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ENDOCRINE METHODS AND TECHNIQUES

Galectin-3 and HBME-1 improve the accuracy of core biopsy in indeterminate thyroid nodules

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Abstract Core needle biopsy (CNB) has been recently described as an accurate second-line test in thyroid inconclusive cytology (FNA). Here we retrospectively investigated the potential improvement given by Galectin-3, Cytokeratin-19, and HBME-1 on the accuracy of CNB in thyroid nodules with prior indeterminate FNA report. The study included 74 nodules. At CNB diagnosis, 15 were cancers, 40 were benign, and 19 had uncertain/non-diagnostic CNB report. The above immunohistochemical (IHC) panel was analyzed in all cases. After surgery, 19 malignant and 55 benign lesions were found. All 15 cancers and all 40 benign nodules diagnosed at CNB were confirmed at final histology. Regarding the uncertain CNB group, 4 (21 %) were malignant and 15 (79 %) benign. When we considered all the series, the most accurate IHC combination was Galectin-3 plus HBME-1, while HBME-1 was the most sensitive marker in those nodules with uncertain CNB report. The combination of CNB plus IHC could indentify 19/19 cancers and 53/55 benign lesions. Sensitivity and specificity of CNB increased from 79 to 100 % and from 73 to 96 %, respectively, by adding IHC. CNB can diagnose the majority of thyroid nodules with previous indeterminate FNA cytology, while the accuracy of CNB is increased by adding Galectin-3, Cytokeratin-19, and HBME-1 panel. We suggest to adopt CNB as a second-line approach to indeterminate thyroid FNA, and apply IHC in those lesions with uncertain/non-diagnostic CNB report. This approach should improve the pre-surgical diagnosis of patients. These results should be confirmed in larger prospective series.

Keywords Thyroid · Nodule · Core needle biopsy (CNB) · Galectin-3

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Introduction

Cytologic examination by fine-needle aspiration (FNAC) is the main tool in evaluating both palpable and non-palpable thyroid nodules due to its high accuracy, reproducibility, and cost effectiveness [1, 2]. However, while FNAC is very reliable in the majority of cases, a significant rate (i.e., up to 20 %) of cytologies is read as indeterminate neoplasm and represents the gray zone of FNAC [1, 2]. In these lesions, cytologic evaluation cannot discriminate malignant from benign tumors and a diagnostic surgery is traditionally warranted. Nevertheless, because about four in five of these nodules is benign at diagnostic surgery, the pre-surgical identification of parameters associated with cancer is strongly required [3, 4].

Several papers reported that immunohistochemistry (IHC) for various markers should assess the risk of

malignancy in thyroid nodules. In particular, IHC for Galectin-3, Cytokeratin-19, and HBME-1 has been reported quite accurate to detect or exclude malignancy in nodules with prior indeterminate FNAC [5]. While Galectin-3 panel was applied on several cytologic preparations (i.e., cell block or liquid-based) from nodule's aspiration [6–8], these markers have not been investigated in thyroid microhistologic samples from core needle biopsy (CNB). By CNB samples, a large percentage of nodules that are read as indeterminate at FNAC may be re-assessed as diagnostic, with high tolerability and good comfort for patients [9]. Then, CNB has been recently described as an accurate complementary test in those thyroid lesions with prior not conclusive FNAC [10–14], and is included in current guidelines for those nodules with prior inadequate cytologic report [1]. To date, whether the Galectin-3 panel holds an improving role on the accuracy of CNB microhistologic specimens has not been described.

Here we aimed to retrospectively investigate the potential role of Galectin-3, Cytokeratin-19, and HBME-1 panel to increase the accuracy of CNB in diagnosing thyroid nodules previously classified as indeterminate neoplasm at FNAC.

Materials and methods

Patients

In the period from June 2012 to December 2013, a series of 85 thyroid nodules underwent CNB at Ospedale Israelitico of Rome following indeterminate FNAC report. All these lesions had been cytologically read by two expert cytopathologists (LG, AC) and classified according to the Italian consensus for thyroid cytology [15]. A part of these patients ($n = 10$) were lost at follow-up. Of the remaining cases, one (1.2 %) CNB sample was inadequate (that did not permit microhistologic examination nor immunohistochemistry application) and was excluded. Then, the final series of the study included 74 nodules (mean size 15.4 ± 8.6 mm, ranging from 4 to 48 mm) from 74 patients (61 females and 13 males, mean age 48 ± 14.5 years, range 19 to 83 years); of these, 15 were cancers at CNB, 19 had uncertain microhistology (i.e., uncertain follicular neoplasm), and 40 nodules had benign CNB report. Patients with cancer at CNB underwent surgery for malignancy; patients with uncertain CNB were operated upon to obtain a final histologic diagnosis; subjects with benign CNB were submitted to surgery because of goiter with moderate/severe compressive symptoms. Histologic follow-up was available in all cases and lesions were classified based on WHO system [16].

