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The role of molecular markers in the management of thyroid nodules

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Riassunto

Ruolo dei marcatori molecolari nel trattamento dei noduli tiroidei

Introduzione. I noduli tiroidei sono una patologia molto comune, ma solamente in pochi casi essi rivelano un fenotipo maligno. Insieme all'ecografia, l'agoaspirazione della lesione con ago sottile è uno dei principali strumenti utilizzati per comprendere la natura di un nodulo. Il materiale così prelevato viene osservato al microscopio per l'esame citologico, che però in circa il 22–30% dei casi non è sufficiente a raggiungere una diagnosi definitiva di benignità o malignità. In questi casi spesso il ricorso alla chirurgia per una lobectomia diagnostica rappresenta l'unica via percorribile. L'esame istologico sul nodulo rimosso potrebbe rivelare due possibili risultati: se si trattasse di una lesione benigna, l'intervento chirurgico avrebbe potuto essere evitato; se si rivelasse essere un tumore maligno, sarebbe necessario un altro intervento per rimuovere l'altro lobo, e ridurre così il rischio di recidive.

Il ruolo dei test molecolari volti ad individuare mutazioni somatiche già a livello del materiale citologico è stato largamente studiato negli ultimi anni. Tuttavia, la loro utilità clinica è tuttora oggetto di dibattito.

Obiettivo dello studio. Il presente è uno studio prospettico e unicentrico che si propone a) di stimare la distribuzione delle principali alterazioni molecolari riscontrate nelle lesioni tiroidee a livello delle diverse categorie diagnostiche citologiche e b) di valutare la loro utilità clinica in fase preoperatoria.

Materiali e metodi. Sono stati raccolti in totale 680 campioni citologici. L'analisi molecolare per la valutazione di alterazioni sui geni *BRAF*, *NRAS*, *HRAS* e *KRAS* è stata condotta con PCR seguita da sequenziamento genomico diretto. L'analisi citologica è stata eseguita in cieco da due citopatologi in maniera indipendente.

Risultati. In totale 630 campioni sono stati considerati idonei per le successive analisi. Secondo il sistema di classificazione della citologia tiroidea del gruppo SIAPEC, i noduli sono stati ripartiti nelle categorie diagnostiche come segue: 24 TIR1 (non diagnostico, 4%), 425 TIR2 (benigno,

67%), 114 TIR3 (indeterminato, 18%), 11 TIR4 (sospetto per malignità, 2%) e 56 TIR5 (maligno, 9%). Complessivamente le alterazioni molecolari di *BRAF* sono state riscontrate in 36 noduli (5.7%), prevalentemente appartenenti al gruppo dei TIR5; le mutazioni a carico dei geni *RAS* sono state trovate in un totale di 47 noduli (7.5%), ed erano presenti perlopiù nei TIR2 (5.9%) e nei TIR3 (16.7%).

Nel corso dello studio, 180 noduli sono stati chirurgicamente rimossi. Di questi 180, 96 sono risultati tumori maligni (52%). Nel 54% di questi era stata riscontrata almeno una alterazione molecolare a livello citologico. In particolare, le mutazioni di *BRAF* sono risultate specifiche al 100% per la malignità. Inoltre, queste erano statisticamente associate a fattori di prognosi sfavorevoli del tumore. Questa associazione non è stata invece riscontrata per le mutazioni di *RAS*, che oltretutto erano presenti in due noduli risultati poi benigni. Nelle categorie citologiche TIR2 e TIR3, il rischio di malignità osservato (14% and 45% rispettivamente) era piuttosto elevato. In ogni caso la presenza di una mutazione di *RAS* sul campione citologico è risultata altamente indicativa di una neoplasia maligna ad architettura follicolare.

Conclusioni. Con questo studio è stata ottenuta una stima affidabile della reale frequenza delle mutazioni a carico dei geni *BRAF* e *RAS* nei noduli tiroidei, e la loro relativa distribuzione nelle diverse categorie citologiche. Valutando i noduli con risultato istologico si è osservato che per i casi TIR4 e TIR5 la sola analisi citologica è sufficiente ad ottenere una elevata specificità. Nei noduli indeterminati, invece, un test molecolare può essere utile a definire la natura della neoplasia, anche se con una specificità non assoluta. In conclusione, questo studio dimostra che un protocollo che affianchi l'analisi molecolare a quella citologica non solo è applicabile come pratica routinaria, ma dovrebbe essere considerato attentamente in particolare per i noduli indeterminati.

Abstract

The role of molecular markers in the management of thyroid nodules

Background. Thyroid nodules are frequently encountered in the general population. In spite of this high incidence, the prevalence of thyroid cancer among thyroid nodules is rather low. Fine–needle aspiration (FNA) cytology is the most widely used tool for determining the nature of a nodule, together with ultrasound examination. However, this test fails to reach reliable results in about 25–30% of cases, for which a diagnostic lobectomy represents the only applicable solution. Whenever the nodule turns out to be benign at histological examination, the surgical approach proves to be unnecessary. On the contrary, for a subset of patients with a diagnosis of malignancy a second step surgery is required to avoid the risk of tumor recurrence. The role of molecular markers in helping clinical decisions and triaging patients to the appropriate surgical approach has been investigating for the last ten years. However, the real prevalence of the most common genetic alterations in thyroid nodules as well as their clinical significance is still unclear.

Objectives. This is a prospective single–institution study aimed to a) evaluate how the most frequent molecular alterations are distributed among the cytological categories of thyroid nodules and b) assess their preoperative utility in defining the nature of the nodule.

Materials and methods. A series of 680 FNA biopsies were consecutively collected and analyzed for the presence of alterations in *BRAF*, *NRAS*, *HRAS* and *KRAS* genes by using a high resolution melt analysis followed by direct sequencing. For all cases the cytological diagnosis was made independently by the same two pathologists in a blind way.

Results. A total of 630 cases were included in the final series. According to the Italian system for the classification of thyroid cytology, they were diagnosed as follows: 24 TIR1 (non diagnostic, 4%), 425 TIR2 (benign, 67%), 114 TIR3 (indeterminate, 18%), 11 TIR4 (suspicious for malignancy, 2%) and 56 TIR5 (malignant, 9%). Overall, molecular alterations in *BRAF* were found in 36 cases (5.7%), and were prevalent in TIR5 group; *BRAF* mutations were also present in one TIR2 and in one TIR3 nodules. Mutations in *RAS* genes were detected in 47 nodules (7.5%), with a frequency of 5.9% and 16.7% in TIR2 and TIR3 respectively.

During project duration, 180 nodules underwent surgical removal. On histology, 96 nodules (52%) resulted as malignant lesions. Among these, 54% had at least one of the tested molecular

alterations. *BRAF* mutations were 100% specific for malignancy, and their presence was statistically associated with poor prognostic factors. *RAS* genes did not show any correlation with tumors features of aggressiveness, on the contrary two of the benign cases were *RAS*–mutant. Malignancy rates in TIR2 and TIR3 categories were rather high (14% and 45% respectively). The preoperative detection of *RAS* mutations was of great value in predicting follicular–patterned malignant lesions, showing a satisfying positive predictive value.

Conclusions. This project defined reliable esteems of the real prevalence of the main molecular alterations in thyroid cytology. Moreover, the surgical cohort revealed that for TIR4 and TIR5 cytology alone had enough specificity and sensitivity, while molecular testing on cytology is effective in defining the risk of malignancy in indeterminate nodules. Therefore, the application of a such low–cost panel of markers on routine thyroid cytology should be considered.

1.Introduction

Palpable thyroid nodules are frequently encountered in the general population, occurring in approximately 5% of women and 1% of men [1,2]. The prevalence of thyroid nodules raises the 19–68% when population is screened with high–resolution ultrasound techniques [3,4]. Not all thyroid nodules represent a relevant entity from a clinical point of view, with a prevalence of cancer ranging from 7 to 15%, depending on numerous factors including mainly population, sex, age and history of radiation exposure [5,6]. Once a nodule is detected, the main objective of the clinician is to identify its nature, or in other words to exclude malignancy. According to the latest guidelines of the American Thyroid Association (ATA), ultrasound provides important information, including gland size, nodule size, location and sonographic features (solid or cystic components, echogenicity, margins, calcifications, vascularity); moreover, the presence of any suspicious cervical lymph nodes in the central or lateral compartments can be assessed. The results of ultrasound evaluation can be associated with a certain risk of malignancy [7,8], leading to a further evaluation of the nodule by fine–needle aspiration (FNA) biopsy. This test for the examination of thyroid nodules is cost–effective and highly reliable, particularly when performed under ultrasound guidance [9,10]. However, a definitive diagnosis can be made only after histological examination. Nevertheless, the aim of FNA cytology is to reduce the rate of surgery for benign disease, without missing any malignancy. For such reasons the results of FNA biopsies should be evaluated with all the clinical, laboratory and imaging data [11].

1.1.Follicular–derived thyroid cancer

Thyroid cancer is the most common endocrine malignancy and its incidence is increasing. Thyroid cancer has generally a good clinical prognosis, with survival rates of more than 95% after 20 years from diagnosis. However, disease recurrence and persistence rates are quite high [12].

Primary thyroid tumors are frequently epithelial tumors originating from follicular cells, and rather rarely they derive from parafollicular cells, resulting in medullary carcinomas [13]. The

follicular–derived thyroid tumors include benign forms, such as follicular adenoma (FA), malignant well–differentiated tumors, mainly papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), and malignant poorly differentiated (PDTC) and undifferentiated or anaplastic (ATC) carcinomas.

PTC is the most common form of thyroid cancer, accounting for 85–90% of all thyroid carcinoma. Microscopically, conventional PTC has a papillary growth pattern, papillae with well–developed central fibrovascular core lined by layers of cells with crowded, oval nuclei (Figure 1a, 1b). Often PTC contain also a variable proportion of follicular areas. Tumor cells are usually cuboidal or columnar, with nuclei containing peculiar characteristics such as eosinophilic inclusions and grooves.

PTC tend to invade lymphatic vessels, leading to regional node metastases. These are extremely common (50% of more) at initial presentation. Distant metastases to lungs and bones are rare, occurring in 5–7% of cases [13]. Poor prognostic factors in PTC are older age at diagnosis, male sex, large tumor size, extrathyroidal growth, presence of less differentiated or solid areas and vascular invasion.

Some histological subtypes of PTC are thought to be associated with poor prognosis, but it is still controversial. Tall cell variant (TCV) PTC represents about 10% of all PTC (Figure 1c). These tumors have an extensive papillary pattern, and usually show extrathyroidal extension. The prognosis for this variant is less favorable than for conventional PTC [12].

Follicular variant (FV) PTC accounts for 15–20% of all PTC (Figure 1d). Its diagnosis is easier when nuclear features are well recognizable and tumor has an infiltrative growth pattern [14]. Encapsulated lesions, without signs of tumor capsule invasion, with unclear or imperfect nuclear features are the most controversial. Several studies demonstrated a poor diagnostic agreement for these lesions even among expert thyroid pathologists [15–17]. Differently from conventional and TCVPTC, FVPTC are difficult to be diagnosed at cytological examination, because they rarely show nuclear characteristics of PTC; often FVPTC are hidden into indeterminate category.

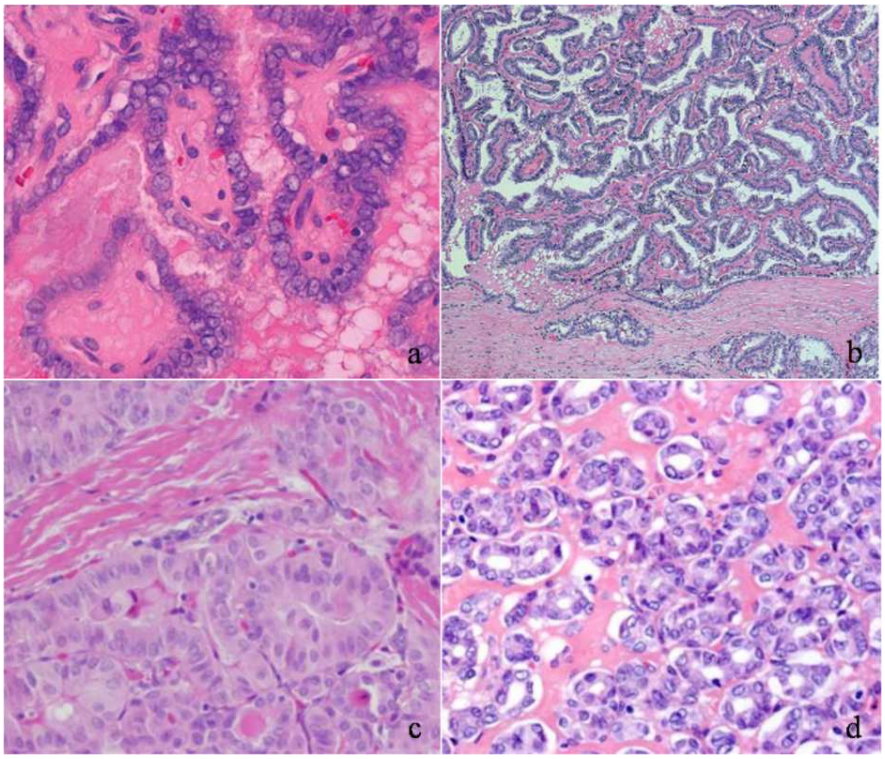

Figure 1. Histological images of PTC, hematoxylin/eosin stain. a) PTC nuclear features (X100) [13]; **b)** classical PTC (X100) [18]; **c)** tall cell variant PTC (X20); **d)** follicular variant PTC (X20).

