Expert Opinion

BRAF mutation in cytology samples as a diagnostic tool for papillary thyroid carcinoma

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Introduction: Thyroid cancer is a rare disease that needs to be differentiated from the more frequent benign nodular goiter. The current, primary technique for distinguishing between benign and malignant nodules is by a fine-needle biopsy (FNB) cytological examination. This type of examination, unfortunately, often provides inconclusive results, and in recent years the introduction of molecular markers for the preoperative diagnosis of thyroid nodules has been proposed.

Areas covered: This review covers current and emerging research in the diagnostic application of the *BRAF* mutation in papillary thyroid carcinomas. It considers the available literature related to the usefulness of preoperative *BRAF* mutation analysis as a diagnostic tool to refine inconclusive cytology. It also considers the available techniques used to detect this specific mutation.

Expert opinion: Many effective methods are now available to detect *BRAF* mutation in FNB material. Thanks to its high specificity, this genetic alteration is now considered a useful diagnostic marker for patients who have indeterminate thyroid nodule cytology and is a useful tool for thyroid nodule management despite its low sensitivity limiting its application. The authors believe that, in the future, the screening of genetic alterations will enter standard clinical practice as an adjunctive tool to conventional cytology, and larger studies will provide a better definition of the best, most cost-effective combinations of markers and methods.

Keywords: BRAF mutation, fine-needle aspiration cytology, papillary thyroid cancer, thyroid cancer, thyroid cancer diagnosis

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1. Introduction

Thyroid cancer is the most common endocrine malignancy. Although its incidence has increased over the last few decades, it remains a rare disease, accounting for only 1% of all new cancers worldwide.

Thyroid cancer is generally indolent and its clinical appearance is that of a nodule slowly growing in size. Hence, a relevant clinical issue in the management of thyroid nodule is to distinguish benign from malignant nodules. This clinical problem is made more relevant by the high incidence of benign nodular goiter. Whereas thyroid cancer is rare, benign nodular goiter constitutes a very common clinical finding. Its prevalence is extremely variable, depending on different factors such as the age of the subject and geographical, environmental and genetic factors.

1.1 Epidemiology of benign and malignant thyroid nodules

The prevalence of palpable thyroid nodules in adults has been estimated to vary from 1 to 9% in iodine-sufficient areas [1-4]. However, ultrasonography allows the detection of a large number of clinically silent thyroid nodules termed

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Article highlights.

- Thyroid cancer is a rare disease whose initial clinical appearance is that of a nodule, which needs to be diagnosed among the much more common benign thyroid nodules. So far, fine-needle biopsy cytology (FNBC) is the most accurate and cost-effective method for evaluating thyroid nodules. However, inconclusive results, such as unsatisfactory, indeterminate or those suspicious for malignancy, represent a large gray zone even for skilled pathologists.
- Patients with these cytologic findings have to undergo diagnostic surgery, which will detect thyroid malignancy only in some of them. For this reason, molecular markers have been proposed to improve the differential diagnosis between benign nodules and malignant thyroid tumors.
- Activating mutations of the *BRAF* gene occur in a broad range of human cancers. Their frequency in thyroid cancer is second only to melanoma. The valine-to-glutamate substitution at residue 600 ($BRAF^{V600E}$) is nearly the only *BRAF* mutation found in about half of papillary thyroid cancer (PTC) with a histological subtype-dependent frequency. $BRAF^{V600E}$ is a very specific marker of PTC as it is not present in follicular or medullary thyroid cancer or in benign thyroid diseases.
- Several methods have been developed and applied successfully to searching for *BRAF* mutations in thyroid specimens. A sensitive method is needed because contamination with wild-type *BRAF* from non-tumor cells occurs regularly in FNB samples. Besides dideoxy sequencing of polymerase chain reaction products, some detection methods for $BRAF^{V600E}$ are restriction fragment length polymorphism, mutant allele-specific polymerase chain reaction amplification, real-time LightCycler PCR, also called Mutector assay, real-time quantitative gap ligase PCR, dual priming-based multiplex PCR analysis and pyrosequencing.
- A $BRAF^{V600E}$-based assay has 100% specificity, but its sensitivity is limited by the restricted expression of this oncogene to PTC. The frequent finding of PTC in indeterminate cytology makes $BRAF^{V600E}$ useful in this cytological category. In FNBC suspicious for malignancy, this assay can be used as a confirmatory one. The utility of adjunctive methods of cancer identification in inadequate and benign FNBC is consistent with the risk of malignancy and the rate of false negatives, respectively. The low rate of false positives limits the utility and the cost-effectiveness of *BRAF* mutation testing or other diagnostic tests for thyroid cancer in benign FNBC. *BRAF* mutation is also associated with a more aggressive disease, thus it can be used as a prognostic marker.
- The large amount of new data prompted a substantial change in the guidelines for the management of thyroid cancer on this issue. Now *BRAF* mutation is considered to be a diagnostic marker that is useful for patients with indeterminate FNBC to help thyroid nodule management.

