Measurement of Thyroid Tumor Markers on Fine-Needle Washouts

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13.1 Introduction

Differentiated thyroid carcinomas (DTC), such as papillary (PTC) and follicular (FTC), produce thyroglobulin (Tg). Then, DTC patients can be followed up by periodic evaluation of serum Tg levels and examination by local and whole-body imaging [1]. A rate up to 50% of these patients has metastatic neck lymph nodes at their initial presentation and/or during postoperative follow-up [1]. While ultrasonography (US) can detect neck lesions suspicious for DTC metastases, fine-needle aspiration (FNA) under US guide is generally performed to prove the metastatic involvement of these lesions. This approach is essential to allow a tailored surgical excision [1]. Because FNA samples from neck lymph node may be not adequate, the measurement of Tg in the fluids from FNA (FNA-Tg) is essential in combination with cytology [2, 3]. Specifically, FNA-Tg achieves high relevance in those cases involving small and/or partially cystic lymph nodes.

Based on the experience on FNA-Tg, the determination of calcitonin (CT) in washout fluids (FNA-CT) from thyroid nodules suspected for medullary thyroid carcinoma (MTC) has been recently described [4]. This approach was based on the limits of conventional cytology in detecting MTC [5]. In fact, poor sensitivity of cytology (i.e., 55–65%) was recorded in single- and multicenter series [6–8], and FNA-CT can reduce false-negative and inconclusive cytologic results with a sensitivity near to 100% [4]. These data prompted the board of ATA guidelines to recommend the use of FNA-CT in patients suspected for MTC [9].

The treatment of choice of hyperfunctioning parathyroid (HP), such as adenomas, hyperplasia, or more rarely carcinomas, is represented by surgical removal. Then, their identification and localization are pivotal to better address the therapy. In this context, various potential limits of different imaging techniques (i.e., ultrasonography, scintigraphy, magnetic resonance) have been reported [10]. The determination of parathyroid hormone in FNA washout fluids (FNA-PTH) was proposed as an improving tool in localizing HP, with controversial results [11]. Generally, Tg, CT, and PTH are measured in blood, and their determination in fluids other than serum/plasma has been developed in the last years. Although studies have reported overall satisfactory results, a good standardization of procedures has not yet been reached, and further efforts should be made in order to better define pre-analytical, analytical, and post-analytical aspects.

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L. Giovanella (ed.), *Atlas of Thyroid and Neuroendocrine Tumor Markers*,
https://doi.org/10.1007/978-3-319-62506-5_13

13.2 FNA-Tg in Lymph Nodes Suspicious for Metastases from Differentiated Thyroid Carcinoma

The measurement of FNA-Tg in cervical lymph nodes/masses suspected to be metastases from DTC was firstly described in 1992 by Pacini et al. who demonstrated that the sensitivity of FNA-Tg was significantly higher than that of cytology (85%) [2]. Also, no FNA-Tg false-positive results were recorded. Later, several studies confirmed similar results, and a meta-analysis of 24 studies including 2865 lymph nodes reported overall sensitivity of 95% and specificity of 94% for FNA-Tg, with significant heterogeneity [12].

Even if many articles with concordant findings have published, a better standardization of analytical methods and cutoff levels is required. The cutoff of FNA-Tg to discriminate metastatic lymph nodes from negative ones has been largely debated; in the first studies, the threshold values ranged from 0.9 to 50 ng/mL [12]. The metastases diagnosed in the presence of thyroid gland and those detected in athyreotic patients were analyzed, and different FNA-Tg cutoffs were proposed in the presence (36 ng/mL) or absence (1.7 ng/mL) of thyroid [13, 14]. However, two different papers found a unique accurate threshold of 0.93 ng/punction [15] and 28.5 ng/mL [16, 17]. In general, both sensitivity and specificity of FNA-Tg were higher in surgically treated DTC than in those waiting for surgery [18, 19]. In this context, the use of high-sensitive Tg assays should provide more accurate data. Snozek and colleagues [20] measured FNA-Tg by an immunochemiluminometric assay (ICMA) with functional sensitivity of 0.1 ng/mL; 96% of non-malignant samples had values ≤1 ng/mL, and 100% of metastatic lesions had levels >1 ng/mL. Furthermore, using a high-sensitive immunoradiometric assay (IRMA) with functional sensitivity 0.2 ng/mL, a cutoff of 1.1 ng/mL provided 100% sensitivity, specificity, and accuracy [20]. These results were confirmed by others [14, 21–23]. As an ancillary information, a potential improvement of previous rhTSH was reported in patients with positive AbTg [24, 25]. Table 13.1 details the findings of main studies.

