The Diagnostic Utility of CyclinD1, Galactin3, and CK19 in Differentiation between Benign and Malignant Thyroid Nodules: A Retrospective Study

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The diagnostic utility of cyclinD1, galactin3, and CK19 in differentiation between benign and malignant thyroid nodules: a retrospective study
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\textbf{Background and objective} Thyroid nodule management depends mainly on fine needle aspiration cytology to bear out its nature. Yet, in particular cases, differentiation between benign and malignant thyroid nodules represents a diagnostic problem. Our aim was to investigate the diagnostic utility of cyclinD1, galactin3, and CK19 immunostains in resolving this issue, as the financial and emotional load of malignancy management is particularly considerable.

\textbf{Materials and methods} Our study included 60 patients presented with nodular thyroid enlargement. After surgery, 30 cases were diagnosed histologically as follicular adenomas and 30 cases were diagnosed as papillary thyroid carcinomas. Immunohistochemical study of the aforementioned markers was performed in both groups. The best cut-off level and the diagnostic performance of single or combinations of these immunohistochemical markers were calculated by using the receiver operating characteristic curve analysis.

\textbf{Results} Signal intensities for galactin3, CK19, and cyclinD1 were significantly greater in papillary thyroid carcinoma compared with follicular adenomas. The combination of K19/cyclinD1 exhibited the highest performance to assess the diagnosis of malignancy with overall diagnostic accuracy was 99.2%, with 90.3% sensitivity, 100% specificity, 100% positive predictive value, and 90.8% negative predictive value.

\textbf{Conclusion and recommendation} Our findings advocate that a combination of K19/cyclinD1 markers may increase the reliability of identification of thyroid malignancy. We recommend more studies on cyclinD1 expression in wider thyroid lesions and more precise statistical results with a higher number of cases. \textit{Egypt J Pathol} 38:1–5 © 2018 Egyptian Journal of Pathology.

Keywords: cyclinD1, cytokeratin19, follicular adenoma, papillary thyroid carcinoma

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\textbf{Introduction}

Although nodular thyroid disease has a common prevalence, which varies according to the detection method (2–6% by palpation, 19–35% by ultrasound, and 8–65% in autopsy data) (Dean and Gharib, 2008), the thyroid cancer incidence is rare (1% of new cancer diagnosed each year), yet this incidence has been increasing since the past 40 years worldwide (Lim et al., 2017). In Egypt, thyroid cancer represents ~1.5% of all cancers as well as ~30% of endocrine malignancies, with about threefold incidence predominance in females (Ahmed and Aboelnaga, 2015).

Preoperative assessment of nodular thyroid disease hangs on fine needle aspiration cytology, mainly in which, about 10–20% of the results will be uncertain. Numerous studies have estimated the diagnostic performance of many immunohistochemical markers in differentiation between benign and malignant thyroid lesions such as galactin1, galactin3, cytokeratin19 (CK19), Hercept Brevi fora Mesotheliat-1 (HBME-1), and cyclinD1. In spite of these markers having been detected mainly in malignant lesions, they were also shown to have a noticeable expression in benign thyroid lesions (Flanagan, et al., 2008; Monika et al., 2016). Therefore, a new immunohistochemical marker or a combination of markers is vital to discriminate benign from malignant thyroid lesions. Owing to the previously mentioned data, our study was designed to investigate the diagnostic utility of cyclinD1, CK19, and galactin3, individually or in a combination, in trying to resolve this diagnostic dilemma.

\textbf{Materials and methods}

\textbf{Patient selection}

This study was implemented in the Department of Pathology at Banha University and Department of Pathology at Assiut University from January 2015 to January 2017, after approval of the study protocol by the local ethical committee. We retrospectively went through the histopathology database for collecting cases of nodular thyroid lesions that underwent total thyroidectomy or lobectomy between January 2010 and January 2015. We gathered the patient demographic data comprising age, sex, and the type of operation from patient medical files.

\textbf{Tissue specimens}

Tissue blocks were sectioned at 4–5 μm and stained with routine hematoxylin and eosin stain to settle the reported histological diagnosis and grading. Sections suitable for immunostaining were selected.