This box summarizes key points contained in the article.

incidentalomas. Using this tool, the prevalence of thyroid nodules is found to vary from 20 to 67% in unselected populations and can be even higher in older subjects and in areas with insufficient iodine intake [5-7].

Most thyroid nodules are benign in nature and thyroid carcinoma accounts for ~ 5% of nodules [8,9]. This percentage changes largely in endemic goiter areas, where the abundance of multinodular goiters reduces the ratio of malignant nodules in favor of benign nodules. The incidence rate of thyroid cancer has increased in both sexes over the last few decades, doubling in the last 20 years [10]. This can be attributed mainly to papillary thyroid carcinoma (PTC) and particularly its follicular variant (fvPTC), while follicular thyroid carcinoma (FTC) has declined and poorly differentiated and anaplastic carcinoma has remained very rare [8]. PTC remains the most frequent thyroid cancer, accounting for ~ 85 – 90% of all cases.

2. Clinicopathological evaluation of thyroid nodules

2.1 Clinical assessment

Generally, only clinically evident nodules or incidentalomas with a diameter > 1 cm should be evaluated, as smaller incidentalomas only rarely acquire clinical significance [11]. The evaluation of a patient with thyroid nodules comprises a careful history, a meticulous clinical examination consisting of inspection and palpation of the neck, and searching for signs or symptoms of altered thyroid function. The ultrasonographic evaluation, hormonal status evaluation, search for serological autoimmunity and cytological analysis supplement the clinical assessment. The clinical assessment can divide the thyroid nodules into groups that have a suspicion of malignancy; however, it misses the goal of differentiating benign nodules from malignant neoplasms [12]. Thyroid ultrasound is a first-line diagnostic procedure, useful for detecting and characterizing nodular thyroid disease. Ultrasound features associated with malignancy are hypoechogenicity, microcalcifications, irregular margins, absent halo sign, solid pattern and intranodular vascularization [13,14]. However, these patterns taken singly have a low specificity. When multiple patterns are considered, the specificity increases but the sensitivity decreases [15]. Taking these limits into consideration, ultrasound evaluation of thyroid nodules finds its main application in the selection of those nodules that require cytology examination and to guide the needle biopsy [16].

2.2 Fine-needle biopsy cytology

So far, cytologic examination of fine-needle biopsy (FNB), performed with or without aspiration, is the most accurate and cost-effective method for evaluating thyroid nodules. Although this procedure has improved with time, it still has

some relevant limits: the sampling is sometimes insufficient for correct evaluation; some lesions are impossible to classify as benign or malignant; and the method is highly operator-dependent, with accuracy being assured only with a very experienced pathologist. Although ultrasound-guided biopsy and on-site assessment of specimen adequacy at the time of biopsy reduce the rate of non-diagnostic tests, ~ 5% of fine-needle biopsy cytology (FNBC) remains inadequate [17,18]. Differential diagnosis is sometimes difficult or impossible, and these nodules are classified as indeterminate or suspicious for malignancy. Another important issue is the way the cytology findings are reported to the clinician. The vast array of diagnostic nomenclatures now in use sometimes leads to a non-univocal interpretation. A commonly accepted categorical reporting system makes the cytology diagnosis easier to understand by the clinicians who have to use it for decision-making. Simplification of thyroid reporting into categories has been proposed by different authors and has been accepted by different institutions [19,20]. These classifications, gaining growing consensus, are based on four to six or more categories, including: unsatisfactory or inadequate for insufficient or degraded aspirates; obviously benign; indeterminate for follicular patterned lesions that cannot be fully classified, requiring histological assessment for actual classification; suspicious for malignancy; and obviously malignant [21,22]. Using this five-tier system for FNBC classification, the aspirates classified as unsatisfactory, indeterminate or suspicious represent a large gray zone even for skilled pathologists (**Table 1**) [19,23-25]. Patients with these cytologic findings have to undergo diagnostic surgery, which will detect thyroid malignancy only in some of them. For this reason, ancillary tools have been sought in recent years in order to improve the differential diagnosis between benign and malignant thyroid nodules. At present, immunologic and, more recently, molecular markers are the most promising tools to better characterize nodules with uncertain cytologic diagnosis.