Form a clinical point of view, FVPTC generally show an indolent behavior. Anyway, it has been demonstrated that for FVPTC the invasion of tumor capsule represents a key factor in determining the prognosis [19]. In particular, encapsulated non–invasive FVPTC lack any evidence of lympho–vascular invasion and have an extremely low metastatic potential, being associated with a favorable outcome [19–22]. Considering this, to avoid over–diagnosis and overtreatment of these indolent tumors, their reclassification has been recently proposed [23], with the new nomenclature of "Non–invasive follicular thyroid neoplasm with papillary–like nuclear features" (NIFTP) (Figure 2).

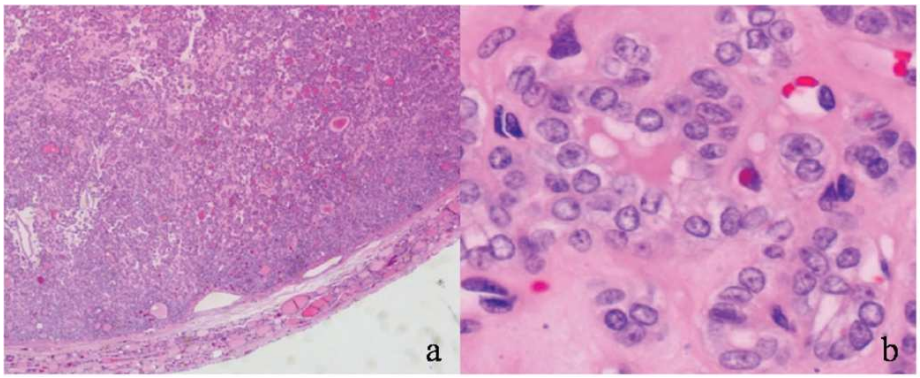

Figure 2. Histological images of a NIFTP, hematoxylin/eosin stain [24]. **a)** the thin fibrous capsule is illustrated (X2); **b)** the same lesion shows nuclear features typical of PTC (X40).

FTC represent 5–15% of all thyroid cancers. They show a follicular differentiation but lack peculiar nuclear characteristics. At presentation FTC is usually a solitary and encapsulated tumor, and its main diagnostic criteria is the invasion of the capsule and/or the vascular invasion. The latter is an indicator of poor prognosis [25]. The majority of FTC are minimally invasive, with a slight invasion of tumor capsule invasion; they rarely cause distant metastasis. On the other hand, widely invasive FTC is much less common, but about 80% of these tumors cause distant metastasis, leading to high mortality rate [12].

PDTC and ATC account for approximately 5%–10% of thyroid cancers. Patients affected form these cancers have a mean survival of 3.2 and 0.5 years after presentation respectively [26]. They represent two well distinct entities, but it seems that most of them arise from preexisting PTC [27,28].

1.2.Thyroid cytology

The evaluation of FNA cytology is determined by specific criteria and follows a system of diagnostic categories, each one associated with a different risk of malignancy.

The most widely used classification systems for the evaluation of FNA cytology are a) the Bethesda system (USA) [29] b) the Royal college of Pathology Guidance (UK) [30] and c) the Italian system for reporting of thyroid cytology [11].

A comparison among these three reporting systems is shown in Table 1: a close similarity among these schemes is evident.

SIAPEC-AIT2013	USA Bethesda	UK RCPath
TIR 1 Non-diagnostic	I. Non-diagnostic	Thy1/Thy1c
TIR 1c Non-diagnostic cystic	Cystic fluid only	Non-diagnostic for cytological diagnosis
		Unsatisfactory, consistent with cyst
TIR 2 Non-malignant	II. Benign	Thy2/Thy2c
		Non-neoplastic
TIR 3A Low-risk indeterminate lesion (LRIL)	III. Atypia of undetermined significance or follicular lesion u.s. AUS/FLUS	Thy 3a
		Neoplasm possible—atypia/ non-diagnostic
TIR 3B High-risk indeterminate lesion (HRIL)	IV. Follicular neoplasm or suspicious for a follicular neoplasm	Thy 3f
		Neoplasm possible—suggesting follicular neoplasm
TIR 4 Suspicious of malignancy	V. Suspicious of malignancy	Thy 4
		Suspicious of malignancy
TIR 5 Malignant	VI. Malignant	Thy 5 Malignant

Table 1. Comparison among cytology classification systems [11].

Expected risk of malignancy and suggested clinical actions for the Italian classification system are summarized in Table 2. The seven cytology categories of the Italian classification are explained in detail in the next few paragraphs.

TIR 1 category includes either inadequate and non–representative specimens. The inadequacy usually depends on technical issues, such as staining artifacts, or on the presence of obscuring blood. On the other hand, a specimen is considered non–representative when an insufficient number of cells are collected from the nodule and the scarcity of material does not allow a reliable evaluation. It has been demonstrated that the institutional prevalence of TIR1 is strongly operator–dependent [31,32]; the overall rate of TIR1 per institution should remain below 10%. Whenever a specimen with a low cell representativeness is characterized by the presence of a cystic component, it is classified as TIR1C.

Code	Diagnostic category	Expected risk of malignancy (%)	Suggested actions
TIR1	Non-diagnostic	Not defined	Repeat US-guided FNA after at least 1 month
TIR1C	Non-diagnostic-cystic	Low (variable on the basis of clinical findings)	Evaluate the clinical setting and/or repeat FNA
TIR2	Non-malignant/ benign	<3	Follow-up
TIR3A	Low-risk indeterminate lesion (LRIL)	<10[a]	Repeat FNA/ clinical follow-up
TIR3B	High-risk indeterminate lesion (HRIL)	15–30[a]	Surgery
TIR4	Suspicious of malignancy	60–80	Surgery (consider frozen section)
TIR5	Malignant	>95	Surgery

[a] Expected rate of malignancy for the TIR3 subcategories is mainly found on clinical experience and is only partially based on the evidence of the published data

Table 2. Italian system for reporting of thyroid cytology [11].

TIR2 category comprises the major part of cytology specimens, accounting for approximately 70–80% of cases. This category is consistent with benign nodules, including adenoma, colloid/hyperplastic nodules, and thyroiditis. Specifically, TIR2 samples show a good cell representativeness with varying proportions of colloid and normal follicular cells organized as macrofollicles. The false negative rate for a diagnosis of TIR2 is expected to not exceed 3%.

TIR3 category includes 10–25% of nodules and it is the most discussed and controversial. Indeed, TIR3 nodules are characterized by a microfollicular pattern, and at histology they might be consistent with adenomatous hyperplasia, follicular adenoma but also with follicular carcinoma or follicular variant of papillary thyroid carcinoma. This is the main reason for which these nodules are often defined as “indeterminate”. The risk of malignancy associated with TIR3 nodules ranges from 5 to 30%. With the purpose of stratifying the risk rate and reduce that range among TIR3 cases, a subclassification in TIR3A and TIR3B has been introduced in the latest guidelines. TIR3A cases, defined as low–risk lesions, are characterized by an increased cellularity, microfollicular structures and scant colloid. Theoretically, the malignancy rate among TIR3A cases should remain below 10%. Samples with high cellularity, microfollicular pattern and scant/absent colloid (suggesting for a follicular neoplasm) are diagnosed as TIR3B, as well as nodules with mild and focal cyto–nuclear atypia. The risk of malignancy of TIR3B nodules is

estimate to reach 30%. According to the Italian guidelines, TIR3A should undergo repeat FNA, whereas TIR3B should be referred for diagnostic surgery, similarly to AUS/FLUS and FN/SFN of Bethesda system.

TIR4 category accounts for about 5% of cases, and is consistent with a high risk of malignancy, reported as 60–80%. TIR4 nodules include highly suspected specimens with clear nuclear atypia, for which, however, the diagnosis of malignancy is not fully justifiable.

Finally, TIR5 nodules have a definitive diagnosis of malignancy (Figure 3). A TIR5 cytology corresponds probably to a papillary thyroid carcinoma, or more rarely to medullary, poorly differentiated or anaplastic thyroid carcinoma. This category accounts for 4–8% of cases, with an associated risk of malignancy higher than 95%.

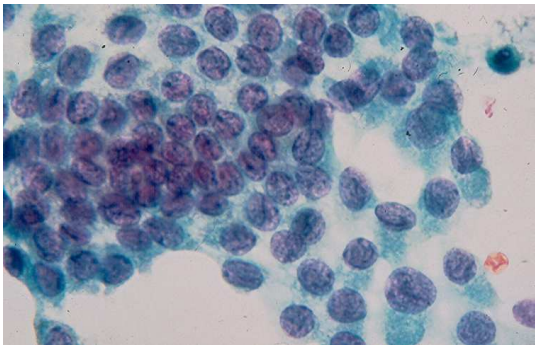

Figure 3. Image of a cytological specimen with diagnosis of TIR5. (Papanicolaou stain, X40) [33].

Of note, the reported risk of malignancy associated with each category is an esteem which rarely is confirmed by real series of cases [34–37]. Indeed, the malignant rates are calculated on the basis of surgical outcomes, and surgical cohorts are necessarily biased by selection effects.

Moreover, as FNA cytology fails to reach a definitive diagnosis in about 25–30% of cases [38], the indeterminate category has attracted much attention because of the necessity to better characterize these nodules before referring at surgery. This challenge concerns the presence of subtle cyto–morphological and cyto–nuclear features which are shared by several follicular patterned lesions, including either benign and malignant forms, such as hyperplastic nudules, follicular adenoma, follicular carcinoma, and the follicular variant of PTC [39]. Indeed, whenever a nodule proves to be benign at histology, surgery can be considered an overtreatment. On the

other hand, if a diagnostic lobectomy is performed and the nodule turns out as malignant, a completion thyroidectomy may be required to prevent the risk of recurrence, and patient is sent to a second–step surgery. To overcome this diagnostic issue, several molecular markers have been proposed as auxiliary instruments supporting and aiding cytology evaluation.

1.3.Molecular markers and molecular tests for thyroid cytology

In follicular–derived thyroid tumors, the most frequent molecular alterations occur in the MAP kinase (MAPK) and PI3K/AKT signaling pathways, including mainly *BRAF* and *RAS* point mutations, and RET/PTC and PAX8/PPARg translocations (Table 3).

Thyroid tumor	*Molecular alteration*
Papillary thyroid carcinoma	*BRAF*^{V600E} (classical and TCV) *BRAF*^{K601E} (FV) *H–, N–, K–RAS* *TERT* promoter *RET/PTC* *PAX8/PPARg*
Follicular thyroid carcinoma	*BRAF*^{K601E} *H–, N–, K–RAS* *TERT* promoter *PAX8/PPARg*
Poorly differentiated and undifferentiated thyroid carcinoma	*BRAF*^{V600E} *H–, N–, K–RAS* *TERT* promoter *PIK3CA* *EIF1AX*
Follicular thyroid adenoma	*BRAF*^{K601E} *H–, N–, K–RAS* *PAX8/PPARg*

Table 3. Molecular alterations frequently detected in thyroid cancer [40–43].

These alterations are mutually exclusive, however they often coexist with additional mutations in other oncogenes such as *PIK3CA*, *EIF1AX* and the promoter of *TERT* gene, particularly in more advanced forms. Each molecular alteration detected on cytology can reach a certain degree of sensitivity and specificity. For instance, *BRAF* p.V600E mutation represents the most common alteration found in PTCs (mainly in conventional type), reaching the 45% of frequency [44]. Not

only this mutation is highly specific for malignancy, but also it has been associated with a worse prognosis in terms of lymph node involvement, distant metastases and higher risk of recurrence [28,45–48]. However, its sensitivity is rather low, since a *BRAF* wild–type result on cytology can by no means certainly rule out malignancy [49]. On the other hand, mutations in *RAS* genes are not highly specific nor sensitive, occurring either in malignant and in benign neoplasms. Researchers have been discussing for many years about the role of *RAS* mutations in thyroid cytology, and the controversy involves especially their detection in indeterminate nodules.

Nowadays, the introduction of innovative and promising technologies allows to extensively explore the molecular characteristics of a tumor, studying gene panels rather than single alterations. An ideal molecular test applied on cytology samples should be a) informative, providing reliable data able to rule in or rule out malignancy and b) feasible and cost–effective, in terms of nucleic acids input, costs and time [50].