Written consent was obtained for all participants. The study was approved by Ethical Committee of Ospedale Israelitico, Rome (IT).

Core needle biopsy (CNB)

All CNB were performed at Ospedale Israelitico of Rome. As we previously described [11, 12, 17], CNB was performed using a 21-G Menghini cutting needle (Biomol, Hospital Service, Rome, IT) under ultrasound guide in freehand manner. The obtained core samples were fixed by buffered formalin 10 % immediately just following biopsy. No minor nor major complications with CNB were recorded.

Microhistology and immunohistochemistry

Formalin fixed tissue cores were automatically processed and embedded in paraffin. Serial four-micron sections were collected on polarized slides and stained with hematoxylin-eosin for morphologic evaluation. Microscopic diagnosis was reported as papillary thyroid cancer (PTC) when the typical features were present, as follicular hyperplasia when microfollicular pattern was seen next to non-neoplastic parenchyma, and as follicular neoplasm when microfollicular pattern was separated from non-neoplastic parenchyma by fibrous capsule [11]. As previously described [11], the core sample included nodular tissue, extranodular parenchyma, and nodule's capsule, when present. CNB specimens were reported as uncertain/indeterminate when microfollicular arrangement lacked of nuclear alteration and the samples did not allow defining the relationship between nodule and normal parenchyma [11].

Following microscopic diagnosis, for each case, additional sections were cut from the paraffin blocks (four micron), collected on polarized slides, and submitted for automatized immunohistochemistry with antibodies for Galectin-3 (clone 9C4), Cytokeratin-19 (clone A53-B/A2.36), and HBME-1 (clone HBME-1) revealed by peroxidase using biotin-free method (Thermo Fisher Scientific, CA, USA). Results were described as previously reported for immunohistochemical analysis: immunoreactivity was considered positive if >10 % of follicular epithelial cells stained [18]. The immunoreactivity was scored as negative, focally positive (less than 25 % of lesion cells), or diffusely positive (more than 25 % of lesion cells) [19]. All immunostained slides were blindly evaluated by two expert examiners (AC, ES), and discordant cases were mutually resolved.

Follow-up

Final postoperative histologic assessment was the gold standard in all 74 cases included in the study. All nodules were classified according to the most recent WHO criteria [16].

Statistical analysis

Sensitivity, specificity, positive (PPV), and negative (NPV) predictive values, and accuracy were obtained according to Galen & Gambino predictivity tests, using Graph Pad Prism (Graph Pad Software Inc., USA).

Results

At final histology, 19 cancers (18 follicular variant and one classic variant of PTC) and 55 benign lesions were found. In the cancer group, mean age was 43 ± 15 years and mean size was 14 ± 8 mm; in the benign group, 48 ± 14 years of patients' age and 16 ± 9 mm of lesions size were recorded.

Regarding CNB, all 15 PTC and 40 benign nodules diagnosed at microhistologic examination were histologically confirmed after surgery; of the uncertain CNB, 4/19 (21 %) were malignant (four cases of follicular variant of PTC) and the remaining 15/19 (79 %) were benign. Table 1 details clinical and pathological features of the 19 patients with uncertain CNB. In the 55 nodules with diagnostic microhistology, CNB had 100 % PPV and NPV; when we considered the indeterminate cases as negative, sensitivity and specificity of CNB achieved 79 and 73 %, respectively.

Immunohistochemical panel of Galectin-3, HBME-1, and Cytokeratin-19 was performed in all 74 cases. Of these, 19 positive Galectin-3, 23 positive HBME-1, and 49 positive Cytokeratin-19 cases were found. When considering the overall panel of these three markers, 17 cases were triple positive, 23 triple negative, and 34 had positive IHC results in various degrees. The higher sensitivity and NPV were found for HBME-1. The most accurate combination of IHC markers in predicting malignancy was Galectin-3 plus HBME-1 (Table 2). Focal and diffuse positivity of IHC markers in the 19 cancers was observed in 3 and 13 cases for Galectin-3, 4, and 15 cases for HBME-1, 2. and 16 cases for Cytokeratin-19, respectively. Figure 1 illustrates CNB samples with application of IHC.

