THE VALIDITY OF IMMUNOCYTOCHEMISTRY IN DIFFERENTIATING BETWEEN BENIGN AND MALIGNANT THYROID NEOPLASMS PREOPERATIVELY USING FNAC
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ABSTRACT

BACKGROUND
Fine Needle Aspiration Cytology (FNAC) of thyroid neoplasms has its own pitfalls when used alone. This study was undertaken to evaluate whether overexpression of galectin-3 can make a presurgical diagnosis of malignant thyroid neoplasms on FNAC (by immunocytochemistry). TTF-1 was also included in the study to identify the thyroid origin of cells.

MATERIALS AND METHODS
The study sample included patients with solitary thyroid nodules who underwent FNAC and subsequent surgery at our hospital. The study design was based on diagnostic test evaluation. Immunocytochemistry was performed with galectin-3 and TTF-1 on fresh FNA smears by the standard protocol. Grading was done for galectin-3 positivity based on intensity of immunostaining from grade 1 to 3. Postoperative histological diagnosis represented the gold standard.

RESULTS
39 solitary thyroid nodules were studied. TTF-1 was positive in 37 out of 39 cases. Galectin-3 was positive in all malignant cases. It showed a sensitivity of 94% and a specificity of 91% in detecting malignancy. Also, grade 3 immunopositivity for Gal-3 was seen only in papillary carcinoma.

CONCLUSION
Galectin-3 expression is thus a reliable marker of malignancy (particularly in papillary carcinoma) and grading of Gal-3 immunostaining correlates with malignant transformation. The study also reiterates the efficacy of immunocytochemistry on cytological smears.

KEYWORDS
FNAC, Immunocytochemistry, Galectin-3, TTF-1, Thyroid Malignancies.

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BACKGROUND
The most common endocrine malignancy is carcinoma of the thyroid gland. Fine-Needle Aspiration Cytology (FNAC) is the most commonly used diagnostic modality for the preoperative assessment of these neoplasms. However, FNAC when used alone has its own pitfalls particularly in the diagnosis of papillary carcinoma. This study addresses the issue of whether preferential over expression of galectin-3 (Gal-3) in thyroid malignancies can serve to make a presurgical diagnosis of malignant thyroid neoplasms on FNAC as demonstrated by immunocytochemistry. Thyroid Transcription Factor-1 (TTF-1) was used to identify the thyroid origin of cells. The objective of our study was to estimate the sensitivity, specificity and predictive values of galectin-3 in detecting malignant thyroid neoplasms on FNAC smears.

The application of Immunocytochemistry (ICC) in the diagnostic workup of difficult cases has paralleled the progress seen in surgical pathology and has added a new dimension for cyologic diagnosis.

Cytomorphology forms the basis for determining the most appropriate technique to use to arrive at a precise diagnosis. The most important task for the cytopathologist is to examine conventional Romanowsky or Papanicolaou-stained smears carefully, so that a differential diagnosis and hence the appropriate question to be answered by immunocytochemistry is generated. The cytology specimens can be processed with air drying or immediate fixation in alcohol or they can be processed as cytcentrifuge or cell block preparations. Some of the important prerequisites for adequate ICC studies are a well-spread film of cells on a glass slide, adequate fixation, removal of blood and proteinaceous material and a sensitive, reproducible method of immunocytochemistry.

In a large thyroid cancer diagnostic marker panel study reported to date, it was found that Galectin-3 could be the most accurate stand-alone marker for differentiated thyroid cancer diagnosis when compared with a panel of 56 other...
molecular markers. According to this study, the most useful markers for differentiated thyroid cancer diagnosis were Gal-3, Cytokeratin 19, vascular endothelial growth factor, androgen receptor, p16, Aurora-A and Hector Battifora Mesothelial Antigen-1 (HBME-1).

Galectins are a large family of proteins that bind β-galactosides on cell glycoproteins and glycolipids. Gal-3 is a structurally unique 31-kDa member of the galectin family. Gal-3 has been identified in the nucleus, cytoplasm and extracellular space in humans. It is expressed by human macrophages, neutrophils, mast cells and Langerhans cells. It has a role in the regulation of apoptosis, cell motility and T-cell growth and has also been implicated in the progression of thyroid cancer. Overexpression of Gal-3 in thyroid follicular cells have been found to bring about changes in cellular phenotype including the development of anchorage-independent growth, increased proliferation and loss of contact inhibition suggesting that Gal-3 is a regulator of normal cell proliferation and that overexpression of Gal-3 results in malignant transformation and metastasis.