Several molecular tests have been developed and commercialized for thyroid cytology: some of them detect mutations and some others analyze gene expression profiles. The performance of each test in terms of positive and negative predictive values (PPV and NPV) is not an intrinsic property, but should be calculated on the basis of the pretest probability of malignancy, which considerably varies among populations, institutions and cytology categories [51,52]. As a consequence, the test results obtained by a research group should not be extended to other conditions. Considering this premise, the characteristics and performance rates of three molecular tests among the most applied on indeterminate thyroid cytology are summarized in Table 4.

Test	Afirma[a]	ThyraMIR/ThyGenX[b]	TyroSeq[c]
Company	Veracyte	Interpace Diagnostics	University of Pittsburg
Method	microarray technology analyzing 167–gene mRNA expression	ThyGenX: multiplex PCR; detection of mutations in a 7–gene panel (*BRAF* and *RAS* genes, rearrangements of *RET/PTC* and *PAX8/PPARg*) ThyraMIR: miRNA expression analysis	Next–generation sequencing platform detecting mutations in several genes (*BRAF*, *RAS* genes, *CTNNB1*, *GNAS*, *TP53*, *TERT*, *TSHR* and others) and rearrangements (*RET*, *PPARg*, *NTRK*, *ALK*)
Sample*	2 dedicated FNA passes	1 dedicated FNA pass	1–2 drops from first pass
Cost*	$4875 Afirma GEC/MTC $975 Afirma MTC $475 Afirma *BRAF*	$1675 for ThyGenX alone $3300 for ThyraMIR	$3200
Sensitivity	83–100%	69%	90%
Specificity	7–52%	86%	93%
PPV	14–57%	71%	83%
NPV	75–100%	85%	96%

Table 4. Test characteristics. The table summarizes the main characteristics of Afirma, ThyraMIR/ThyGenX and ThyroSeq (v.2) molecular tests.

*Information from a review by Nishino [50]
[a]data obtained from a comparison among several studies on indeterminate cytology performed by Afirma [50,51,53,56,57]
[b]from Labourier et al. [58]
[c]from Nikiforov et al. [59]

2.Aim

The landscape in which the present study is placed is various and multi–sided. The value of cytological examination in the assessment of thyroid nodules is incomparable, nevertheless molecular biology finds easily its place as ancillary discipline aimed to overcome some important limitations of cytology. The common goal is to provide patients with an effective preoperative screening, thus giving a global idea of the pathology, able to guide diagnosis, surgical approach and therapy. This need becomes more evident when cytological specimens obtained by FNA biopsy yield a diagnosis of indeterminate.

Advances in the understanding of molecular mechanisms underlying thyroid tumorigenesis [60] led to the development of molecular tests for thyroid cytology. Several studies demonstrated the importance of an integrated approach, combining either genotyping and gene expression analysis. This strategy is frequently precluded because of the scarcity of nucleic acids obtainable from cytology specimens. In addition, such methodologies are generally very expensive, and their efficiency depends on a series of factors which are peculiar of each specific population and institution.

Therefore, in order to define the prevalence and the clinical role of the more frequent molecular alterations found in thyroid nodules, this project aims to molecularly characterize a rather homogeneous, consecutive series of cytological specimens from thyroid nodules.

The molecular test is performed by a cheap technology commonly used in laboratories of molecular pathology, a real–time PCR followed by high resolution melting analysis and direct sequencing. The molecular alterations analyzed are mainly point mutations in genes belonging to the MAPK signaling pathway: *BRAF*, *HRAS*, *NRAS* and *KRAS*. Moreover, in a subset of cases, mutational status of the promoter of *TERT* gene is evaluated.

The project is designed to have some important characteristics of strength, never found in previous studies:

- prospective, consecutive, non–selected series of cases, in order to obtain a real picture of the prevalence of molecular markers;
- homogeneity of samples collection, since FNA is performed by the same operator;
- homogeneity in cytology diagnoses, because microscopic evaluation is performed independently by the same two cytopathologists, and medical reports written accordingly;
- molecular analysis performed blindly;
- analysis performed on needle rinse material from FNA, obtained independently from slides preparation without waste of diagnostic material.

3.Materials and methods

3.1.Patients population

A total of 680 samples have been consecutively collected from patients with thyroid nodules undergoing routine FNA biopsy at the Unit of Endocrinology 1 of the Azienda Ospedaliero Universitaria Pisana (Pisa, Italy) between June 2013 and September 2014. No selection criteria have been adopted, all FNA biopsies were performed by the same operator. Information about size and location were collected for each nodule, as well as patients age and sex. Cytological diagnosis was formulated independently and blindly following the Italian system [11]. During this project, a subgroup of nodules have been surgically excised: information about histological outcome [61] and clinical–pathological characteristics have been collected, with the last update in September 2016.

3.2.Samples collection and DNA purification

After nodule aspiration and slides preparation, additional material has been recovered by rinsing the syringe with phosphate–buffered saline (PBS), flushing repeatedly the fluid into a 1.5 ml clean tube. Samples have been processed by a mild centrifugation ($5x10^3$ rpm for 15 minutes), and supernatant was discarded. Cell pellets were resuspended in a preservative and hemolytic solution (ThinPrep PreservCyt Solution, Hologic Inc., Marlborough, MA, USA) in order to remove blood components. Then, tubes were centrifuged again ($5x10^3$ rpm for 15 minutes), and supernatant was removed. Cell pellets were immediately used for DNA purification or stored at –20°C until DNA extraction. DNA was purified using QIAamp DNA Mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Cell pellet was resuspended in PBS, 200µl, before adding proteinase K and lysis buffer. Final elution volume was set at 45 µl. DNA quality and quantity were evaluated by a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, Wilmington, DE, USA). Whenever the DNA concentration was higher than 10ng/µl, samples underwent dilution with nuclease–free water.

3.3.Molecular analysis.

All samples have been analyzed for the presence of somatic molecular alterations in *BRAF* (exon 15), *NRAS* (exon 3), *HRAS* (exon 3) and *KRAS* (exon 2) genes. A real–time PCR was set–up using a pre–prepared reaction mix (Hot StarTaq Master Mix, Qiagen), a DNA–binding dye (EvaGreen, Biotium Inc., Fremont, CA, USA) and specific primer pairs (Table 5). For all samples reactions were performed using 4µl DNA. After PCR reaction, products were evaluated by high resolution melt (HRM) analysis. Melt curves of samples were compared with that of known positive and negative controls, included in each experimental session together with a no template control. For samples with altered melt curves, PCR products were purified and analyzed by direct sequencing on a AbiPrism 3130 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA).

Gene [Ref Ensemble], exon	Primer pairs
BRAF [ENSG00000157764] exon 15	F: 5'– TCC TTT ACT TAC TAC ACC TCA GAT –3' R: 5'– AGT GGA AAA ATA GCC TCA AT –3'
NRAS [ENSG00000213281] exon 3	F: 5'– TGG TGA AAC CTG TTT GTT GG –3' R: 5'– TGG CAA ATA CAC AGA GGA AGC –3'
HRAS [ENSG00000174775] exon 3	F: 5'– GGA AGC AGG TGG TCA TTG AT –3' R: 5'– AGT ACA GGT GAA CCC CGT GA –3'
KRAS [ENSG00000133703] exon 2	F: 5'– TCA TTA TTT TTA TTA TAA GGC CTG CTG –3' R: 5'– AGA ATG GTC CTG CAC CAG TAA –3'

Table 5. Primer pairs.

In a subset of cases (cases with TIR3, TIR4, TIR5 cytology and nodules of any category with *BRAF* or *RAS* mutations) the analysis of the promoter of *TERT* gene was also performed by PCR followed by direct sequencing [62].

3.4.Statistical analysis.

The presence of statistical correlations among the considered parameters was evaluated using Statistica Software (Dell Software, Round Rock, TX, USA) proceeding as follows: $\chi 2$ test to

compare categorical variables; t–test to compare quantitative variables (after assessment of the normal distribution of the continuous variables). Statistical significance was set at p–value <0.05.

The performance of the molecular test was calculated for TIR3 nodules considering the following parameters:

a) true positive (True P): cases positive for a mutation and histologically malignant;
b) false positive (False P): cases with mutations and benign at histology;
c) true negative (True N): cases without mutations and benign at histology;
d) false negative (False N): cases without mutations and malignant;

using the following formulae (where the prevalence (Prev) is calculated as the ratio between the malignant cases and all the cases):

$$Sensitivity\ (Sens) = \frac{True\ P}{(True\ P + False\ N)} \times 100$$

$$Specificity\ (Spec) = \frac{True\ N}{(True\ N + False\ P)} \times 100$$

$$Positive\ predictive\ value\ (PPV) = \frac{Sens \times Prev}{(Sens \times Prev) + \ [(1 - Spec) \times (1 - Prev)]} \times 100$$

$$Negative\ predictive\ value\ (NPV) = \frac{Spec \times (1 - Prev)}{[Spec\ \times (1 - Prev)] + \ [(1 - Sens) \times Prev]} \times 100$$

4.RESULTS

4.1.Cytological diagnosis, population

Twenty–nine out of 680 FNA biopsies were negative lymphnode aspirates; nine nodules had a cytological pattern compatible with lymphocytic thyroiditis; three were diagnosed as lymphoma and one as schwannoma. Eight cases were TIR1C. Overall, these 50 cases have been excluded from the original series. The remaining 630 nodules, from 536 patients, formed the final series of cases. There were 120 men (22.4%) and 416 women (77.6%) (sex ratio 1:3.5); mean age was 48.7 years.

Nodules were distributed among the cytological categories as follows: 24 TIR1 (4%), 425 TIR2 (67%), 114 TIR3 (18%, 79 TIR3A and 35 TIR3B), 11 TIR4 (2%) and 56 TIR5 (9%) (Figure 4).

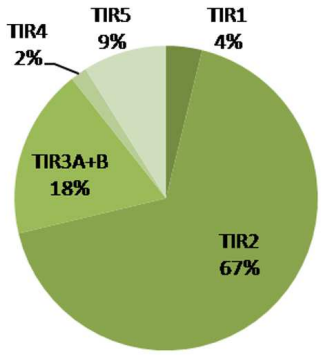

Figure 4. Cytological diagnosis of the 630 nodules.

4.2.Molecular analysis.

All the 680 cases have been evaluated with molecular analysis. The 50 nodules that have been subsequently excluded from this project were wild–type for all the considered markers, except for one sample, which was inadequate.

Examples of altered melting curves and electropherograms obtained by direct sequencing are reported in Figures 5 and 6.

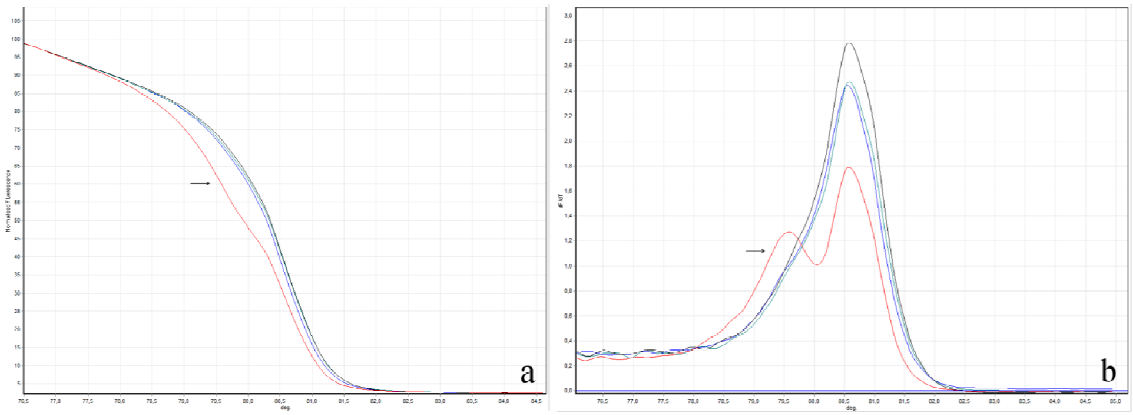

Figure 5. HRMA curves. a) Normalized fluorescence decreases as temperature increases. The black arrow indicates a sample showing a curve with an altered melting profile; **b)** the same condition seen with the derivative graph. Here it is more evident that the sample with the altered curve (black arrow) has two populations of PCR products with different melting properties. This image represents the HRMA for *NRAS* codon 61.