\textbf{Immunohistochemistry}

In the current study, we implemented three immunohistochemistry (IHC) markers cyclinD1, galactin3, and CK19 on selected tissue sections of 30 cases of follicular adenoma (FA) and 30 cases of papillary thyroid carcinoma (PTC).
IHC was proceeded in 4μm tissue sections and antigen retrieval with citrate buffer at pH 6 for 10 min. We waited up to 20 min for the slides to cool and then slides were washed properly with Tris Buffered Saline (TPS; Mix 6.1 g of Tris Base, 8.8 g of NaCl in 1000 ml of distilled water. Adjust the pH to 9.0 with concentrated HCl and then add 0.5 ml of Tween 20). Endogenous peroxidase activity was blocked with H2O2 followed by blocking of nonspecific antibody sites by biotin. The sections were incubated for 1h with a solution of the following antibodies: (a) anti-Ck19 Ab: mouse monoclonal Ab (ab7754; Abcam, Cambridge, Massachusetts, USA); (b) antigalactin3 Ab: mouse monoclonal Ab clone (ab2785; Abcam); and (c) anti-cyclinD1 monoclonal antibody (ab16663; Abcam). The concentration of the antibodies was 1/100 for Ck19 and cyclin D1 and 1/50 for galactin3.

After primary antibody incubation, the excess antibody was washed off with Tris Buffer Saline, followed by incubation with a biotinylated secondary antibody for 25 min, which reacts with the primary antibody. Then, the solution was incubated with enzyme-labeled streptavidin for 25 min using ABC Detection IICC Kit (ready to use, ab93697; Abcam). Diaminobenzidine (ab143166; Abcam), was used as the chromogen. Hematoxylin (ab143166; Abcam), was used as a nuclear counter stain. For every IHC run, negative and positive controls were used according to the manufacturer’s guidelines.

**Interpretation of immunohistochemical sections**

Each immunohistochemical marker was scored from 0 to 6 by the addition of the percentages of immunopositive cells (range: 0–3; 0 = 0, 0 ≤ 1–33, 2 = 34–66, and 3 = 67–100%) to the staining intensity (range: 0–3; 0 = none, 1 = low, 2 = moderate, and 3 = high). This score was determined after calculating the mean of the three fields of the lesion.

**Statistical analysis**

All calculations were achieved using the commercially accessible ‘SPSS version 16’ statistical software (SPSS Inc., Chicago, Illinois, USA). To perform statistical analysis, we separated the patients into two groups: FA group and PTC group. The diagnostic performance of single or combined immunomarkers and the documentation of the best cut-off points for the diagnosis of cancer were assessed using the receiver operating characteristic (ROC) curves including the estimation of the area under the ROC curve (AUC). The specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) for single and combination of several markers were evaluated from cross-tabs based on the cut-off points. The significance was calculated using the χ2-test. P value less than 0.05 was reflected as significant.

**Results**

This study included 63% (n = 38) women and 36.7% (n = 22) men. The mean age of patients with FA was 45 years (range: 15–76 years) and 41 years for patients with PC (range: 11–73 years).

Although in PCs, Ck19 expression was cytoplasmic with increased intensity at apical membrane (Fig. 1b), in FA, absent to low expression was seen (Fig. 1a). For galactin3, it had cytoplasmic with perinuclear condensation expression in the tumor cell and the stroma associated with cancers and adenomas (Fig. 1c and d). CyclinD1 signal was exclusively nuclear in 70–90% of malignant epithelial cells, whereas benign epithelial and stromal cells showed weak cytoplasmic expression (Fig. 1e and f).

The scores of cytoplasmic expression of galactin3 and CK19 and the nuclear expression of cyclinD1 were significantly higher in cancer cells of PTC compared with epithelial cells in FA (P < 0.001, χ2-test).

Statistical analysis for single and all probable combinations using the three markers to discriminate between FA and PTC is shown in Table 1. For each individual marker, the cut-off has been defined from the ROC curve. The cut-offs allowing to separate negative/low versus positive immunostaining were greater than 3.5 for galactin3, 4.5 for CK19, and greater than 0.5 for cyclinD1 (Table 1 and Graph 1). The diagnostic performance of the markers was individually evaluated by comparing the areas under the ROC. As described in Table 1, the AUC of cyclinD (AUC = 0.991) is greater than the AUCs of the CK19 (AUC = 0.930) and for galactin3 (AUC = 0.878). From cross-tab analyses, cyclinD1 appears to be the most sensitive markers (93.3%) and the most specific one (100%) (P < 0.001, χ2-test).