When we retrospectively evaluated the potential improvement of CNB accuracy furnished by IHC examination, we found that sensitivity of CNB increased from 79 to 100 % and specificity changed from 73 to 96 %

Table 1 Clinical and pathological characteristics of the 19 patients with uncertain/indeterminate CNB report

Gender	Age	Size	Galectin-3	HBME-1	Cytokeratin-19	Final histology
F	43	6	FP	DP	DP	FV-PTC
F	35	7	DP	DP	DP	FV-PTC
F	57	6	N	FP	FP	FV-PTC
F	28	19	N	FP	FP	FV-PTC
F	40	15	FP	N	FP	FA
F	49	14	N	FP	FP	FA
F	40	16	N	N	FP	NH
F	38	16	N	N	FP	FA
M	45	30	N	N	DP	NH
F	72	11	N	N	N	HA
F	43	10	N	N	N	HA
F	61	25	N	N	N	FA
F	35	30	N	N	N	HA
F	72	32	N	N	N	NH-T
M	58	23	N	N	N	HA
M	40	48	N	N	N	NH
F	55	20	N	N	N	NH
F	45	18	N	N	N	NH-T
F	41	7	N	N	N	FA

FC focal positive, *DP* diffuse positive, *N* negative, *FV-PTC* follicular variant of papillary thyroid carcinoma, *FA* follicular adenoma, *NH* nodular hyperplasia, *HA* Hürthle adenoma, *NH-T* nodular hyperplasia with thyroiditis

Table 2 Accuracy of Galectin-3, HBME-1, and Cytokeratin-19, and combined CNB plus immunohistochemistry in all 74 thyroid nodules with previous indeterminate FNAC

	Sensitivity	Specificity	PPV	NPV
Galectin-3	84.2	94.5	84.2	94.5
HBME-1	100	92.7	82.6	100
Cytokeratin-19	94.7	43.6	36.7	96.0
Galectin-3 plus HBME-1	100	89.1	76	100
Galectin-3 plus Cytokeratin-19	94.7	56.4	42.8	96.9
HBME-1 plus Cytokeratin-19	100	58.2	45.2	100
Combination of CNB and IHC				
CNB plus Galectin-3	89.4	94.5	85.0	96.3
CNB plus HBME-1	100	92.7	82.6	100
CNB plus Cytokeratin-19	94.7	56.4	42.9	96.9
CNB plus Galectin-3/HBME-1	100	96.4	90.5	100

Sensitivity and positive predictive value (PPV) of immunohistochemical markers were obtained considering focal, poor, and diffuse positivity as a whole. Specificity and negative predictive value (NPV) were calculated considering the absolute negativity of the tests

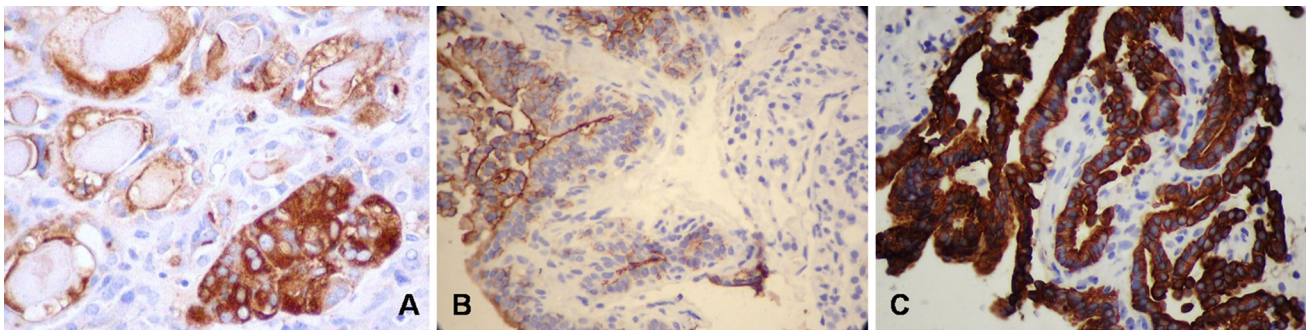

Fig. 1 Microscopic picture of CNB samples from papillary carcinoma with positive IHC: Galectin 3 displays positive cytoplasmic reaction of variable intensity and focal nuclear staining (**a**); HBME-1 shows positive cytoplasmic reaction with luminal enhancement (**b**); Cytokeratin-19 demonstrates strong positive cytoplasmic staining (**c**)

Table 3 Accuracy of IHC in detecting cancers and benignancies in 19 thyroid nodules with uncertain CNB report

	Sensitivity	Specificity	PPV	NPV
Galectin-3	50	93.3	66.7	88.2
HBME-1	100	93.3	75	93.3
Cytokeratin-19	100	66.7	44.4	100

The subgroup of 19 thyroid lesions with uncertain/non-conclusive CNB report included 4 cancers (all follicular variant PTC) and 15 benign nodules

(Table 2). The combination of microhistologic examination with the panel of Galectin-3 and HBME-1 could correctly indentify 19/19 cancers and 53/55 benign lesions. In particular, HBME-1 was the most sensitive and specific marker in discriminating cancers from benign nodules in those 19 nodules with uncertain CNB. The results of IHC markers in these lesions are shown in Table 3. Figure 2 shows main results recorded in the present study.