3. The *BRAF* gene and its physiopathological role

3.1 Physiology of BRAF signaling

The members of the RAF family (ARAF, BRAF and CRAF or RAF-1) are protein-serine/threonine kinases that participate in the Ras-RAF-MEK-ERK signal transduction cascade, also denoted as the mitogen-activated protein kinase (MAPK) cascade [26]. The MAPK cascade plays a pivotal role in many aspects of cell biology in nearly every cell type. The RAF kinase isoforms are composed of three conserved regions: CR1, CR2 and CR3. CR1 contains a RAS-binding domain and a cysteine-rich domain, which can bind two zinc ions. CR2 is a serine/threonine-rich domain that binds to the regulatory protein 14-3-3. CR3 is the protein kinase domain [27]. Under non-stimulatory conditions, RAF resides in the cytosol. Ras induces RAF translocation to the plasma membrane, where it undergoes conformational changes induced by multiple steps, including phosphorylation, dephosphorylation and protein–protein interactions. Whereas maximal activation of CRAF requires serine and tyrosine phosphorylation by both Src and Ras, BRAF is highly activated by oncogenic Ras, is not activated by Src, and Ras and Src do not synergize [28]. BRAF activation leads to phosphorylation of the MAPK-kinases MEK-1/2, which in turn phosphorylate and activate the MAPKs. Several MAPKs have been identified: extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun-amino-terminal kinase (JNK), p38 and ERK5 [29]. Once activated, MAPKs phosphorylate a multitude of target substrates on serine or threonine residues and regulate cellular activities, including gene expression, proliferation, apoptosis, cell differentiation, movement and metabolism [30,31].

3.2 *BRAF* gene mutations in human cancer

More than 65 different missense *BRAF* mutations have been detected in human cancer so far [32]. Most of them occur within the kinase domain or in the glycine-rich loop. A few of these mutations (G466E, G466V, G596R and D594V) hamper BRAF activation by Ras and partly hamper ERK activation [33,34]. The others destabilize the inactive conformation of BRAF, thus stimulating its kinase activity leading to increased ERK activity. A single missense mutation at position 1799 accounts for nearly 90% of all *BRAF* somatic mutations detected in human cancer. This mutation occurs within the activation segment, introducing negative charges and disrupting the hydrophobic interaction between the glycine-rich loop of the N-terminal region and the activation segment of the kinase domain [32,35]. The V600 substitution, 95% of which consists of V600E, accounts for nearly 8% of all human cancer, including melanoma (27 – 70%), thyroid cancer (36 – 53%), colorectal cancer (5 – 22%), serous ovarian cancer (~ 30%), and, at lower frequency (1 – 3%), sarcoma, glioma, liver, stomach, breast and lung cancer [36]. Single point mutations are not the only genetic alterations that activate BRAF found in human tumors. Fusion of the *BRAF* gene with the *AKAP9* gene through a paracentric inversion of the long arm of chromosome 7 was first described by fluorescence *in situ* hybridization analysis (FISH) [37]. This recombination results in an AKAP9/BRAF chimera, where the BRAF autoinhibitory regulatory domain is lost, inducing constitutive activation of the kinase. In only a very few cases was the BRAF autoinhibitory regulatory domain lost by the deletion of 3 nucleotides at position 1799 – 1801, or 18 nucleotides were inserted at position 1799 – 1816, resulting in the insertion of 6 amino acids in the BRAF protein [38,39].

4. *BRAF* mutations in thyroid cancer

In the first study that described the mutations of *BRAF* in human cancer, thyroid tumors were not investigated [32]. However, as soon this tumor was considered, it became clear that this was a principal site for *BRAF* mutations and that their frequency in thyroid cancer was second only to

Table 1. Risk of malignancy of cytological categories, based on histopathological diagnosis.