Gal-3 is predominantly identified in the nucleus and can be transported to the perinuclear and nuclear compartments. In mouse cells, phosphorylated Gal-3 has been identified in both the nucleus and cytoplasm, whereas the nonphosphorylated form remains exclusively within the nucleus. Cell proliferation has been associated with an increased fraction of the phosphorylated and thus the cytoplasmic form of Gal-3 in many studies. In the clinical setting, these studies suggest that it is the cytoplasmic expression of Gal-3 in thyroid tumours, rather than its nuclear expression, that would be of critical importance.

The thyroid phenotype is characterised by the expression of a variety of proteins that are specifically synthesised by the thyroid follicular cells. One such thyroid-specific nuclear protein is TTF-1. Because retained TTF-1 expression is highly specific for thyroid and lung tumours, it has been widely used to discern the primary site of tumour origin in patients with metastatic disease of unknown origin.

In case of thyroid neoplasms, nuclear reactivity for TTF-1 is present in follicular cell–derived benign and malignant lesions and medullary carcinomas. Poorly-differentiated carcinomas often show decreased and focal staining for TTF-1 and most anaplastic carcinomas lack TTF-1 reactivity. When used in combination with thyroglobulin, TTF-1 is an effective marker for thyroid origin.

AIM AND OBJECTIVES
Our objective was to estimate the efficacy parameters (sensitivity, specificity and predictive values) of using galectin-3 immunocytochemistry in detecting malignant thyroid neoplasms on FNAC smears.

MATERIALS AND METHODS
FNAs were sent for pathologic review. The cases were included if the FNAs were of intermediate cytology. The aim was to test the diagnostic potential of galectin-3 using immunohistochemical staining of the corresponding surgical specimens. Galectin-3 was performed on the FFPE tissue blocks using the standard tissue microarray technique. The slides were stained with a primary antibody against galectin-3 (Dako, clone 1A5, 1:50 dilution). The expression of galectin-3 was evaluated by two independent reviewers in a blinded manner. The slides were scored as 0 for negative, 1 for weak, 2 for moderate and 3 for strong nuclear and cytoplasmic expression. The scores were then evaluated as follows: 0–1 (negative), 2 (weak), 3–4 (moderate), 5–7 (strong).

Inclusion and Exclusion Criteria
The cytological diagnosis was made on Papanicolau-stained smears to make sure that the FNA was targeted at the lesion of interest. Immunocytochemistry for galectin-3 and TTF-1 was performed on unstained FNA smears by the following method. The slides were examined under the light microscope with the condenser lowered to check for adequate material and circled underneath in the cellular areas. If adequate material was present on a single slide, both the markers were added to the same slide. If not, two slides were used. The smears were hydrated through descending grades of alcohol and washed in two changes of distilled water. Antigen retrieval was done with EDTA antigen retrieval buffer solution at a pH of 9 for 15-20 minutes in Multi Epitope Retrieval System (MERS). Afterwards, the slides were washed in distilled water and TBS (tri-buffered saline) wash buffer. The endogenous peroxidase was then blocked using H2O2 solution for five minutes and the washing steps were repeated. The slides were then incubated with the primary antibody against thyroid transcription factor-1 and Galectin-3 (Pathnsitu) for 30 minutes in a moist chamber and washed. They were then treated with PolyExcel target binder reagent (Pathnsitu) for 15 minutes and washed again. Incubation with PolyExcel horseradish peroxidase (Pathnsitu) was done for 15 minutes and washed. Finally, the slides were treated with working DAB chromogen (one mL of DAB buffer mixed with one drop of DAB chromogen) for two to five minutes and washed in distilled water. Counterstaining was done with haematoxylin and the slides were dehydrated, cleared and mounted.