Discussion

The indeterminate neoplasms at FNA represent a major dilemma in thyroidology. In fact, while one in four nodules with indeterminate cytology is a cancer at histology, the pre-surgical diagnosis of these lesions is still a challenge [3, 4]. Several studies analyzing molecular tests on cytologic samples have been reported to improve the preoperative diagnosis of thyroid nodules. In particular, a novel gene-expression classifier analyzing 167 genes achieved very high NPV (i.e., 95 %) in this context [5]. These tests are gaining momentum in USA.

The IHC panel of Galectin-3, Cytokeratin-19, and HBME-1 has been largely investigated and the combined use of HBME-1 and Galectin-3 reached the highest specificity [6–8]. Galectin-3 is a component of the b-galactoside-binding lectins involved in the cell–cell and cell–matrix modulation and cell growth and differentiation; Cytokeratin-19 is a member of the family of keratins, which are intermediate filament proteins responsible for the

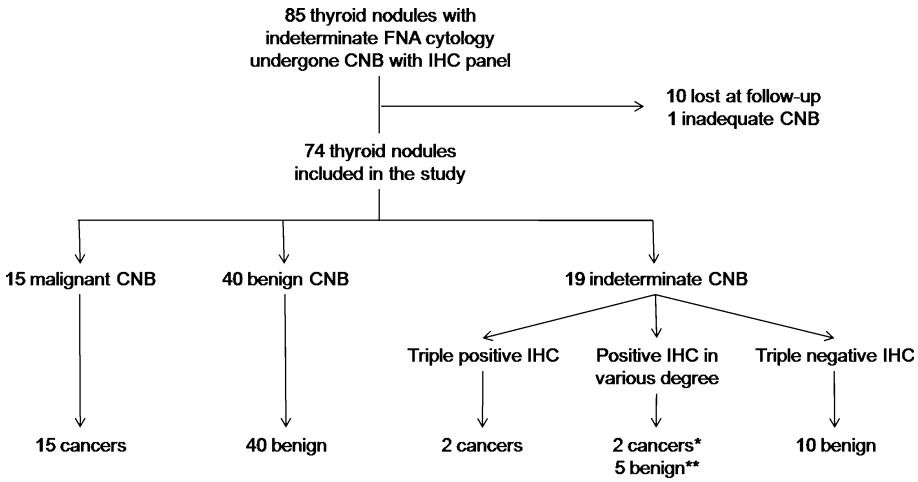

Fig. 2 Study design and main results. CNB, core needle biopsy; IHC, immunohistochemistry. *Both cancers showed focal positivity at HBME-1 and Cytokeratin-19. **The five benign nodules had focal positivity of HBME-1 in one case, poor positivity of Galectin-3 in another case, and poor or diffuse positivity of Cytokeratin-19 in all cases

structural integrity of epithelial cells; HBME-1 is an antibody against membrane antigens of the mesothelioma's cells surface. Interestingly, the initial studies reported a 100 % sensitivity and 94 % specificity of Galectin-3 expression in differentiating follicular carcinoma from follicular adenoma [20]. Unfortunately, the use of a single antibody of this IHC panel did not reach consistent diagnostic accuracy in indeterminate neoplasms because Galectin-3 positivity is found in some cases of goiters and thyroiditis, and in a non-negligible rate of follicular adenomas (i.e., up to 45 %) [21, 22]. In addition, the above mentioned cytologic preparations may allow poor material for ancillary and extensive studies such as IHC examinations [6–8].

In the last decade, CNB has been proposed to assess thyroid lesions with prior indeterminate FNAC [9]. The main results of these studies showed that a large rate of lesions cytologically classified as Thy 3, Class 3, or Category III-IV can be diagnosed as benign or malignant by CNB alone or combined with a second FNAC [10–14]. Nevertheless, no studies evaluating the potential improvement furnished by IHC panels applied to CNB have been performed. The core samples obtained by CNB should be the actual material for extensive studies [23]. In fact, microhistology can assess nuclear changes, architectural alterations of follicular structures, and relations with surrounding tissues. Also, CNB is able to determine whether the nodule capsule is present or lacking [11]. Moreover, the paraffin core sections represent the optimal substrates for ancillary techniques and automated immunostaining, and the feasibility of IHC is maintained with poor cellular or fibrous samples and in small cell aggregates [23, 24]. A core specimen has a diameter up to 500 μm permitting up to 100 immunostaining analyses. Of high relevance, we recently showed that BRAF-mutated PTC can be perfectly detected by IHC for the specific monoclonal antibody VE1 performed in these samples [25]. These data strongly encourage to use VE1 in thyroid CNB to detect BRAF-mutated PTC before surgery.