Based on the above demonstrations, FNA-Tg is included in all international guidelines. The ATA guidelines [1] quoted at Recommendation 32 that "US-guided FNA of sonographically suspicious lymph nodes > 8-10 mm in the smallest diameter should be performed to confirm malignancy if this would change management (strong recommendation, moderate-quality evidence); the addition of FNA-Tg washout in the evaluation of suspicious cervical lymph nodes is appropriate in select patients, but interpretation may be difficult in patients with an intact thyroid gland (weak recommendation, low-quality evidence)." The ATA board suggests FNA-Tg in lymph nodes with cystic changes, inadequate cytology, or cytologic/echographic divergences. Also, ATA underlines that false-positive FNA-Tg may occur in evaluating lymph nodes of central compartment of patients with thyroid. Finally, ATA guidelines highlight the lack of standardization of FNA-Tg procedures or assays with consequent potential difficult in interpreting data [1]. The guidelines by AACE/AME/ETA stated that "In the presence of

Table 13.1 Accuracy of FNA-Tg reported in the literature

First author (year)	Lymph nodes	Method	Cutoff (ng/mL)	Sensitivity	Specificity
Pacini (1992)	23	IRMA	21.7	100	100
Cunha (2007)	18	CLIA	0.9	100	100
Giovanella (2009)	126	IRMA	1.1	100	100
Kim (2009)	91	IRMA	50	100	80
Bournaud (2010)	98	IRMA	0.93	92.3	97.8
Salmashoglu (2011)	255	IRMA	28.5	100	96.4
Snozek (2007)	122	CLIA	1.0	100	96.2
Moon (2013)	528	RIA	1.0	93.2	95.9

IRMA immunoradiometric assay, *CLIA* chemiluminometric immunoassay, *RIA* radioimmunoassay

suspicious cervical lymphadenopathy, FNA biopsy of both lymph node and suspicious nodule(s) is essential (Grade B)" [26]. These guidelines suggest to wash the needle in 1 ml of saline solution, do not indicate a specific cutoff, and underline that in athyreotic DTC patients "the detection of even low thyroglobulin levels by UGFNA should be considered suspicious for malignancy." Guidelines for neck US and US-guided techniques for the management of DTC patient after treatment were published by ETA [27]; there, ETA suggests to report the results of FNA-Tg as "ng/FNA" ("a more suitable result which reflects the quantity of Tg in the needle") and propose to adopt value <1 ng/FNA as normal, values between 1 and 10 ng/FNA to be compared with the results from cytology, and levels >10 ng/FNA as positive for the presence of tumor tissue. Also, at Recommendation 12, they quoted that FNA cytology and FNA-Tg should take into account the stage and histology of cancer, size and location of lymph node, and serum Tg level. The most recent guidelines by AACE/ACE/AME recommend FNA-Tg according to clinical indication [28]. All in all, based on the evidence of literature and according to current guidelines [1, 26–28], FNA-Tg has to be measured in cervical lymph nodes suspicious for metastases from DTC.

13.3 FNA-CT in Thyroid Nodules and Lymph Nodes of Patients with Suspicious Medullary Thyroid Carcinoma

The first studies on this topic were published in 2007 [29, 30]. There, 100% MTC lesions (nodules and lymph nodes) were correctly identified by FNA-CT, and only a minor rate had positive cytology. Initially, Boi et al. [29] proposed an "arbitrary" FNA-CT cutoff of 36 pg/mL (i.e., corresponding to three times the highest value found in non-medullary lesions). Later, a multicenter experience showed that among 34 patients with a primary MTC (i.e., thyroid nodule), 21 (62%) and 34 (100%) were detected at conventional cytology and FNA-CT, respectively [31]. In this paper a cutoff of 39.6 pg/mL was calculated for practice use. Another interesting prospective study [32] compared FNA-CT to basal and pentagastrin-stimulated calcitonin and cytology; the recorded sensitivities were 100% for FNA-CT (using a cutoff of 17 pg/mL), 93.7% for basal calcitonin, 87.5% for stimulated calcitonin, and 12.5% for cytology. Other studies confirmed these results [33–35]. Finally, only one paper searched a reference range for FNA-CT [36]; there, in a series of 78 non-medullary thyroid nodules, the 97.5th upper FNA-CT value was 8.50 pg/mL for saline and 7.43 pg/mL for buffer solution. Table 13.2 details the findings from the major studies.