The slides were reviewed independently by two of the investigators who were blind to the histopathological and cytological diagnosis. Positive staining for TTF-1 was considered when any single epithelial cell showed nuclear immunostaining. Positive staining for galectin-3 was considered when any single epithelial cell showed cytoplasmic or nucleocytoplasmic immunostaining. Grading was done for galectin-3 positivity based on the intensity of staining from 1 to 3 (1-faint positivity, 3-intense positivity, 2-intermediate between 1 and 3) using macrophages as internal controls. The sensitivity, specificity, positive predictive value and negative predictive value of immunocytochemistry was estimated against the gold standard using appropriate statistical methods. Postoperative histological diagnosis was taken as the gold standard. The study had been approved by the Institutional Ethics Committee of our college.
RESULTS
A total of 256 patients with solitary thyroid nodules underwent FNAC at our Cytopathology Department. Of these, only 39 cases had adequate material for immunocytochemistry and subsequent histopathological follow-up. Of these, there were 18 cases of colloid nodules of which four cases showed hyperplastic changes, 15 cases of papillary carcinoma, two cases of thyroiditis, two cases of follicular adenoma, one case of follicular carcinoma minimally-invasive type and one case of carcinoma with thymus-like differentiation (castle) as evidenced by histological follow up. Among the 15 cases of papillary carcinoma, two were of the diffuse sclerosing variant and one was of the follicular variant. All age groups were more or less uniformly affected in papillary carcinoma with maximum cases in the 21-30 yrs. and above 60 yrs. group. There were 29 females and 10 males in the study population.

On immunocytochemistry, positivity for TTF-1 was seen as a uniform nuclear staining (Figure1). 37 out of 39 cases of solitary thyroid nodule were TTF-1 positive and 2/39 cases were negative for TTF-1, one - castle and the other - a colloid nodule. The diagnostic efficacy parameters of using TTF-1 as a marker of thyroid origin were evaluated using Epi Info (Table1).

The distribution of galectin-3 immunostaining is summarised in Figure2. Galectin-3 immunostaining was observed in 15/15 cases of papillary carcinoma (9 of them showing cytoplasmic positivity and 6 showing both nuclear and cytoplasmic positivity), one case each of follicular carcinoma (nucleocytoplasmic positivity), lymphocytic thyroiditis (nucleocytoplasmic positivity) and Hurthle cell nodule (cytoplasmic positivity). Among the negative cases, 3 cases showed nuclear positivity, which is nonspecific and hence not counted among the positive cases.

Macrophages were used as internal controls for grading from 1 to 3 (Figure3a). Among the 15 cases of papillary carcinoma, seven cases showed grade 3 positivity (Figure3b), four cases showed grade 2 (Figure3c) and the rest of the four cases showed grade 1 positivity (Figure3d). The one case of follicular carcinoma showed grade 2 positivity, whereas grade 1 positivity was seen in lymphocytic thyroiditis and Hurthle cell nodule. The diagnostic efficacy parameters of using galectin-3 as a marker of malignancy are summarised in Table2.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value (95% CI)</th>
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</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>0.94 (0.69-1.00)</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.91 (0.69-0.98)</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>0.89 (0.64-0.98)</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>0.95 (0.74-1.00)</td>
</tr>
<tr>
<td>Positive likelihood ratio</td>
<td>10.35 (2.75-39.02)</td>
</tr>
<tr>
<td>Negative likelihood ratio</td>
<td>0.06 (0.01-0.44)</td>
</tr>
<tr>
<td>Prevalence</td>
<td>0.44 (0.28-0.60)</td>
</tr>
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Table 1. TTF-1 Expression as a Marker of Thyroid Origin in FNAC-Diagnostic Efficacy Parameters
DISCUSSION
FNAC is a commonly used first-line investigation in the preoperative evaluation of solitary thyroid nodules. Since most of the malignant lesions of thyroid present as solitary nodules, it is important these preoperative investigations are as efficacious as possible. Accurate evaluation of thyroid lesions on FNAC not only helps in avoiding unnecessary surgical procedures, but also the morbidity associated with it such as removal of parathyroid glands, injury to the recurrent laryngeal nerve and the need for lifelong supplementation of thyroid hormones.

FNAC alone, without the aid of other ancillary techniques poses a challenge in certain situations. This includes inability to distinguish between follicular adenoma and follicular carcinoma since they appear similar cytologically. Sometimes, a benign colloid nodule with hyperplastic changes may be seen predominantly in microfollicular pattern leading to a mistaken diagnosis of a follicular neoplasm. Thus, FNAC sometimes fail to draw a line even between benign and malignant lesions of thyroid. In such cases, the surgeons usually perform more radical procedures to prevent under-treatment. Our study was undertaken as an effort to circumvent this problem.

In our study, FNAC was combined with immunocytochemistry to see if they overcome the drawbacks of using FNAC alone. Two markers, TTF-1 and Galectin-3 were used. The former was used as to make sure that the cells showing positivity for galectin-3 were indeed thyroid follicular cells and the latter as a marker of malignancy in thyroid FNAC.

