
<td>18.20</td>
<td>88.72</td>
<td>87.00</td>
<td>59.60</td>
<td>55.30</td>
</tr>
<tr>
<td>PAX8/PPARγ rearrangements</td>
<td>20.00</td>
<td>100.00</td>
<td>100.00</td>
<td>46.00</td>
<td>60.00</td>
</tr>
</tbody>
</table>

AC: accuracy, NPV: negative predictive value, PPV: positive predictive value, SN: sensitivity, SP: specificity
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(VEGF) and MET.\(^5^9,6^0\) These results provide the molecular basis for the aggressiveness and treatment failure of papillary thyroid cancer in association with \(BRAF\) mutation\(^6^1\) and also suggest the strong association of \(BRAF\) mutation with papillary thyroid cancer mortality in an international multicenter study.\(^6^2\)

**RET/PTC Rearrangement**

The most common types of chromosomal rearrangement in thyroid cancer are the intrachromosomal rearrangement like RET/PTC1 and RET/PTC3.\(^5^5\) RET/PTC rearrangements are also known to activate both the MAPK and phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) pathways and are responsible for thyroid dedifferentiation.\(^6^3\) RET/PTC rearrangements can be useful thyroid cancer diagnostic markers. The preoperative diagnosis of thyroid nodules might be more accurate through the detection of RET/PTC in thyroid FNA specimens, in particular in those that present indeterminate cytology or don’t have a sufficient amount of cells for cytologic assessment.\(^6^4,6^5\) However, RET/PTC rearrangements have also been detected in follicular adenoma and other benign lesions at modest rates that limit their utility as single diagnostic markers.\(^6^6\) A recent meta-analysis indicates that RET/PTC specificity average value is 18% and its positive predictive value is 87%.\(^1^1\) RET/PTC rearrangements are relatively rare compared to \(BRAF\) or \(RAS\) mutation. It is important not to overestimate the value of RET/PTC gene rearrangements as a single marker and caution should be taken for thyroid cancer diagnosis.

**RAS Mutation**

The RAS oncogene family consists of 3 genes (\(HRAS\), \(KRAS\), and \(NRAS\)) that encode small GTPase proteins members of signal transduction.\(^4^6\) Activating mutations in these genes stimulate the MAPK and PI3K/Akt pathways regulating cell growth, proliferation, differentiation, mobility, and mortality.\(^5^7\)

This mutation may be detected in up to 40% of DTCs, with a predominant distribution among FTCs and FVPTCs.\(^5^1\) \(RAS\) mutation is considered to be important for the detection of FVPTC, which are difficult to diagnose by FNA cytology.\(^1^1\) While there might be some false positive nodules of benign follicular adenomas, it seems that \(RAS\)-positive follicular adenomas are precursor lesions for \(RAS\)-positive FTCs or FVPTCs and \(RAS\) mutation obviously predisposes the well-differentiated cancer to dedifferentiation, leading to a more aggressive cancer.\(^6^6\)

\(RAS\) mutation is not sensitive and specific enough to serve as a single marker for prediction of benign or malignant nodules. Therefore, \(RAS\) mutation results must be interpreted with caution. However, \(RAS\) mutational study could prove to be a very powerful adjunctive tool to cytology, as many cytologically indeterminate aspirates harbor \(RAS\) mutations, suggesting the presence of cancer.\(^4^6\)

**PAX8/PPARγ Rearrangement**

Rearrangements between \(PAX8\) and \(PPARγ\) genes, \(PAX8/PPARγ\) rearrangements, a kind of fusion oncogene, are mostly found in follicular tumors (30–40% of FTCs and 2–10% of follicular adenomas) and are rare in non-classical PTC, especially in FVPTC (<5%).\(^6^9\) Though the mechanism by which this gene rearrangement influences malignant transformation is not fully understood, it is assumed to be associated with inhibition of the antiproliferative property of the PPARγ receptor.\(^4^6\) Many investigators suggested that such oncogenic activation subsequently causes the affected nodule to show a malignant behavior. Thus, such lesions should be treated as precancerous.\(^4^6\) However, Sahin et al.\(^7^0\) reported that tumors with \(PAX8/PPARγ\) rearrangements usually carry a favorable prognosis.

**Conclusion**

The accurate diagnosis of thyroid nodule is the cornerstone for the determination of the appropriate treatment for patients. It is very important in many clinical aspects, such as operability, complication rate related
to surgery, prognosis, and patient quality of life. Even though histopathology and FNA cytology are commonly used to diagnose thyroid nodule, they present limitations, which have been summarized in previous studies. Thus, IHC and molecular markers can play a key role in the proper management of thyroid cancer. These modalities combined with conventional diagnostic tools will improve thyroid cancer diagnostic accuracy. Furthermore, testing FNA samples using panels of markers, including several IHC and molecular markers, may allow us to increase the diagnostic accuracy. Today, the cost of these tests continues to drop, which will make the use of IHC and molecular markers easier in a close future. Though they have some limitations such as uneven cost in many different countries, inconsistent sensitivity and specificity, large variation in performance, cost-effectiveness of IHC and molecular markers has been proven in recent study. They have shown a favorable profile, despite these limitations, and IHC is usually cheaper than molecular and other genomic tests. It might be more useful in low-income countries. Besides the diagnostic utility of molecular markers, the elucidation of thyroid cancer molecular basis will improve the therapeutic outcomes.

Conflict of Interest
The authors have no conflict of interest to declare.

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