The promising results obtained in these studies prompted ATA expert board to include that in the MTC guidelines [9]. In these guidelines, FNA-CT is suggested in both lymph nodes and thyroid nodules. Specifically, at Recommendation 19 (grade B), it is reported that "FNA findings that are inconclusive or suggestive of MTC should have calcitonin measured in the FNA washout fluid and immunohistochemical staining of the FNA sample to detect the presence of markers." However, what cutoff level for FNA-CT has to be adopted has not been reported. In addition, AACE/ACE/AME indicates that FNA-CT can be used in enlarged

Table 13.2 Accuracy of FNA-CT reported in the literature

First author (year)	Lesions[a]	Analytic Method	Cutoff (pg/mL)	Sensitivity	Specificity
Boi (2007)	36	CLIA	36	100	100
Kudo (2007)	14	NR	67	100	ND
Diazzi (2015)	60	CLIA	17	100	88.8
Trimboli (2014)	90	CLIA	39.6	100	100
De Crea (2014)	62	CLIA	10.4	89	100

CLIA chemiluminometric immunoassay, *NR* not reported
[a]Thyroid nodules/lymph nodes

lymph nodes of patients with MTC or in suspicious thyroid nodules of patients at risk for MTC or MEN2 syndrome [28].

A consideration for clinical practice should be addressed. The experience of the authors of this chapter suggests to measure serum CT in patients undergoing thyroid FNA and to use FNA-CT in those subjects with elevated serum CT levels. This selection of patients at risk for MTC allows the use of FNA-CT in the same FNA sample and, of high relevance in clinical practice, provides useful information to the cytopathologist [4]. As a potential limitation of this approach, those MTC with no secretion of serum CT have to be taken into account [37].

13.4 FNA-PTH in Lesions Suspected for Hyperplastic Parathyroids

As the first, Doppman et al. reported FNA-PTH in seven enlarged parathyroids [38]. Later, other papers showed the relevance of FNA-PTH to localize parathyroid adenomas with specificity from 75 to 100% and sensitivity from 70 to 100% [11, 38–43]. The accuracy of FNA-PTH was higher than that of cytology [15, 38, 43] and MIBI scintiscan [42–44]. No fixed cutoff has been reported, and no consensus on reference range and upper reference limit exists. In clinical practice, a FNA-PTH/serum PTH ratio ≥ 2 should be considered as positive for parathyroid adenoma (Table 13.3).

13.5 Considerations on FNA-Tg, FNA-CT, and FNA-PTH Testing

The measurement of FNA-Tg, FNA-CT, and FNA-PTH has been recently developed and largely worldwide diffused in the last years. The technique is easy to perform, without a dedicated needle: samples can be collected from FNA for cytology by washing out the needle, after dispensation of the specimen onto the appropriate slides. Despite the achievement of satisfactory results, the determination of thyroid and parathyroid markers in fluids other than blood poses today one of the major challenges to laboratory medicine due to the lack of international standards for the performance and interpretation of the technique. The main technical features and relevant problems are summarized in Table 13.4.

13.5.1 Pre-analytical Factors

The first issue to be addressed is the appropriate sampling: samples should be representative of the lesion in the lymph node or in the thyroid bed [45, 46]. However, unlike the FNA cytology, it is possible to make a diagnosis by using FNA markers even though no epithelial cells were aspirated, since Tg, CT, and PTH present high levels both inside and in the neighboring area of the lesion [47].

Second, when determining the concentration of a marker in fluids other than serum/

Table 13.3 Accuracy of FNA-PTH reported in the literature

First author (year)	Lesions	Method	Cutoff (pg/mL)	Sensitivity	Specificity
Sacks (1994)	45	IRMA	20	82	100
Kiblut (2004)	170	CLIA	1000	87	75
Conrad (2006)	66	ECLIA	1000	80	100
Kwak (2009)	18	IRMA	PTH-FNA > PTH-serum	92.9	100
Boi (2012)	43	CLIA	103	100	100
Kuzu (2016)	57	NR	PTH-FNA > PTH-serum	89	100

IRMA immunoradiometric assay, *CLIA* chemiluminometric immunoassay, *ECLIA* electrochemiluminometric immunoassay, *NR* not reported

Table 13.4 Measurements of FNA-Tg, FNA-CT, and FNA-PTH: technical features

	FNA-Tg	FNA-CT	FNA-PTH
Overall reliability	High	High	High
Solution to be used	Saline, 1 mL	Saline, 1 mL	Saline, PTH-free serum
Cutoff to be adopted	< 1 μg/L negative > 10 μg/L positive	< 10 ng/L negative > 36 ng/L positive	FNA/serum PTH ratio > 2
Potential false positives	Ectopic normal thyroid tissue	Unknown assay interferences	PTH truncated fragments
Potential false negatives	TgAb, hook effect	Hook effect	Hook effect
Reliability in the presence of inadequate FNA cytology	Unchanged	Unchanged	Unchanged
Pre-analytic factors	Laboratory specialists must be informed of the suspicious DTC Collection, preparation, and management of the sample	Laboratory specialists must be informed of the suspicious MTC Collection, preparation, and management of the sample	Laboratory specialists must be informed of the suspicious IPTH Collection, preparation, and management of the sample
Post-analytic factors	FNA-Tg concentration expressed as ng/FNA units or ng/mL, cutoff	FNA-CT concentration expressed as pg/FNA units or ng/mL, cutoff	FNA-PTH concentration expressed as pg/FNA units or ng/mL, cutoff
Time of work	1 day	1 day	1 day
Costs	Up to 15 €	Up to 20 €	Up to 15 €