A total of 39 cases of solitary thyroid nodules were studied of which 51% (20/39) were benign, 5% were thyroiditis (2/39) and 41% (16/39) cases were malignant. Altavilla et al studied FNAC of 2433 solitary thyroid nodules over 5 years and identified about 66.9% as benign, 10.76% thyroiditis and 1.3% as malignant. The increase in the number of malignant cases can be explained on the basis of aggressive surgical management of neoplastic especially malignant lesions. Females outnumber males in our study group in the ratio of 2.9:1 similar to established data.

On immunocytochemistry, TTF-1 showed characteristic nuclear immunopositivity in 37/39 cases. Two cases were negative, 1 case of carcinoma with thymus-like differentiation and 1 case of a colloid nodule. The former is truly negative for TTF-1 and the false negativity in the latter could be due to the masking of follicular cells by the thick colloid. This setback is unlikely to pose a problem in identifying malignant lesions since they show scant colloid on cytology. According to our study using TTF-1 as a marker of thyroid origin offers a sensitivity of 97%, specificity of 100%, positive predictive value of 100% and negative predictive value of 50%. The low-negative predictive value is due to the very low number of true negative cases in our study population. This re-establishes older studies, which claim that TTF-1 is a sensitive marker for thyroid neoplasms similar to Thyroglobulin (TG). Also, its nuclear expression facilitates easy interpretation.

Galectin-3 (Gal-3) is the prime marker used in our study as a marker to differentiate malignant from benign thyroid lesions. Gal-3 immunostaining can be seen in three patterns- nuclear, cytoplasmic and combined nucleocytoplasmic staining. Out of these, cytoplasmic and nucleocytoplasmic staining represent true malignant transformation, whereas nuclear staining of Gal-3 is nonspecific and can be seen in normal cells (hence taken as negative in our study). Accordingly, Gal-3 positivity was seen in 100% (16/16) cases of malignancy and 9% (2/22) cases of benign thyroid lesions. The malignant lesions positive for Gal-3 include 15 cases of papillary carcinoma and 1 case of follicular carcinoma. The benign Gal-3 positive cases include one case of Hurthle cell nodule and one case of lymphocytic thyroiditis. Studies have described Gal-3 positivity in Hurthle cells and in inflammatory states as in thyroiditis. Thus, according to our study, Galectin-3 as a marker of thyroid malignancy on FNAC smears showed a sensitivity of 94%, specificity of 91%, positive predictive value of 89% and a negative predictive value of 95%. During the last few years, several studies have been published on the diagnostic efficacy of Gal-3 immunocytochemistry, most of them done on cell blocks. According to one study, Gal-3 immunocytochemical staining had a sensitivity of 60%, specificity of 100%, positive predictive value of 100% and negative predictive value of 83.3% for thyroid malignancies. This study had made use of cell blocks. Another study by Raggio et al showed a sensitivity of 55%, specificity of 100%, negative predictive value of 70% and 78% diagnostic accuracy on FNAC smears. Studies have also been done showing that the intensity of Gal-3 expression was more in cytology smears compared to cell block sections. A pilot study by Lei Zhang also showed good correlation between immunohistochemical and immunocytochemical staining of Gal-3 in 49 out of 50 cases.

Grading of Gal-3 immunostaining was done retrospectively to see if they correlate with malignant transformation. All cases showing grade-3 positivity were cases of papillary carcinoma. The one case of follicular carcinoma showed grade-2 positivity, whereas the two benign cases that were Gal-3 positive showed grade-1 positivity (Figure 4). Thus, grading may correlate with malignancy. Similar grading studies have been done and
have obtained similar results. A study by Gianmario et al graded Gal-3 based on percentage of positively stained cells and came up with similar results.

The disadvantages of immunocytochemistry include insufficient aspirate, an aspirate from non-target areas, limited sample availability limiting marker panels, background staining in protein rich aspirates, false positivity due to endogenous biotin, etc. In addition to these, insufficient sample size and inability to study the effect of ICC on preceding FNAC diagnosis are the significant drawbacks of this study.

CONCLUSION

Galectin-3 expression is thus a reliable marker of malignancy in fine needle aspiration cytology material particularly in the confirmation of papillary carcinoma of thyroid and strong Gal-3 expression should therefore prompt immediate surgical removal. It is also probable that a much better efficacy could have been reached if a larger sample was studied. Care should also be taken to assess immunocytochemistry in the light of Pap-stained cytology smears to avoid misinterpreting positivity of macrophages and Hurthle cells. This study further demonstrates its efficacy when smears themselves were used rather than cell block sections.

REFERENCES