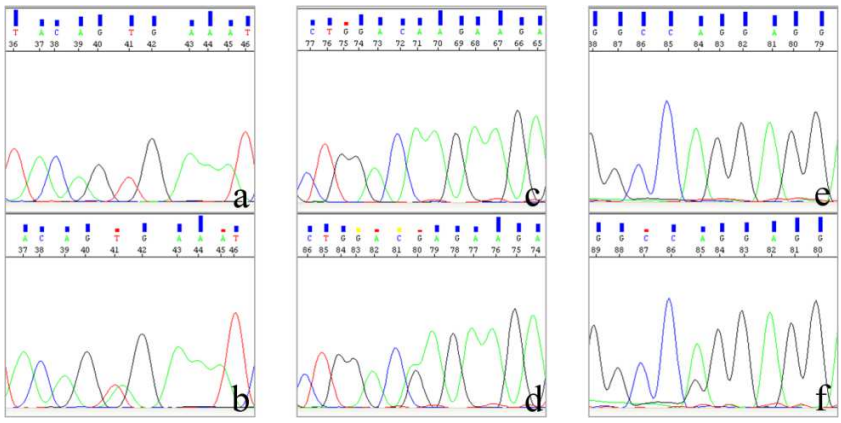

Figure 6. Sequencing electopherograms. a) The sequence of *BRAF* codons 599–601 does not show alterations; **b)** the codon 600 of *BRAF* with the substitution A>T (p.V600E)**; c)** wild–type sequence of *NRAS* codon 61; **d)** substitution G>A in *NRAS* codon 61 (p.Q61R); **e)** wild–type sequence of *HRAS* codon 61 and **f)** substitution G>A in codon 61 (p.Q61R)**.**

Among the remaining 630 nodules, *BRAF* mutations were found in 36 cases (5.7%), *NRAS* mutations in 36 (5.7), *HRAS* mutations in 7 (1.1%) and *KRAS* mutations in 4 cases (0.6%). All mutations in *BRAF* gene were p.V600E, except for one p.K601E (in a TIR2 nodule) and one T599R+V600E complex mutation (in a TIR5 nodule). The prevalent mutation found in *NRAS* codon 61 was the p.Q61R, in 32 cases, followed by the p.Q61K, in four cases. In codon 61 of *HRAS* there were three p.Q61R and four p.Q61K mutations. Mutation in *KRAS* codons 12/13 were: two p.G12V, one p.G12D and one p.G13D.

According to the cytological category, *BRAF* mutations were prevalent in TIR5 group, as well as mutations in *RAS* genes were more frequent in TIR3 category. The results of genotyping are schematized in Figure 7 and reported in detail in Table 6.

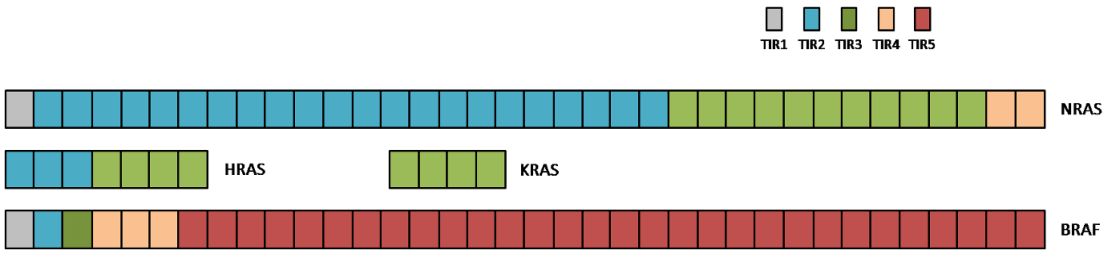

Figure 7. Distribution of the detected molecular alterations according to the cytological categories.

On TIR3, TIR4 and TIR5 nodules the genotype analysis was extended also to the promoter of *TERT* gene. *TERT* promoter mutation was found in eight cases out of 181 (4.4%). All these cases had the C228T substitution (C>T at –124 bases from the ATG start site); they were one TIR3A, one TIR4 (also mutated for *NRAS* p.Q61R), and six TIR5 (three of which were also mutated *BRAF* p.V600E). Later, the analysis of *TERT* promoter was extended also to the nodules mutated for *BRAF* or *RAS* genes, independently from their cytological class, but no further mutations in *TERT* promoter have been found among these cases.

Cytology	***BRAF*** n, (%)	***NRAS*** n, (%)	***HRAS*** n, (%)	***KRAS*** n, (%)	**All *RAS*** n, (%)	wild–type n, (%)
TIR1 n=24	1 (4.1)	1 (4.1)	0	0	1 (4.1)	22 (91.7)
TIR2 n=425	1* (0.2)	22 (5.2)	3 (0.7)	0	25 (5.9)	399 (93.9)
TIR3 n=114	1 (0.9)	11 (9.7)	4 (3.5)	4 (3.5)	19 (16.7)	94 (82.4)
TIR4 n=11	3 (27.3)	2 (18.2)	0	0	2 (18.2)	6 (54.5)
TIR5 n=56	30 (53.6)	0	0	0	0	26 (46.4)
All n=630	36 (5.7)	36 (5.7)	7 (1.1)	4 (0.6)	47 (7.5)	547 (86.8)

Table 6. Cytological group of all cases and their molecular status.

**BRAF* p.K601E mutation

4.3.Follow–up and histology.

During this project, a total of 40 patients (8.9%) underwent repeat FNA, obtaining the following results: 32 nodules with the same cytological report (21 TIR2, ten TIR3A, one TIR3B); four nodules with a worse diagnosis (two from TIR2 to TIR3A, one from TIR3A to TIR3B and one from TIR3A to TIR4); four nodules with a better diagnosis (from TIR3A to TIR2).

Moreover, 180 nodules out of 630 have been surgically excised (28.6%). After histological examination, the 180 nodules have been diagnosed as follows: 86 benign nodules (48%), among which 24 follicular adenoma (27.9%) and 62 nodules in nodular goiter (72.1%), and 94 cases (52%) diagnosed as malignant, 71 PTC (75.5%), nine FTC (9.6%), one ATC (1.1%) and 13 metastatic lesions from PTC (13.8%).

In the group of 86 benign nodules, microcarcinomas have been found during histological examination in 20 cases (23.3%) (Table 7).

Characteristics of microcarcinomas		**n, (%)**
Site	extra–nodule	16 (80%)
	parenchyma in nodule	4 (20%)
Histology	FVPTC	11 (55%)
	CV/TCVPTC	8 (40%)
	MTC	1 (5%)
Multifocality	multifocal	5 (25%)
	unifocal	15 (75%)
Extrathyroidal invasion	yes	4 (20%)
	no	16 (80%)
Lymphnode metastasis	yes	2 (10%)
	no	18 (90%)

Table 7. Clinical pathological characteristics of incidental microcarcinomas.

Of these microcarcinomas, three were found incidentally in the contralateral lobe; 13 were found in the parenchyma of the same lobe of the nodule; four were detected in the context of the nodule. Mean size of unifocal carcinomas was 4.4 ± 3.1 mm. Four microcarcinomas showed extrathyroidal infiltration, and two caused regional node metastases.

Tables 8 and 9 indicate how malignant and benign lesions were distributed among cytological categories.

Cytology	Surgical cohort **n, (%)**	Malignant outcome **n, (%)**	Benign outcome **n, (%)**
TIR1 n=24	8 (33.3)	2/8 (25)	6/8 (75)
TIR2 n=425	57 (13.4)	8/57 (14)	49/57 (86)
TIR3 n=114	56 (49.1)	25 (44.6)	31 (55.4)
TIR4 n=11	10 (90.9)	10/10 (100)	0
TIR5 n=56	49 (87.5)	49/49 (100)	0
All n=630	180 (28.6)	94/180 (52.2)	86/180 (47.8)

Table 8. Surgical outcome of 180 nodules.

Histology	*Cytology*					
	TIR1 n=8	**TIR2** n=57	**TIR3** n=56	**TIR4** n=10	**TIR5** n=49	**All** n=180
BENIGN (n=86)						
Nodule in goiter	6	42	14	0	0	62 (72.1%)
Follicular Adenoma	0	7	17	0	0	24 (27.9%)
MALIGNANT (n=94)						
PTC	0	8	18	7	38	71 (75.5%)
FTC	0	0	7	2	0	9 (9.6%)
ATC	0	0	0	1	0	1 (1.1%)
Metastasis	2	0	0	0	11	13 (13.8%)

Table 9. Histological outcome according to cytological categories.

All the nodules belonging to TIR4 and TIR5 groups had a malignant outcome. Considering the histological diagnoses, the observed risk of malignancy in TIR2 and TIR3 categories was 14% and 45% respectively. Of note, almost all PTC in TIR2 and TIR3 categories were FVPTC. The distribution of PTC variants among TIR2, TIR3, TIR4 and TIR5 nodules is showed in Table 10.

PTC Variant	*Cytology*				
	TIR2	**TIR3**	**TIR4**	**TIR5**	**All**
	n=8	n=18	n=7	n=38	n=71
Classical	1	2	4	30[a]	37 (52.1%)
Follicular	7	14	2	3	26 (36.6%)
Tall cell	0	0	0	4	4 (5.6%)
Others	0	2[b]	1[c]	1[b]	4 (5.6%)

Table 10. Distribution of PTC variants according to cytological category.

[a]six classical PTC had tall–cell areas
[b]solid variant
[c]macrofollicular variant

From a molecular standpoint, 51 out of 94 malignant nodules (54.3%) had at least one mutation, while there were only two mutated cases (both in *KRAS* gene, p.G12D and p.G13D, and both were TIR3) among the 86 benign nodules (2.3%). The presence of any type of mutation in cytology specimens was highly predictive of malignant tumors (p–value 0.00001). Either *BRAF* mutations alone (p–value 0.00001) and *RAS* family mutations alone (p–value 0.0002) had a great power in predicting malignancy. Sensitivity, specificity and predictive power of the molecular test are discussed in detail in the next paragraph.

In cases that turned out as malignant, mutations were distributed among cytological categories as shown in Table 11.

Molecular marker	*Cytology*					
	TIR1 n=2	**TIR2** n=8	**TIR3** n=25	**TIR4** n=10	**TIR5** n=49	**All** n=94
BRAF	1	0	1	3	26	31
NRAS	0	4	6	2	0	12
HRAS	0	1	2	0	0	3
KRAS	0	0	1	0	0	1
***TERT* promoter**	0	0	1	1*	5**	7
wild–type	1	3	14	5	20	43

Table 11. Molecular alterations in malignant cases of the surgical cohort and their distribution among cytological groups.

**TERT* promoter mutation coexisted with *NRAS* mutation
***two out of 5 cases had coexistence of *TERT* promoter and *BRAF* mutations

Among the 52 TIR2 cases that underwent surgery, 8 nodules resulted as malignant, and they were 7 FVPTC and one cystic CVPTC. Five out of 7 FVPTCs had a *RAS* mutation (71%). Moreover, among *RAS* positive cases, two were invasive FVPTC and 4 presented multiple foci.

For TIR3 category, 56 nodules have been removed, and there were 31 benign and 25 malignant tumors on histology. The 25 carcinomas included seven FTCs and 18 PTCs: two conventional PTCs, 14 FV, two solid variant PTCs. Two FTCs out of seven had a *RAS* mutation (29%), and one had *TERT* promoter mutation (14%). *RAS* mutations have been found also in seven out of 18 PTCs (39%), and all of them were FV. Moreover, one of the two CVPTCs had *BRAF* mutation.

All TIR4 nodules of the surgical cohort resulted as malignant: seven PTC, two FTC and one ATC. The latter had the coexistence of *TERT* promoter and *NRAS* p.Q61R mutations. Three out of four CVPTC had *BRAF* p.V600E mutation (75%) and the macrofollicular variant PTC had *NRAS* mutation.

In the same way, all TIR5 nodules that underwent surgical removal were malignant tumors. Eleven out of 49 cases resulted as metastatic lesions; four of these had *BRAF* mutation (36.4%). Almost all TIR5 nodules turn out as CVPTC (30 cases), and 17 of them (57%) were *BRAF* mutated. In addition, all TCVPTC (100%) and one infiltrative FVPTC (33%) had the *BRAF* p.V600E mutation.

FVPTCs deserve a particular mention. Overall, in the surgical cohort there were 26 FVPTCs:

- 5 infiltrative (19.2%) (two TIR3B, one TIR4 and two TIR5 at cytology);
- 7 encapsulated with invasion of tumor capsule (26.9%) (four TIR2 and three TIR3A at cytology);
- 14 encapsulated non–invasive (53.9%) (three TIR2, eight TIR3A, one TIR3B, one TIR4 and one TIR5 at cytology).

Given the advent of NIFTP as a new pathological entity, the 14 encapsulated non–invasive FVPTCs have been carefully revised by two pathologists independently. Only four of them (28.6%) met the cyto–nuclear requirements for the diagnosis of NIFTP. The mutational status of all FVPTCs is schematized in Figure 8.

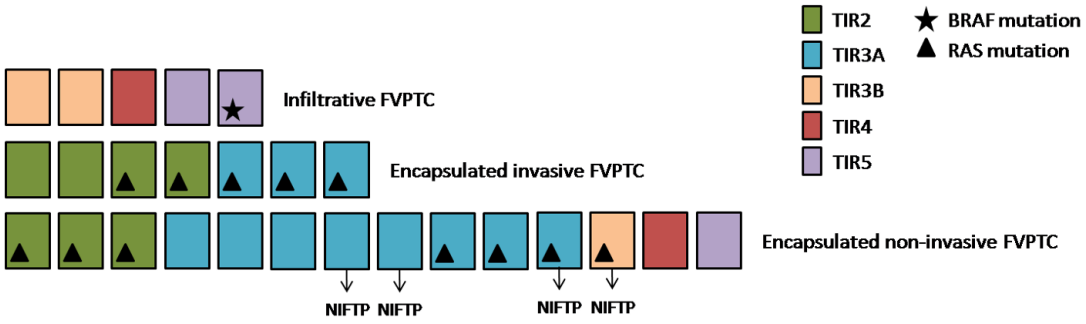

Figure 8. Relationship between invasiveness, cytological group and molecular markers in FVPTCs. Among the non–invasive encapsulated tumors, four can be defined as NIFTP (black arrows).