plasma, we have to consider the so-called matrix effects that are changes of the medium in which the marker is measured and could represent confounding factors [43, 44]. However, despite the demonstration of the matrix effect in some studies, the most advanced IMA for serum markers (i.e., Tg, CT, and PTH) do not seem to be affected by this type of interference, obtaining comparable results with the use of saline, marker free-serum, and kit buffer [43, 45, 46]. Consequently, saline solution is widely employed in current practice.

Third, plain tubes should be employed preferentially as lithium-heparin tubes slightly reduced the FNA-Tg concentration when compared to plain tubes in one study [3]. Also, the volume of fluid used to wash the FNA needle ranges from 0.5 to 3.0 mL with 1.0 mL as most widely utilized one (Table 13.4).

Fourth, CT and PTH are poorly stable peptides requiring precautions for preservation (i.e., need to be kept on ice through the entire process) [42, 48]. Finally, the sample could require a pretreatment such as mixing and centrifugation in order to discard cellular debris coming from blood and tissue contamination [49].

All in all, variability in marker measurement in FNA washouts should be reduced by using saline solution in fixed volume (i.e., 1 mL) and a plain tube and using special pre-analytical precautions when measurement of unstable molecules (i.e., CT, PTH) is required. Figure 13.1 illustrates the initial preparation of the sample to be used for measurement of Tg, calcitonin, or PTH in washout from FNA.

13.5.2 Analytical Factors

Many analytical problems (i.e., "hook effect," immunoassay interference, and analytical variability) are similar when Tg/CT/PTH are measured in serum/plasma or different fluids, respectively. However, measuring thyroid/parathyroid markers in fluids other than blood is more problematic due to the lack of experimental data to support the validity of results and absence of formal support for this application by commercial manufacturers. Then, full analytical validation to regulatory standards by laboratories is required [4, 49]. Additionally, the possible influence of the Tg autoantibodies in the determina-

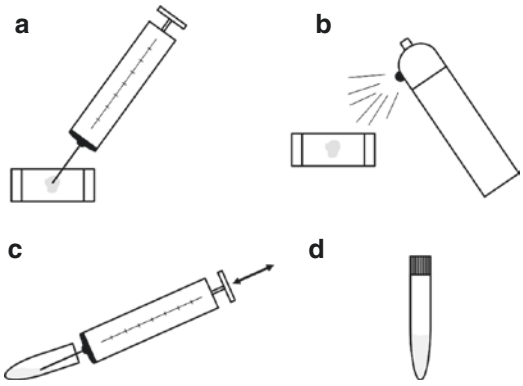

Fig. 13.1 Preparation of sample for the measurement of thyroglobulin, calcitonin, and PTH in fluids from FNA of neck lymph nodes, thyroid nodules, or suspicious parathyroids

tion of FNA-Tg was evaluated with inconclusive results; anyway the influence of TgAb on the clinical performance of FNA-Tg is limited, and Tg levels remained detectable in washouts from patients with malignant lesions [13, 14].

13.5.3 Post-analytical Factors

The marker measured in the FNA fluid is not the true concentration, but it reflects the dilution of the marker left in the needle in the arbitrary selected volume of the washout fluid. So, some authors and also the ETA guidelines suggest expressing Tg, CT, and PTH in ng/FNA units [24]. Nevertheless, several studies reported FNA marker in ng/mL, allowing for the comparison of the FNAB-marker and serum marker levels.

Moreover, in the interpretation of FNA marker levels, it is important to consider the clinical context of the patient such as pre-/post-thyroidectomy, histologic diagnosis, and serum TSH concentration [49].

Conclusion

Measuring endocrine biochemical markers in FNA washout fluids rapidly emerged as a powerful and relatively cheap tool to refine challenging clinical diagnosis in patients with thyroid/parathyroid tumors. In particular, both FNA-Tg and FNA-CT measurements are now included in current clinical guidelines. Nevertheless, we underline that results should be used in conjunction with information from the clinical evaluation of the patient and other diagnostic tools. A close cooperation between laboratory specialists and clinicians involved in thyroid/parathyroid diseases' care is mandatory to define the most appropriate pre-analytical procedures, to select accurate interpretation criteria, and to properly address cases with conflicting results. In our personal experience, the presence of laboratory specialist during FNA procedures was relevant, especially during the introduction of these techniques in daily clinical practice, to define an accurate work flow.

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