For markers combinations, the data revealed that the combination of K19/cyclinD1 exhibits the highest performance to assess the diagnosis of malignancy (AUC = 0.992, cut-off > 2.7) (Table 1 and Graph 2). The sensitivity, specificity, PPV, and NPV were also calculated for the panel of combined markers. Combinations of galactin3/CK19 markers, and CK19/cyclinD1 markers improve specificity (100 and 93.3%, respectively) and sensitivity (96.6 and 100%, respectively). However, based on statistical analysis, the combination of K19/cyclinD1 is the best one associating with higher overall diagnostic accuracy than that of K19/galactin3 (AUC = 0.992 vs. 0.958, respectively; see Table 1).

**Discussion**

Morphological pictures by conventional histology and fine needle aspiration cytology are the routine to reach the final diagnosis in thyroid nodules. In some challenging cases, the pathologists face difficulties to discriminate benign from malignant thyroid nodules (Arcolia et al., 2017).

This drives us to investigate galactin3, CK19, and cyclinD1 immunohistochemical markers to determine their efficiencies in the diagnoses of malignant thyroid lesions.

CK19 is a member of cytokeratin family essential for the structural integrity of the epithelium. Many authors have described strong and diffuse CK19 immunoreactivity in the PC versus absent to low immunoreactivity in benign thyroid lesions (Flanagan et al., 2008; Krzeslak et al., 2008; Liu et al., 2015). Galactin3 is a member of the galactin family (Kopitz et al., 2014; Ippel et al., 2016) and is linked to the pathogenesis of well-differentiated thyroid carcinoma.
Right column represents follicular adenoma (FA), whereas the left column represents papillary thyroid carcinoma (PTC). (a) FA negative for cytokeratin19 (ABC, ×400); (b) PC showing cytoplasmic and apical cytokeratin19 expression (ABC, ×400); (c) FA showed focal positivity for galactin3 immunostain (ABC, ×100); (d) PC showing diffuse cytoplasmic with perinuclear condensation galactin3 expression (ABC, ×400); (e) FA negative for cyclinD1 (ABC, ×200); and (f) PC showing nuclear cyclinD1 (ABC, ×400).

<table>
<thead>
<tr>
<th>Markers</th>
<th>AUC</th>
<th>Cut-off</th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK19</td>
<td>0.930</td>
<td>&gt;4.5</td>
<td>100</td>
<td>80</td>
<td>100</td>
<td>83.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gal3</td>
<td>0.878</td>
<td>&gt;3.5</td>
<td>90</td>
<td>70</td>
<td>87.5</td>
<td>75</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CyclinD1</td>
<td>0.891</td>
<td>&gt;5.0</td>
<td>100</td>
<td>93.3</td>
<td>100</td>
<td>93.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CyclinD1 and CK19</td>
<td>0.892</td>
<td>&gt;2.7</td>
<td>100</td>
<td>93.3</td>
<td>100</td>
<td>93.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CyclinD1 and Gal3</td>
<td>0.980</td>
<td>&gt;2.3</td>
<td>83.3</td>
<td>96.6</td>
<td>96.2</td>
<td>85.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CK19 and Gal3</td>
<td>0.986</td>
<td>&gt;3.8</td>
<td>100</td>
<td>96.6</td>
<td>100</td>
<td>96.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CyclinD1, CK19, and Gal3</td>
<td>0.986</td>
<td>&gt;2.5</td>
<td>83.4</td>
<td>93.4</td>
<td>93.4</td>
<td>93.4</td>
<td>&lt;0.001</td>
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AUC, area under the curve; CK19, cytokeratin 19; gal-3, galactin3; NPV, negative predictive value; PPV, positive predictive value.

(Gashari et al., 1999; Inohara et al., 1999; Bartolazzi et al., 2001; Carpi, et al., 2010; Liu et al., 2014; Sumana et al., 2015).