4.4.Statistical analysis and test performance

Clinical–pathological characteristics of the malignant nodules are reported in detail in Table 12, together with the results of the statistical analysis; the 13 metastatic lesions have been excluded from this evaluation. Moreover, three groups have been made up: *BRAF* mutant, *RAS* genes mutant (*HRAS*, *NRAS* and *KRAS* considered all together) and wild–type cases; then, statistical comparisons have been performed between *BRAF* mutant and wild–type and between *RAS* mutant and wild–type.

BRAF mutant nodules were generally smaller (mean tumor size 21.1 mm) than wild–type (mean 26 mm) and *RAS* mutant tumors (mean 30.3 mm). Patients' age was higher in nodules with *BRAF* mutations, but there were not statistical differences. Mutations in *RAS* genes were not significantly associated with pathological characteristics of tumors, while *BRAF* mutations correlated with: infiltration of tumor capsule (p–value 0.03), invasion of thyroid parenchyma (p–value 0.0005), invasion of thyroid capsule (p–value 0.000001), infiltration of perithyroidal soft tissue (p–value 0.0003), multifocality (p–value 0.02) and regional lymphnode involvement at presentation (p–value 0.004).

Clinical–pathological characteristics		*BRAF*			All *RAS*			All cases **(n=81)**
		mut	**wt**	*p–value*	**mut**	**wt**	*p–value*	
Age (mean)	[years]	43.6	40.2	ns	39.9	40.2	ns	41.3 ± 14.8
Sex	Male	11	12	ns	5	18	ns	23 (28.4%)
	Female	15	43		11	47		58 (71.6%)
Tumor size (mean)	[mm]	21.1	26.0	ns	30.3	26.0	ns	25.3 ± 16.9
Tumor capsule invasion	Yes	25	39	**0.03**	9	55	ns	64 (79.0%)
	No	1	16		7	10		17 (21.0%)
Thyroid parenchyma invasion	Yes	23	22	**0.001**	4	41	ns	45 (55.6%)
	No	3	33		12	24		36 (44.4%)
Thyroid capsule invasion	Yes	23	15	**0.000**	3	35	ns	38 (46.9%)
	No	3	40		13	30		43 (53.1%)
Extrathyroidal extension	Yes	17	9	**0.000**	1	25	ns	26 (32.1%)
	No	9	46		15	40		55 (67.9%)
Multifocality	Yes	20	28	**0.02**	9	39	ns	48 (59.3%)
	No	6	27		7	26		33 (40.7%)
Lymphnode metastases	Yes	15	11	**0.004**	1	25	ns	26 (32.1%)
	No	11	44		15	10		55 (67.9%)

Table 12. Statistical analysis. Associations between the main clinical pathological tumor characteristics and mutations in *BRAF* or *RAS* genes.

Since only seven cases had *TERT* promoter mutations, this molecular marker has not been considered as a parameter in the statistical analysis. Patients with *TERT* promoter mutant nodules were considerably older (mean age 57.3 years) than other ones. Moreover, all *TERT* mutant tumors had pathological features of aggressiveness, except for one FTC (a TIR3 nodule on cytology, without known concomitant mutations), which was minimally invasive and did not caused metastases. In the entire surgical cohort, only one nodule had a coexistence of *TERT* promoter and *RAS* mutations: it was an ATC. In addition, two nodules had either *TERT* promoter and *BRAF* mutations: they both were CVPTCs; one of these two patients developed disease recurrence after 18 months from initial surgery.

For TIR3 group, the performance of molecular test was calculated in terms of specificity, sensitivity, PPV and NPV, considering that in the surgical cohort the prevalence of malignancy among TIR3 nodules was 44.6%. Specificity was 94%, sensitivity 44%, PPV and NPV were 85.5% and 67.6%, respectively. A graph representing how PPV varies according to the

prevalence has been built using the values of sensitivity and specificity observed in TIR3 group (Figure 9).

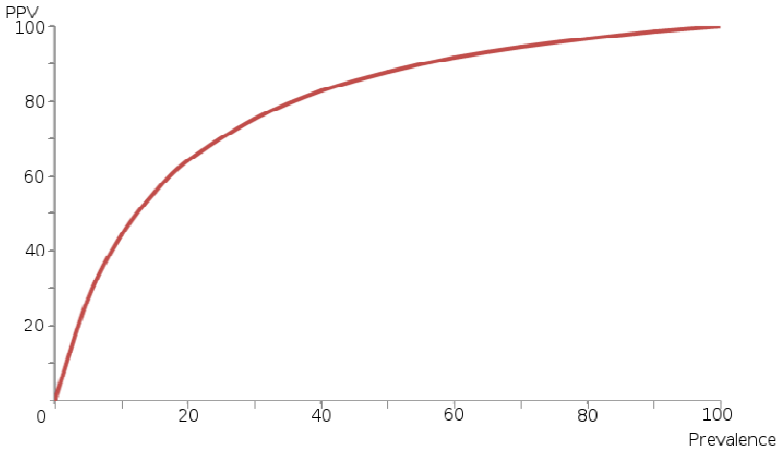

Figure 9. Molecular test performance. The curve indicates how PPV varies according to the prevalence of malignancy in the studied population.

Moreover, in order to make a comparison between the performance of the present molecular test and that of ThyroSeq and Afirma commercial tests on indeterminate nodules, a comparative PPV graph was obtained by using data of sensitivity and specificity already available in previous studies (Figure 10).

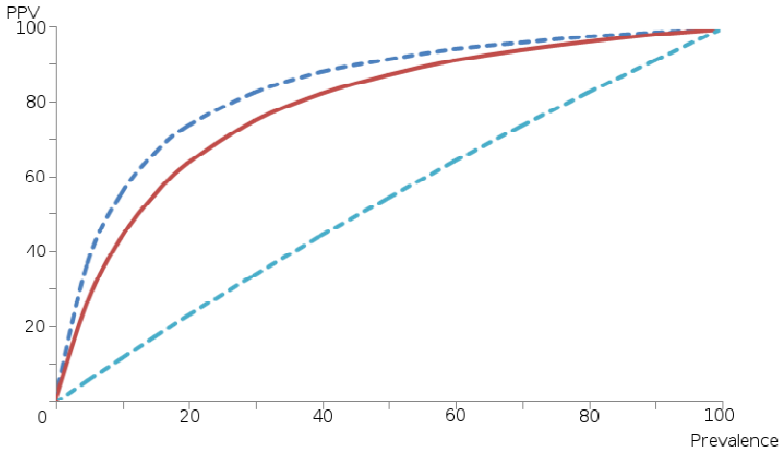

Figure 10. Comparison of molecular tests performance. The curves indicates how PPV varies according to the prevalence of malignancy in the studied population; the blue curve refers to ThyroSeq results [59]; the red curve belongs to the test proposed in the present study (showed also in Figure 9); the light–blue curve represents the performance reported for Afirma test [50].

Considering the prevalence of malignancy in TIR3 group observed in this study, the PPV would have been of 90% with ThyroSeq and 49.5% with Afirma.

5.Discussion

The preoperative evaluation of thyroid nodules represents a real clinical challenge. Indeed, thyroid nodules are frequently detected in the general population, but only 5% of all nodules turn out as malignant [63]. The challenge for a clinician is therefore to determine the best clinical strategy for each patient, avoiding either over– and under–diagnosis. The FNA biopsy allows the stratification of about 60–80% of nodules, which can be effectively classified as benign or malignant [38]. The remaining nodules are difficult to be approached, and often a diagnostic lobectomy represents the only practicable solution [64].

Recently, many studies investigating thyroid cancer genetics led to the application of molecular biomarkers in the clinical management of this nodules. The role of molecular tests as ancillary instruments in the perioperative decision making is becoming increasingly accepted [65,39,66]. One of the main limitations to the application of these molecular tools is the high cost [67,68], which sometimes involves controversy related to cost–effectiveness issues. Another crucial point limiting a wide use of molecular tests on thyroid cytology is the fact that the performance of each test is deeply influenced by the prevalence of malignancy within a specific cytology category in each different institution.

In this project a total of 630 thyroid nodules have been evaluated for a panel of molecular markers by using a quite cheap technique, namely a standard direct sequencing. The main aim was to describe the prevalence of each genetic alteration in a non–selected series of cases, and then to investigate whether and how these alterations could support the clinical management of thyroid nodules.

Taking into consideration the results of cytological examination (Figure 4), the frequency of each category observed among the 630 nodules is perfectly in line with the ranges indicated by the Italian group in the paper introducing the latest cytology classification [11]. Indeed, the incidence of TIR1 nodules is much lower than 10%, benign cytology represents about 70% of cases and TIR3 nodules are below 20%. Also the nodules diagnosed as suspicious (TIR4) are below 5%, as

indicated by the Italian group. The indicated range for malignant nodules is 4–8%, and in this series it reaches 9%.

From a molecular standpoint, the general frequency of *BRAF* mutation was to 5.7% and *RAS* genes were mutated in 7.5% of cases. This is consistent with Moses and collaborators [69], which reported an overall frequency of 5.8% for *BRAF* mutations and 5.3% for *NRAS* mutations in a series of 400 thyroid nodules. It is not easy to find in the published literature data obtained from non–selected series of FNA biopsies, and in particular there are few information about how mutational rates are distributed among cytological categories. For instance, *BRAF* mutations have been found in 81% of malignant, 59% of suspicious nodules and in none follicular neoplasm by Collet et al. [70]; in 69% of malignant and in 10.5% of suspicious/indeterminate nodules by Pizzolanti et al. [71].

Interestingly, the only *BRAF* mutation found in a TIR2 nodule was a p.K601E; the patient did not undergo surgery, according to the low–risk associated with this specific mutation and in absence of other clinical indications [72,73]. Indeed, this nodule does not represent necessarily a false negative of cytology, since *BRAF* p.K601E mutation has been found also in benign thyroid tumors [42,74]. As a consequence, a conservative approach con be considered for cytologically benign lesions harboring this molecular alteration [72,74].

In the present study, *RAS* mutations showed an incidence of 5.9% in TIR2 and 16.7% in TIR3 nodules. Up to our knowledge, this is the first appraisal of the prevalence of *RAS* mutations in these specific cytological groups obtained in the context of a truly unselected cohort of patients.

Among the 40 patients who underwent repeat FNA biopsy, the majority (80%) maintained the same cytological diagnosis; the 10% of patients had a downgraded diagnosis, while another 10% had a worsened pathological condition.

During this study, 180 nodules have been surgically removed: after histological analysis, 86 (48%) turned out as benign and 96 (52%) as malignant.

Literature data report that microcarcinomas are detected after surgery for benign thyroid disease with an incidence of about 12–13% [75]; Gürleyik et al. reported a prevalence rate of 9.4% in 395 patients undergoing thyroidectomy [76]; Askitis et al. found 33 microcarcinomas reviewing a

cohort of 228 patients treated with surgery for various benign thyroid diseases (14.5%) [77]. In the present study, microcarcinoma have been incidentally found at histological examination in 20 out of 86 benign nodules (23.3%). The majority of microcarcinomas were PTCs (95%) and presented as unifocal lesion (75%) in the extra–nodule parenchyma (80%) (Table 7). Even if microcarcinomas were indolent and patients underwent surgery because of a benign nodule, 4 out of 20 microcarcinomas (20%) had extrathyroidal invasion, and two of them caused also regional lymphnode metastases. Previous studies on incidental microcarcinomas conducted retrospectively showed lower incidences of extrathyroidal invasion as well as lower rates of local lymphnode involvement [76].

Evaluating the correlations between cytology and histology in the surgical cohort, all suspicious and malignant cases were malignant on final histology. Besides this, in TIR2 group the malignant outcome was higher than expected (14%); this is probably due to the presence of clinically suspicious conditions that unbalanced the decisions of clinicians toward a surgical approach, independently from the cytological diagnosis. This issue constitutes a bias that likely causes a misrepresentation of the real risk of malignancy found among benign cytology. Similarly, the malignancy rate among cytologically indeterminate nodules reached the 45%: comparable results have been obtained by other researchers [34–37,78]. A balanced strategy could be to render the risk of malignancy observed for each category as a range, where the lowest value is obtained considering all non–operated nodules as benign, and the higher one is the malignancy rate obtained from the surgical cohort. In this way, the risk of malignancy observed would be of 2–14% for TIR2 and 22–45% for TIR3.