Here we aimed to investigate, in a series of 74 cytologically indeterminate nodules, the potential role of Galectin-3, Cytokeratin-19, and HBME-1 panel to improve CNB accuracy. In agreement with our previous experience and several other studies [10–14], CNB correctly diagnosed 74 % of nodules (15 malignant and 40 benign), while a minor rate of 26 % was inconclusive. Of the latter, other four cancers could be detected by IHC because they showed positivity of HBME-1 and Cytokeratin-19. The combined use of CNB-IHC diagnosed correctly 72/74 (97.3 %) lesions with prior indeterminate FNAC, being two benign nodules poorly or focally positive at Galectin-3 or HBME-1. All in all, the most accurate combination was CNB plus Galectin-3 and HBME-1. Of very high interest, the diagnostic surgery could have been avoided in those patients with benign nodule by this combination. This high NPV achieves high relevance in clinical practice because the chances of reducing unnecessary surgery are substantial [26]. Furthermore, a good prognosis of differentiated thyroid cancers with preoperative Thy 3 report has been recently shown by our group and another study [27, 28].

Our series was enrolled before 2014 and all FNAC were classified by Italian consensus which included one indeterminate class ("TIR 3") [15]. In 2014, this system was updated [29] and the indeterminate cytologies have been classified into two subcategories (i.e., "TIR 3A" and "TIR 3B") which are quite similar to the Bethesda classes III and IV [30]. When we reviewed our series according to this new system, we found a cancer rate of 12 % in TIR 3A and 42 % in TIR 3B; in these two subgroups, CNB alone showed a diagnostic accuracy of 76 and 57 %, while CNB plus Galectin-3/HBME-1 achieved an accuracy of 88 and 97 %, respectively. These findings advise for future prospective studies in these subclasses of indeterminate lesions of Italian reporting system [29].

Our data corroborate that CNB samples should be the actual specimens for IHC profiling. It has to be underlined that the use of Galectin-3, Cytokeratin-19, and HBME-1 panel in different cytologic preparations from conventional FNAC (i.e., cell block or liquid-based preparations) achieved lower diagnostic accuracy and it did not significantly improve the reliability of cytologic examination [6–8, 21, 22]. We reported that microhistologic samples have higher accuracy. Similar data were reported by Carpi et al. [31] which investigated Galectin-3 expression in a series of 12 cancers and 73 benignancies undergone thyroid "large needle aspiration biopsy" following indeterminate cytology. There, the biopsy sample was processed to obtain cell block preparation for IHC, and the combined morphologic-phenotypic examination had 92 % sensitivity, 97 % specificity, and 95 % accuracy [31]. It has to be emphasized that the herein described "thin" core biopsy (i.e., by 21-G needle) is less invasive and more comfortable than the "large" one (i.e., by 16 to 18-G needle).

Some potential limitations of the present study should to be briefly discussed. In fact, here we presented a retrospective study and high exclusion rate (11/85 nodules, 13 %) from the initial series was recorded. First, the rate of uncertain CNB reports is higher with respect to other studies [10, 13, 14]. Also, our cancer group included only PTC, while one would expect to find several other malignancies such as follicular thyroid cancers (FTC) as well. These two issues may be due to the above high exclusion rate, the overall lower prevalence of FTC [32], and other potential selection bias depending on the small sample of patients we enrolled. This should be taken into account in analyzing the present results.

CNB can diagnose the larger majority of thyroid nodules with previous indeterminate FNAC results, being inconclusive only one in four CNB reports. The use of IHC panel of Galectin-3 and HBME-1 in these microhistologic specimens improves the accuracy of CNB alone allowing diagnosis of almost all lesions. Based on these data, we encourage to adopt CNB as a second-line approach to thyroid nodules with indeterminate FNAC cytology, and to apply the above IHC panel in those lesions which remain uncertain at microhistologic CNB assessment. This approach should improve the pre-surgical diagnosis of thyroid indeterminate lesions and reduce unnecessary thyroid surgery. These results should be confirmed in larger prospective series.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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