CyclinD1 is a protein product of the bcl-1 gene and is responsible for controlling cell cycle through regulating G1 to S transition in the cell cycle. Cyclin D1 accumulation leads to excessive proliferation of cells because of a G1 phase shortening (Sporny et al., 2005). Overexpression may be a result of the multiplying of the gene, viral insertions in the cyclin's gene area, translocation of particles of chromosomes, or owing to an increased sensitivity of malignant cells on an external
transcription is detected in malignant mantle-cell lymphomas (Musial et al., 2002). A specific translocation (t11:14) (q13.32) in the aforementioned lymphomas is accountable for an increased synthesis of cyclinD1, which leads to the connection of its gene to the gene of the heavy chain immunoglobulins. So an increased transcription and translation of cyclinD1 gene happens, which is accountable for an increased cyclinD1 expression in lymphoma cells (Sporny et al., 2005).

The value of the clinical use of these markers is controversial because positivity was also reported in benign cases, for example, in the study of Mataraci et al. (2012), CK19 expression was found in adenomatous nodular hyperplasia and FA and Mehrotra et al. (2004) showed that galactin3 was expressed in a large proportion of FAs, multinodular goiters, and Hashimoto's thyroiditis.

The main task of the present study is to find a new immunohistochemical marker that might be more helpful to sharpen up differential diagnosis between benign and malignant cases. CyclinD1 had been widely studied in several types of cancers and could be a good candidate to be tested in the thyroid pathology. One of the greatest challenges of the current study is the assessment of the diagnostic utility of cyclinD1 as a complementary biomarker in the differential diagnosis of thyroid nodular lesions. In this context, we studied the diagnostic performance of cyclinD1 individually and in combination with CK19 and galactin3 as markers to be used in clinical practice.

Our data showed that the expression of cyclinD1, galactin3, and CK19 was significantly higher in PTC than in FA. This is confirmed by the finding of Arcolia et al. (2017) for CK19 and galactin3 and the work by Sporny et al. (2005) on cyclinD1 who detected that the nuclear reaction of cyclinD1 was 100% in the malignant epithelium in contrast to 0% in the normal thyroid follicles. This goes along with the current study, as nuclear cyclinD1 was exclusively expressed in malignant epithelial cells.

As a single marker, cyclinD1 and CK19 displayed a higher specificity (100%) than galactin3 (90%). CyclinD1 showed the highest sensitivity (93.3%). Regarding the combination of cyclinD1 and CK19 to improve the discrimination between malignant and benign thyroid neoplasms, the specificity was 100%, sensitivity was 93.3%, PPV was 100%, NPV was 93.8%, and the overall diagnostic accuracy was 99.2%. So, when we combined the two markers, we made a slight improvement in the overall diagnostic accuracy for malignancy from 99.1, if we use cyclinD1 alone, to 99.2%, if combined with CK19. This association of positivity for cyclinD1 and CK19 proved to be the most relevant combination in the distinction between PTCs and FAs. Hence, our data advocate the concept of the use of combinations of immunomarkers in clinical practice to diagnose thyroid carcinomas.

Evidently, many studies advocate also a panel of markers that might be more helpful than the use of a single one to improve diagnostic accuracy, as provided in the coming lines.

In this context, de Matos et al. (2012) and Arcolia et al. (2017) recommend a combination of the immune signals stimulation, for example, hormones. CyclinD1 is submitted to amplification in neoplasms of a digestive system, parathyroids, breast, liver, larynx, head and neck, ovarian, lungs and bladder carcinomas. Increased
markers of galectin3, CK19, and HBME-1 with sensitivity of 95 and 85%, respectively, and specificity was 97% for both studies. Mataraci et al. (2012), also advised the use of the same combination, especially in case of overlapping between afofollicular variant of PTC and FA or follicular carcinoma.

Prasad et al. (2005) found a combination of fibronectin-1, galectin3, and HBME-1 to be useful in the diagnosis of thyroid neoplasm. Dunderovic et al. (2015) concluded that galectin3 was the most sensitive marker to differentiate FA from follicular carcinoma, and found that CK19 and HBME-1 were more expressed in papillary carcinoma as compared with follicular carcinoma. Cheung et al. (2001), recommended a panel of CK19, HBME-1, and ret markers in the analysis of difficult thyroid nodules, explaining that focal CK19 expression could be seen in benign lesions, but diffuse positivity was found in papillary carcinoma, and HBME-1 expression specified malignancy but not papillary differentiation.

In summary, the diagnostic problems in thyroid pathology are still present in many laboratories, and this paper can be potentially useful for improving information.

Our recommendation is that such combination of markers should be validated in a larger series of tissues, including various subtypes of thyroid lesions.

Conflicts of interest
There are no conflicts of interest.

References