Another important point of discussion is that the vast majority of malignancies hidden into TIR2 and TIR3 categories were follicular–patterned neoplasm, namely FTC and FVPTC. Moreover, the eight TIR2 nodules that resulted as malignant were seven FVPTCs and one CVPTC, which was likely misdiagnosed on cytology because of an extensive cystic component. *RAS* mutations were detected in five out of seven FVPTCs (71.4%), thus indicating a high correlation between *RAS* positivity and this specific histological type, as previously reported [79]. Indeed, it has been demonstrated that FNA cytology has a lower sensitivity than *RAS* testing in detecting FVPTC, independently from their histological subtype (non–invasive, invasive or infiltrative) [80].

Considering the indeterminate group, the 25 nodules diagnosed as malignant were CVPTC (two, 8%), FVPTC (14, 56%), solid variant PTC (two, 8%) or FTC (seven, 28%). *RAS* mutations were detected in two FTCs (29%) and in seven FVPTCs (50%). Also in these cases, the detection of *RAS* mutations on cytology was predictive of FTC or FVPTC histotypes. The only two *RAS* positive indeterminate cases that proved benign on histology were *KRAS*–mutant. This is consistent with previous findings demonstrating that *KRAS* mutations detected on cytology are less likely to label malignant lesions than *H*– and *NRAS* mutations [81].

All in all, as schematized in the diagram in Figure 11, the *RAS* mutant indeterminate nodules had a considerably higher probability to hide a cancer than *RAS* wiltype cases. Indeed, malignancy rates observed in the two group are outstandingly different: in the mutant arm, the indeterminate nodules hiding malignancy on histology were nine out of 11 (82%), in contrast to what observed in wild–type arm, where 14 out of 43 nodules (33%) were malignant.

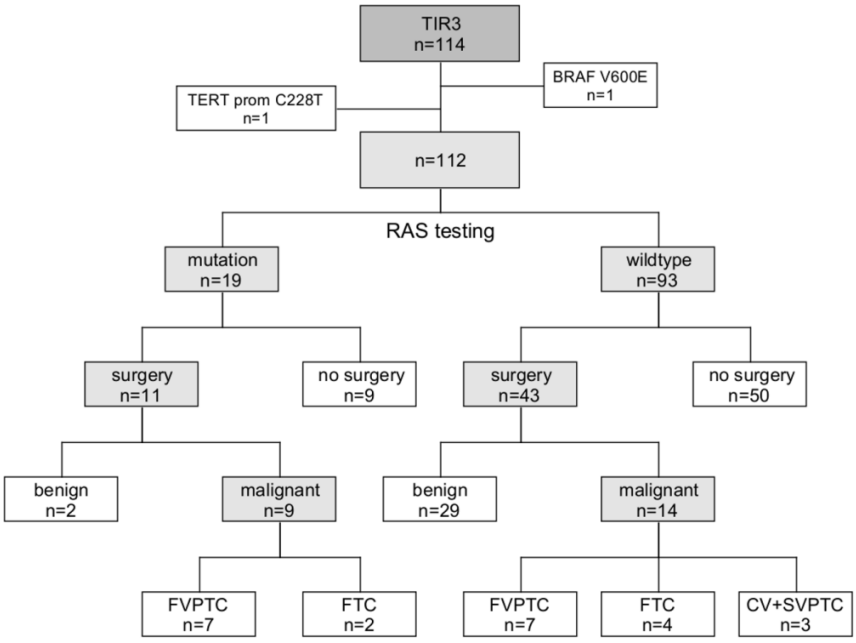

Figure 11. Diagram showing the results obtained for *RAS* testing in indeterminate nodules. On the top, the nodule harboring *BRAF* mutation and the one with *TERT* promoter mutation; the remaining 112 nodules have been divided into *RAS* mutated (on the left) and *RAS* wild–type (on the right).

In our cohort, only four out of 14 encapsulated non–invasive FVPTCs (28.6%) met the cyto–nuclear characteristics for the diagnosis of NIFTP (Figure 8). This is consistent with the findings by Jiang et al, who reclassified only eight cases as NIFTPs among 25 FVPTC potentially suitable (32%) [82]. Moreover, all NIFTP were indeterminate nodules at cytology (three TIR3A and one TIR3B). To date, the molecular landscape of NIFTPs has been barely investigated; we found that two out of four NIFTP had a *RAS* mutation.

The role of alterations in *RAS* genes in thyroid cytology has been largely discussed during the last decade [83]. In particular, they are detected in 30–45% of FVPTC and FTC, but their specificity for malignancy is considered quite low, because *RAS* mutations are also present in 20–25% of benign thyroid neoplasms [84]. The main issues supporting the role of *RAS* testing in thyroid cytology are the following:

- *RAS* mutations have a widely recognized oncogenic role in human cancers;
- several studies demonstrated that *RAS* positive nodules on cytology have a high probability to turn as malignant after surgery [65,79,85], as shown also in the present study;
- *RAS* mutations occur more frequently in malignant thyroid tumors than in follicular adenoma;
- thyroid nodules harboring *RAS* mutations that prove benign on histology are mainly follicular adenomas, which are likely to progress to FTC or FVPTC [86];
- the *RAS* mutational rates reported for PDTC and ATC are of 20–40% and 10–20% respectively: it is reasonable hypothesize that they originate from well–differentiated tumors acquiring additional oncogenic alterations, thus leading to tumor progression.

All these points suggest that a surgical approach for *RAS* positive nodules may represent the best strategy to avoid disease progression and local involvement.

However, there are also evidences that promote the opposite strategy:

- given that *RAS* mutated nodules are more likely to result as malignant tumors, these are usually low–risk lesions, without aggressive behavior; also in the present study, *RAS*

mutations did not show statistical correlations with pathological features of aggressiveness (see Table 12);

- *RAS* mutated tumors maintain generally a normal expression of thyroid iodide–handling genes [60];
- a recent study by the group of Alexander [87] underlined the low–risk nature of a series of cytologically benign, non–excised *RAS* positive nodules that showed an indolent behavior during a long–term radiographic follow–up (over 8 years).

A recent commentary by Mingzhao Xing summarizes the main findings obtained in the latest publications about the clinical role of *RAS* testing in cytology [84]. He concludes, also on the basis of the results by Alexander's group, that *RAS* positive benign nodules can be safely managed with a non–surgical approach. On the other hand, *RAS*–mutant indeterminate nodules should undergo hemithyroidectomy, and then, if additional molecular tests are negative (and here Xing cites Afirma test), they can be conservatively followed.

Of note, the results by the group of Alexander are based on the observation of only five cases [87]. Moreover, the statements by Xing are partially in contrast with the current literature affirming the importance of tumor capsule invasion as a parameter strongly affecting the risk of recurrence observed in FVPTC [23,88]. Two studies conducted by our group on miRNA and mRNA expression in follicular–patterned thyroid tumors clearly demonstrate that a phenotypic signature exists discriminating infiltrative–like tumors from adenoma–like ones: *RAS* mutated tumors (as well as *BRAF* p.K601E–mutant) are grouped among the infiltrative–like lesions, independently from their histological identity [24,89,90]. This leads to the hypothesis that *RAS*–mutant FVPTC have intrinsically a phenotype prone to progression, and it is unknown whether (and how long) it will remain silent or it will flare up. Long–term follow–up of large series of molecularly characterized nodules will be needed to demonstrate this hypothesis.

In addition, the results obtained from the present study deny that a conservative strategy could have been suitable for patients health. Indeed, two out of five *RAS*–mutant TIR2 nodules resulted as invasive FVPTCs, and four showed multiple tumor foci: invasiveness and multifocality are both parameters increasing the risk of disease recurrence [88,91]. Moreover, of the seven FVPCT which were *RAS*–mutant indeterminate nodules, only two have been reclassified as NIFTP, and

thus, on the basis of current knowledge [23], the remaining five patients would have taken benefit from completion thyroidectomy after the initial hemithyroidectomy.

In conclusion, the genotype analysis conducted on a large series of thyroid cytology specimens revealed that:

a) the general prevalence of *RAS* mutations in TIR2 and TIR3 nodules was 5.9% and 6.7%, respectively;
b) *BRAF* mutation was found in 27.3% of TIR4 and in 53.6% of TIR5 nodules; in addition, it was detected in one TIR2 (p.K601E) and in one indeterminate nodule;
c) *TERT* promoter mutations, tested only in TIR3, TIR4 and TIR5 specimens, showed a rather low frequency (4.4%); despite this low mutational rate, its detection helped to identify a TIR3 malignant nodule;
d) among the 180 nodules that were surgically removed, 86 (48%) were diagnosed as benign and 94 (52%) as malignant;
e) all TIR4 and TIR5 nodules turned out as malignant on histology; the risk of malignancy observed in TIR2 (14%) and TIR3 (45%) categories was exceedingly high due to clinical/surgical selection biases;
f) more than a half of malignant tumors had a preoperative detection of a genetic alteration (54.3%); the presence of any mutation was strongly associated with a malignant outcome, and *BRAF* mutations played a key role in this evaluation;
g) as expected, *BRAF* mutations were associated with clinical–pathological features of tumor aggressiveness, on the contrary for *RAS* mutations no correlations have been observed;
h) malignant lesions hidden into TIR2 and TIR3 groups were mainly follicular–patterned lesions, namely FTC and FVPTC; the preoperative detection of *RAS* mutations was of great value in predicting these pathologies (Figure 11), showing a high specificity;
i) in TIR3 category the mutational panel gave promising results in supporting clinical decisions, mainly in terms of PPV (Figure 9).

To sum up, this study shed light on the possible application of a basic molecular panel in routine cytology evaluation, and it showed encouraging results even using a cheap technology. Indeed,

making a comparison between the performance of the mutational panel proposed in the present project in indeterminate nodules and two well–known commercial molecular tests (Figure 10), it becomes evident that it can represent an effective and valuable tool supporting cytology assessment. A rough cost–effectiveness analysis highlights that a thorough molecular evaluation of all TIR2 specimens would not be worthwhile; this is true also for TIR4 and TIR5 nodules, for which cytology alone showed high specificity. For these categories, clinical indication could still represent the main criteria for performing molecular analysis on well selected cases. On the contrary, molecular testing could be convenient and clinically useful for indeterminate nodules that still constitute a real clinical challenge and deserve a characterization as complete as possible in order to provide patients with the best clinical treatment.

6.References

1. Tunbridge WM, Evered DC, Hall R, Appleton D, Brewis M, Clark F, Evans JG, Young E, Bird T, Smith PA (1977) The spectrum of thyroid disease in a community: the Whickham survey. Clinical endocrinology 7 (6):481-493

2. Vander JB, Gaston EA, Dawber TR (1968) The significance of nontoxic thyroid nodules. Final report of a 15-year study of the incidence of thyroid malignancy. Annals of internal medicine 69 (3):537-540

3. Guth S, Theune U, Aberle J, Galach A, Bamberger CM (2009) Very high prevalence of thyroid nodules detected by high frequency (13 MHz) ultrasound examination. European journal of clinical investigation 39 (8):699-706. doi:10.1111/j.1365-2362.2009.02162.x

4. Tan GH, Gharib H (1997) Thyroid incidentalomas: management approaches to nonpalpable nodules discovered incidentally on thyroid imaging. Annals of internal medicine 126 (3):226-231

5. Hegedus L (2004) Clinical practice. The thyroid nodule. The New England journal of medicine 351 (17):1764-1771. doi:10.1056/NEJMcp031436

6. Mandel SJ (2004) A 64-year-old woman with a thyroid nodule. Jama 292 (21):2632-2642. doi:10.1001/jama.292.21.2632

7. Brito JP, Gionfriddo MR, Al Nofal A, Boehmer KR, Leppin AL, Reading C, Callstrom M, Elraiyah TA, Prokop LJ, Stan MN, Murad MH, Morris JC, Montori VM (2014) The accuracy of thyroid nodule ultrasound to predict thyroid cancer: systematic review and meta-analysis. The Journal of clinical endocrinology and metabolism 99 (4):1253-1263. doi:10.1210/jc.2013-2928

8. Smith-Bindman R, Lebda P, Feldstein VA, Sellami D, Goldstein RB, Brasic N, Jin C, Kornak J (2013) Risk of thyroid cancer based on thyroid ultrasound imaging characteristics: results of a population-based study. JAMA internal medicine 173 (19):1788-1796. doi:10.1001/jamainternmed.2013.9245

9. Carmeci C, Jeffrey RB, McDougall IR, Nowels KW, Weigel RJ (1998) Ultrasound-guided fine-needle aspiration biopsy of thyroid masses. Thyroid : official journal of the American Thyroid Association 8 (4):283-289. doi:10.1089/thy.1998.8.283

10. Danese D, Sciacchitano S, Farsetti A, Andreoli M, Pontecorvi A (1998) Diagnostic accuracy of conventional versus sonography-guided fine-needle aspiration biopsy of thyroid nodules. Thyroid : official journal of the American Thyroid Association 8 (1):15-21. doi:10.1089/thy.1998.8.15

11. Nardi F, Basolo F, Crescenzi A, Fadda G, Frasoldati A, Orlandi F, Palombini L, Papini E, Zini M, Pontecorvi A, Vitti P (2014) Italian consensus for the classification and reporting of thyroid cytology. Journal of endocrinological investigation 37 (6):593-599. doi:10.1007/s40618-014-0062-0

12. Katoh H, Yamashita K, Enomoto T, Watanabe M (2015) Classification and General Considerations of Thyroid Cancer. Annals of Clinical Pathology 3 (1)

13. LiVolsi VA (2011) Papillary thyroid carcinoma: an update. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc 24 Suppl 2:S1-9. doi:10.1038/modpathol.2010.129

14. LiVolsi V, Baloch Z (2009) The Many Faces of Follicular Variant of Papillary Thyroid Carcinoma. Pathology Case Reviews 14 (6):214-218

15. Clary KM, Condel JL, Liu Y, Johnson DR, Grzybicki DM, Raab SS (2005) Interobserver variability in the fine needle aspiration biopsy diagnosis of follicular lesions of the thyroid gland. Acta cytologica 49 (4):378-382

16. Elsheikh TM, Asa SL, Chan JK, DeLellis RA, Heffess CS, LiVolsi VA, Wenig BM (2008) Interobserver and intraobserver variation among experts in the diagnosis of thyroid follicular lesions with borderline nuclear features of papillary carcinoma. American journal of clinical pathology 130 (5):736-744. doi:10.1309/AJCPKP2QUVN4RCCP

17. Hirokawa M, Carney JA, Goellner JR, DeLellis RA, Heffess CS, Katoh R, Tsujimoto M, Kakudo K (2002) Observer variation of encapsulated follicular lesions of the thyroid gland. The American journal of surgical pathology 26 (11):1508-1514

18. Al-Brahim N, Asa SL (2006) Papillary thyroid carcinoma: an overview. Archives of pathology & laboratory medicine 130 (7):1057-1062. doi:10.1043/1543-2165(2006)130[1057:PTCAO]2.0.CO;2

19. Faquin WC, Wong LQ, Afrogheh AH, Ali SZ, Bishop JA, Bongiovanni M, Pusztaszeri MP, VandenBussche CJ, Gourmaud J, Vaickus LJ, Baloch ZW (2016) Impact of reclassifying noninvasive follicular variant of papillary thyroid carcinoma on the risk of malignancy in The Bethesda System for Reporting Thyroid Cytopathology. Cancer cytopathology 124 (3):181-187. doi:10.1002/cncy.21631

20. Piana S, Frasoldati A, Di Felice E, Gardini G, Tallini G, Rosai J (2010) Encapsulated well-differentiated follicular-patterned thyroid carcinomas do not play a significant role in the fatality rates from thyroid carcinoma. The American journal of surgical pathology 34 (6):868-872. doi:10.1097/PAS.0b013e3181dbee07

21. Strickland KC, Howitt BE, Marqusee E, Alexander EK, Cibas ES, Krane JF, Barletta JA (2015) The Impact of Noninvasive Follicular Variant of Papillary Thyroid Carcinoma on Rates of Malignancy for Fine-Needle Aspiration Diagnostic Categories. Thyroid : official journal of the American Thyroid Association 25 (9):987-992. doi:10.1089/thy.2014.0612

22. Vaccarella S, Dal Maso L, Laversanne M, Bray F, Plummer M, Franceschi S (2015) The Impact of Diagnostic Changes on the Rise in Thyroid Cancer Incidence: A Population-Based Study in Selected High-Resource Countries. Thyroid : official journal of the American Thyroid Association 25 (10):1127-1136. doi:10.1089/thy.2015.0116

23. Nikiforov YE, Seethala RR, Tallini G, Baloch ZW, Basolo F, Thompson LD, Barletta JA, Wenig BM, Al Ghuzlan A, Kakudo K, Giordano TJ, Alves VA, Khanafshar E, Asa SL, El-Naggar AK, Gooding WE, Hodak SP, Lloyd RV, Maytal G, Mete O, Nikiforova MN, Nose V, Papotti M, Poller DN, Sadow PM, Tischler AS, Tuttle RM, Wall KB, LiVolsi VA, Randolph GW, Ghossein RA (2016) Nomenclature Revision for Encapsulated Follicular Variant of Papillary Thyroid Carcinoma: A Paradigm Shift to Reduce Overtreatment of Indolent Tumors. JAMA oncology 2 (8):1023-1029. doi:10.1001/jamaoncol.2016.0386

24. Borrelli N, Denaro M, Ugolini C, Poma AM, Miccoli M, Vitti P, Miccoli P, Basolo F (2017) miRNA expression profiling of "noninvasive follicular thyroid neoplasms with papillary-like nuclear features" compared with adenomas and infiltrative follicular variants of papillary thyroid carcinomas. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc 30 (1):39-51. doi:10.1038/modpathol.2016.157

25. Podda M, Saba A, Porru F, Reccia I, Pisanu A (2015) Follicular thyroid carcinoma: differences in clinical relevance between minimally invasive and widely invasive tumors. World journal of surgical oncology 13:193. doi:10.1186/s12957-015-0612-8

26. Smallridge RC, Marlow LA, Copland JA (2009) Anaplastic thyroid cancer: molecular pathogenesis and emerging therapies. Endocrine-related cancer 16 (1):17-44. doi:10.1677/ERC-08-0154

27. Nikiforov YE (2004) Genetic alterations involved in the transition from well-differentiated to poorly differentiated and anaplastic thyroid carcinomas. Endocrine pathology 15 (4):319-327

28. Nikiforova MN, Kimura ET, Gandhi M, Biddinger PW, Knauf JA, Basolo F, Zhu Z, Giannini R, Salvatore G, Fusco A, Santoro M, Fagin JA, Nikiforov YE (2003) BRAF mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. The Journal of clinical endocrinology and metabolism 88 (11):5399-5404. doi:10.1210/jc.2003-030838

29. Cibas ES, Ali SZ (2009) The Bethesda System for Reporting Thyroid Cytopathology. Thyroid : official journal of the American Thyroid Association 19 (11):1159-1165. doi:10.1089/thy.2009.0274

30. Physicians BTARCo (2009) Guidelines for the management of thyroid cancer. Report of the Thyroid Cancer Guidelines Update Group. London, UK

31. Kim MJ, Kim EK, Park SI, Kim BM, Kwak JY, Kim SJ, Youk JH, Park SH (2008) US-guided fine-needle aspiration of thyroid nodules: indications, techniques, results. Radiographics : a review publication of the Radiological Society of North America, Inc 28 (7):1869-1886; discussion 1887. doi:10.1148/rg.287085033

32. Pitman MB, Abele J, Ali SZ, Duick D, Elsheikh TM, Jeffrey RB, Powers CN, Randolph G, Renshaw A, Scoutt L (2008) Techniques for thyroid FNA: a synopsis of the National Cancer Institute Thyroid Fine-Needle Aspiration State of the Science Conference. Diagnostic cytopathology 36 (6):407-424. doi:10.1002/dc.20829

33. Nguyen GK, Lee MW, Ginsberg J, Wragg T, Bilodeau D (2005) Fine-needle aspiration of the thyroid: an overview. CytoJournal 2 (1):12. doi:10.1186/1742-6413-2-12

34. Cuhaci N, Arpaci D, Ucler R, Yazgan AK, Kiyak G, Yalcin S, Ersoy PE, Guler G, Ersoy R, Cakir B (2014) Malignancy rate of thyroid nodules defined as follicular lesion of undetermined significance and atypia of undetermined significance in thyroid cytopathology and its relation with ultrasonographic features. Endocrine pathology 25 (3):248-256. doi:10.1007/s12022-014-9298-7

35. Ho AS, Sarti EE, Jain KS, Wang H, Nixon IJ, Shaha AR, Shah JP, Kraus DH, Ghossein R, Fish SA, Wong RJ, Lin O, Morris LG (2014) Malignancy rate in thyroid nodules classified as Bethesda category III (AUS/FLUS). Thyroid : official journal of the American Thyroid Association 24 (5):832-839. doi:10.1089/thy.2013.0317

36. Kholova I, Ludvikova M (2014) Thyroid atypia of undetermined significance or follicular lesion of undetermined significance: an indispensable Bethesda 2010 diagnostic category or waste garbage? Acta cytologica 58 (4):319-329. doi:10.1159/000366498

37. Mathur A, Najafian A, Schneider EB, Zeiger MA, Olson MT (2014) Malignancy risk and reproducibility associated with atypia of undetermined significance on thyroid cytology. Surgery 156 (6):1471-1476; discussion1476. doi:10.1016/j.surg.2014.08.026

38. Bongiovanni M, Spitale A, Faquin WC, Mazzucchelli L, Baloch ZW (2012) The Bethesda System for Reporting Thyroid Cytopathology: a meta-analysis. Acta cytologica 56 (4):333-339. doi:10.1159/000339959

39. Ferris RL, Baloch Z, Bernet V, Chen A, Fahey TJ, 3rd, Ganly I, Hodak SP, Kebebew E, Patel KN, Shaha A, Steward DL, Tufano RP, Wiseman SM, Carty SE, American Thyroid Association Surgical Affairs C (2015) American Thyroid Association Statement on Surgical Application of Molecular Profiling for Thyroid Nodules: Current Impact on Perioperative Decision Making. Thyroid : official journal of the American Thyroid Association 25 (7):760-768. doi:10.1089/thy.2014.0502

40. Landa I, Ibrahimpasic T, Boucai L, Sinha R, Knauf JA, Shah RH, Dogan S, Ricarte-Filho JC, Krishnamoorthy GP, Xu B, Schultz N, Berger MF, Sander C, Taylor BS, Ghossein R, Ganly I, Fagin JA (2016) Genomic and transcriptomic hallmarks of poorly differentiated and anaplastic thyroid cancers. The Journal of clinical investigation 126 (3):1052-1066. doi:10.1172/JCI85271

41. Liu X, Bishop J, Shan Y, Pai S, Liu D, Murugan AK, Sun H, El-Naggar AK, Xing M (2013) Highly prevalent TERT promoter mutations in aggressive thyroid cancers. Endocrine-related cancer 20 (4):603-610. doi:10.1530/ERC-13-0210

42. Trovisco V, Vieira de Castro I, Soares P, Maximo V, Silva P, Magalhaes J, Abrosimov A, Guiu XM, Sobrinho-Simoes M (2004) BRAF mutations are associated with some histological types of papillary thyroid carcinoma. The Journal of pathology 202 (2):247-251. doi:10.1002/path.1511

43. Valderrabano P, Zota VE, McIver B, Coppola D, Leon ME (2015) Molecular Assays in Cytopathology for Thyroid Cancer. Cancer control : journal of the Moffitt Cancer Center 22 (2):152-157

44. Xing M (2007) BRAF mutation in papillary thyroid cancer: pathogenic role, molecular bases, and clinical implications. Endocrine reviews 28 (7):742-762. doi:10.1210/er.2007-0007

45. Bentz BG, Miller BT, Holden JA, Rowe LR, Bentz JS (2009) B-RAF V600E mutational analysis of fine needle aspirates correlates with diagnosis of thyroid nodules. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery 140 (5):709-714. doi:10.1016/j.otohns.2009.01.007

46. Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. CA: a cancer journal for clinicians 60 (5):277-300. doi:10.3322/caac.20073

47. Nakayama H, Yoshida A, Nakamura Y, Hayashi H, Miyagi Y, Wada N, Rino Y, Masuda M, Imada T (2007) Clinical significance of BRAF (V600E) mutation and Ki-67 labeling index in papillary thyroid carcinomas. Anticancer research 27 (5B):3645-3649

48. Riesco-Eizaguirre G, Gutierrez-Martinez P, Garcia-Cabezas MA, Nistal M, Santisteban P (2006) The oncogene BRAF V600E is associated with a high risk of recurrence and less

differentiated papillary thyroid carcinoma due to the impairment of Na+/I- targeting to the membrane. Endocrine-related cancer 13 (1):257-269. doi:10.1677/erc.1.01119

49. Fnais N, Soobiah C, Al-Qahtani K, Hamid JS, Perrier L, Straus SE, Tricco AC (2015) Diagnostic value of fine needle aspiration BRAF(V600E) mutation analysis in papillary thyroid cancer: a systematic review and meta-analysis. Human pathology 46 (10):1443-1454. doi:10.1016/j.humpath.2015.06.001

50. Nishino M (2016) Molecular cytopathology for thyroid nodules: A review of methodology and test performance. Cancer cytopathology 124 (1):14-27. doi:10.1002/cncy.21612

51. Marti JL, Avadhani V, Donatelli LA, Niyogi S, Wang B, Wong RJ, Shaha AR, Ghossein RA, Lin O, Morris LG, Ho AS (2015) Wide Inter-institutional Variation in Performance of a Molecular Classifier for Indeterminate Thyroid Nodules. Annals of surgical oncology 22 (12):3996-4001. doi:10.1245/s10434-015-4486-3

52. Valderrabano P, Leon ME, Centeno BA, Otto KJ, Khazai L, McCaffrey JC, Russell JS, McIver B (2016) Institutional prevalence of malignancy of indeterminate thyroid cytology is necessary but insufficient to accurately interpret molecular marker tests. European journal of endocrinology 174 (5):621-629. doi:10.1530/EJE-15-1163

53. Alexander EK, Schorr M, Klopper J, Kim C, Sipos J, Nabhan F, Parker C, Steward DL, Mandel SJ, Haugen BR (2014) Multicenter clinical experience with the Afirma gene expression classifier. The Journal of clinical endocrinology and metabolism 99 (1):119-125. doi:10.1210/jc.2013-2482

54. Brauner E, Holmes BJ, Krane JF, Nishino M, Zurakowski D, Hennessey JV, Faquin WC, Parangi S (2015) Performance of the Afirma Gene Expression Classifier in Hurthle Cell Thyroid Nodules Differs from Other Indeterminate Thyroid Nodules. Thyroid : official journal of the American Thyroid Association 25 (7):789-796. doi:10.1089/thy.2015.0049

55. Harrell RM, Bimston DN (2014) Surgical utility of Afirma: effects of high cancer prevalence and oncocytic cell types in patients with indeterminate thyroid cytology. Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists 20 (4):364-369. doi:10.4158/EP13330.OR

56. Lastra RR, Pramick MR, Crammer CJ, LiVolsi VA, Baloch ZW (2014) Implications of a suspicious afirma test result in thyroid fine-needle aspiration cytology: an institutional experience. Cancer cytopathology 122 (10):737-744. doi:10.1002/cncy.21455

57. McIver B, Castro MR, Morris JC, Bernet V, Smallridge R, Henry M, Kosok L, Reddi H (2014) An independent study of a gene expression classifier (Afirma) in the evaluation of

cytologically indeterminate thyroid nodules. The Journal of clinical endocrinology and metabolism 99 (11):4069-4077. doi:10.1210/jc.2013-3584

58. Labourier E, Shifrin A, Busseniers AE, Lupo MA, Manganelli ML, Andruss B, Wylie D, Beaudenon-Huibregtse S (2015) Molecular Testing for miRNA, mRNA, and DNA on Fine-Needle Aspiration Improves the Preoperative Diagnosis of Thyroid Nodules With Indeterminate Cytology. The Journal of clinical endocrinology and metabolism 100 (7):2743-2750. doi:10.1210/jc.2015-1158

59. Nikiforov YE, Carty SE, Chiosea SI, Coyne C, Duvvuri U, Ferris RL, Gooding WE, Hodak SP, LeBeau SO, Ohori NP, Seethala RR, Tublin ME, Yip L, Nikiforova MN (2014) Highly accurate diagnosis of cancer in thyroid nodules with follicular neoplasm/suspicious for a follicular neoplasm cytology by ThyroSeq v2 next-generation sequencing assay. Cancer 120 (23):3627-3634. doi:10.1002/cncr.29038

60. Cancer Genome Atlas Research Network (2014) Integrated genomic characterization of papillary thyroid carcinoma. Cell 159 (3):676-690. doi:10.1016/j.cell.2014.09.050

61. World Health Organization (2004) Pathology and Genetics of Tumor of Endocrine Organs. World Health Organization Classification of Tumors. IARC Press:Lyon

62. Macerola E, Loggini B, Giannini R, Garavello G, Giordano M, Proietti A, Niccoli C, Basolo F, Fontanini G (2015) Coexistence of TERT promoter and BRAF mutations in cutaneous melanoma is associated with more clinicopathological features of aggressiveness. Virchows Archiv : an international journal of pathology 467 (2):177-184. doi:10.1007/s00428-015-1784-x

63. Yeung MJ, Serpell JW (2008) Management of the solitary thyroid nodule. The oncologist 13 (2):105-112. doi:10.1634/theoncologist.2007-0212

64. Balentine CJ, Domingo RP, Patel R, Laucirica R, Suliburk JW (2013) Thyroid lobectomy for indeterminate FNA: not without consequences. The Journal of surgical research 184 (1):189-192. doi:10.1016/j.jss.2013.05.076

65. De Napoli L, Bakkar S, Ambrosini CE, Materazzi G, Proietti A, Macerola E, Basolo F, Miccoli P (2016) Indeterminate Single Thyroid Nodule: Synergistic Impact of Mutational Markers and Sonographic Features in Triaging Patients to Appropriate Surgery. Thyroid : official journal of the American Thyroid Association 26 (3):390-394. doi:10.1089/thy.2015.0311

66. Le Mercier M, D'Haene N, De Neve N, Blanchard O, Degand C, Rorive S, Salmon I (2015) Next-generation sequencing improves the diagnosis of thyroid FNA specimens with indeterminate cytology. Histopathology 66 (2):215-224. doi:10.1111/his.12461

67. Lee L, How J, Tabah RJ, Mitmaker EJ (2014) Cost-effectiveness of molecular testing for thyroid nodules with atypia of undetermined significance cytology. The Journal of clinical endocrinology and metabolism 99 (8):2674-2682. doi:10.1210/jc.2014-1219

68. Xing M, Haugen BR, Schlumberger M (2013) Progress in molecular-based management of differentiated thyroid cancer. Lancet 381 (9871):1058-1069. doi:10.1016/S0140-6736(13)60109-9

69. Moses W, Weng J, Sansano I, Peng M, Khanafshar E, Ljung BM, Duh QY, Clark OH, Kebebew E (2010) Molecular testing for somatic mutations improves the accuracy of thyroid fine-needle aspiration biopsy. World journal of surgery 34 (11):2589-2594. doi:10.1007/s00268-010-0720-0

70. Collet JF, Lacave R, Hugonin S, Poulot V, Tassart M, Fajac A (2016) BRAF V600E detection in cytological thyroid samples: A key component of the decision tree for surgical treatment of papillary thyroid carcinoma. Head & neck 38 (7):1017-1021. doi:10.1002/hed.24393

71. Pizzolanti G, Russo L, Richiusa P, Bronte V, Nuara RB, Rodolico V, Amato MC, Smeraldi L, Sisto PS, Nucera M, Bommarito A, Citarrella R, Lo Coco R, Cabibi D, Lo Coco A, Frasca F, Gulotta G, Latteri MA, Modica G, Galluzzo A, Giordano C (2007) Fine-needle aspiration molecular analysis for the diagnosis of papillary thyroid carcinoma through BRAF V600E mutation and RET/PTC rearrangement. Thyroid : official journal of the American Thyroid Association 17 (11):1109-1115. doi:10.1089/thy.2007.0008

72. Afkhami M, Karunamurthy A, Chiosea S, Nikiforova MN, Seethala R, Nikiforov YE, Coyne C (2015) Histopathologic and Clinical Characterization of Thyroid Tumors Carrying the BRAF Mutation. Thyroid : official journal of the American Thyroid Association. doi:10.1089/thy.2015.0227

73. Torregrossa L, Viola D, Sensi E, Giordano M, Piaggi P, Romei C, Materazzi G, Miccoli P, Elisei R, Basolo F (2016) Papillary Thyroid Carcinoma With Rare Exon 15 BRAF Mutation Has Indolent Behavior: A Single-Institution Experience. The Journal of clinical endocrinology and metabolism 101 (11):4413-4420. doi:10.1210/jc.2016-1775

74. Macerola E, Torregrossa L, Ugolini C, Bakkar S, Vitti P, Fadda G, Basolo F (2017) BRAFK601E Mutation in a Follicular Thyroid Adenoma. International journal of surgical pathology:1066896916688083. doi:10.1177/1066896916688083

75. Hay ID, Hutchinson ME, Gonzalez-Losada T, McIver B, Reinalda ME, Grant CS, Thompson GB, Sebo TJ, Goellner JR (2008) Papillary thyroid microcarcinoma: a study of 900 cases

observed in a 60-year period. Surgery 144 (6):980-987; discussion 987-988. doi:10.1016/j.surg.2008.08.035

76. Gurleyik E, Gurleyik G, Karapolat B, Onsal U (2016) Incidental Papillary Thyroid Microcarcinoma in an Endemic Goiter Area. Journal of thyroid research 2016:1784397. doi:10.1155/2016/1784397

77. Askitis D, Efremidou EI, Karanikas M, Mitrakas A, Tripsianis G, Polychronidis A, Liratzopoulos N (2013) Incidental thyroid carcinoma diagnosed after total thyroidectomy for benign thyroid diseases: incidence and association with thyroid disease type and laboratory markers. International journal of endocrinology 2013:451959. doi:10.1155/2013/451959

78. Shrestha RT, Evasovich MR, Amin K, Radulescu A, Sanghvi TS, Nelson AC, Shahi M, Burmeister LA (2016) Correlation Between Histological Diagnosis and Mutational Panel Testing of Thyroid Nodules: A Two-Year Institutional Experience. Thyroid : official journal of the American Thyroid Association 26 (8):1068-1076. doi:10.1089/thy.2016.0048

79. Hwang TS, Kim WY, Han HS, Lim SD, Kim WS, Yoo YB, Park KS, Oh SY, Kim SK, Yang JH (2015) Preoperative RAS mutational analysis is of great value in predicting follicular variant of papillary thyroid carcinoma. BioMed research international 2015:697068. doi:10.1155/2015/697068

80. Lee SR, Jung CK, Kim TE, Bae JS, Jung SL, Choi YJ, Kang CS (2013) Molecular genotyping of follicular variant of papillary thyroid carcinoma correlates with diagnostic category of fine-needle aspiration cytology: values of RAS mutation testing. Thyroid : official journal of the American Thyroid Association 23 (11):1416-1422. doi:10.1089/thy.2012.0640

81. Radkay LA, Chiosea SI, Seethala RR, Hodak SP, LeBeau SO, Yip L, McCoy KL, Carty SE, Schoedel KE, Nikiforova MN, Nikiforov YE, Ohori NP (2014) Thyroid nodules with KRAS mutations are different from nodules with NRAS and HRAS mutations with regard to cytopathologic and histopathologic outcome characteristics. Cancer cytopathology 122 (12):873-882. doi:10.1002/cncy.21474

82. Jiang XS, Harrison GP, Datto MB (2016) Young Investigator Challenge: Molecular testing in noninvasive follicular thyroid neoplasm with papillary-like nuclear features. Cancer 124 (12):893-900. doi:10.1002/cncy.21802

83. Howell GM, Hodak SP, Yip L (2013) RAS mutations in thyroid cancer. The oncologist 18 (8):926-932. doi:10.1634/theoncologist.2013-0072

84. Xing M (2016) Clinical utility of RAS mutations in thyroid cancer: a blurred picture now emerging clearer. BMC medicine 14:12. doi:10.1186/s12916-016-0559-9

85. Nikiforov YE, Steward DL, Robinson-Smith TM, Haugen BR, Klopper JP, Zhu Z, Fagin JA, Falciglia M, Weber K, Nikiforova MN (2009) Molecular testing for mutations in improving the fine-needle aspiration diagnosis of thyroid nodules. The Journal of clinical endocrinology and metabolism 94 (6):2092-2098. doi:10.1210/jc.2009-0247

86. Hsiao SJ, Nikiforov YE (2014) Molecular approaches to thyroid cancer diagnosis. Endocrine-related cancer 21 (5):T301-313. doi:10.1530/ERC-14-0166

87. Medici M, Kwong N, Angell TE, Marqusee E, Kim MI, Frates MC, Benson CB, Cibas ES, Barletta JA, Krane JF, Ruan DT, Cho NL, Gawande AA, Moore FD, Jr., Alexander EK (2015) The variable phenotype and low-risk nature of RAS-positive thyroid nodules. BMC medicine 13:184. doi:10.1186/s12916-015-0419-z

88. Ganly I, Wang L, Tuttle RM, Katabi N, Ceballos GA, Harach HR, Ghossein R (2015) Invasion rather than nuclear features correlates with outcome in encapsulated follicular tumors: further evidence for the reclassification of the encapsulated papillary thyroid carcinoma follicular variant. Human pathology 46 (5):657-664. doi:10.1016/j.humpath.2015.01.010

89. Basolo F, Giannini R, Ugoloni C, Poma A, Vitti P, Miccoli P (2016) 86(th) Annual Meeting of the American Thyroid Association. Thyroid : official journal of the American Thyroid Association 26 Suppl 1:P1-A129. doi:10.1089/thy.2016.29022.abstracts

90. Poma A, Giannini R, Ugolini C, Basolo F Gene expression profiling of follicular thyroid adenoma, minimally invasive and widely invasive follicular thyroid carcinoma. In: Pathologica JotISoAPaDC, Italian Division of the International Academy of Pathology (ed) 7° Congresso Triennale di Anatomia Patologica SIAPEC-IAP, Genova, IT, 2016. Pacini, pp 179-404

91. Kim HJ, Sohn SY, Jang HW, Kim SW, Chung JH (2013) Multifocality, but not bilaterality, is a predictor of disease recurrence/persistence of papillary thyroid carcinoma. World journal of surgery 37 (2):376-384. doi:10.1007/s00268-012-1835-2