Cytology	Risk of malignancy (%)*
Unsatisfactory	5 – 10
Benign	1 – 2
Indeterminate	20 – 30
Suspicious	50 – 75
Malignant	100

*Data from [19,23-25,100,94].

melanoma. From that moment, a large number of studies investigated several aspects of the *BRAF* oncogene in thyroid cancer, including its prevalence in the different histological subtypes and association with environmental, geographical, ethnic and genetic factors. The valine-to-glutamate substitution at residue 600 (V600E) is nearly the only *BRAF* mutation found in thyroid cancer, with a very few exceptions for the K601E and A598V missense mutations, the *AKAP9/BRAF* recombination, the 1799 – 1801 deletion and the 1799 – 1816 insertion [37-41]. For this reason, the studies on *BRAF* in thyroid cancer almost exclusively consider the V600E mutation. The prevalence of the $BRAF^{V600E}$ mutation in PTC varies widely in different studies, ranging from 29 to 83%. Unlike in other genetic alterations such as *RET* rearrangements, the sensitivity of the detection method used has a minor responsibility for this variability. When the analysis considers separate groups for histological and patient features, the prevalence variability is drastically reduced.

Since 2003, > 100 studies have analyzed the presence of $BRAF^{V600E}$ in the thyroid. The results are generally consistent, and considering only the major ones, the overall prevalence of $BRAF^{V600E}$ in sporadic adult thyroid cancer patients is ~ 46% (1326/2867) in PTC and 18% (48/258) in anaplastic thyroid carcinoma (ATC). None of the 237 FTC, 85 medullary thyroid carcinoma (MTC) or 1082 benign neoplasms considered harbored a *BRAF* mutation. Thus, with the only exception being a few cases of hyperplastic nodules that might represent a precursor lesion of PTC, $BRAF^{V600E}$ in the thyroid is restricted to papillary-patterned cancer and it does not occur in Hashimoto's thyroiditis, benign colloid nodules, thyroid adenomas, FTC, MTC or other types of thyroid tumor [42-45]. Its restricted expression makes $BRAF^{V600E}$ a 100%-specific PTC/papillary-patterned cancer marker within thyroid malignancies.

Although the specificity of $BRAF^{V600E}$ as a PTC marker is 100%, when considering its sensitivity it must be taken into account that PTC is a heterogeneous disease including different histological subtypes with individual clinicopathological characteristics [46,47]. The most frequent subtypes are conventional PTC (cPTC), follicular variant PTC (fvPTC) and tall cell PTC (tcPTC). Many studies that determined the prevalence of *BRAF* mutations showed a subtype-dependent distribution, tcPTC showing the highest prevalence (73%), followed by cPTC (50%) and fvPTC (19%) (**Table 2**). It is notable that the prevalence of *BRAF* mutations decreases from the most to the least aggressive PTC variant. The association of *BRAF* mutation with aggressive subtypes of PTC supports the role of *BRAF* mutation in determining the tumor's aggressiveness [39,48]. In studies where other histotypes have been compared, $BRAF^{V600E}$ was more frequent in Warthin-like PTC (75%) and oncocytic variant of PTC (55%) than in cPTC (46%) and less frequent in hyalinizing trabecular PTC [41,49-50].

Some studies investigated whether the $BRAF^{V600E}$ mutation is present in small PTC and whether its prevalence is different from that of overt PTC. Papillary thyroid microcarcinoma (PTMC) is defined by the World Health Organization as a tumor measuring 1 cm or less in its greatest dimension. PTMC is a common incidental finding, much more frequent than large PTC. Thus, it might represent an early phase in the development of PTC or an individual tumor entity with its own clinicopathological characteristics. The prevalence of $BRAF^{V600E}$ mutation in PTMC of 123 Italian and 60 Korean patients was 71 and 52%, respectively [51,52]. In a Chinese cohort of 230 patients with PTC, 92 were PTMC and 138 overt PTC, and the frequency of the $BRAF^{V600E}$ mutation did not differ between the two groups (67.4 and 65.9%, respectively) [53]. As in large PTC, in PTMC $BRAF^{V600E}$ was more frequent in tumors with papillary pattern than with follicular pattern [50]. More recently, Basolo *et al.* demonstrated a strong association of $BRAF^{V600E}$ with PTC variants (classical and tall cell) and tumor size (> 10 mm) in a very large study of small (≤ 20 mm) PTC [54]. The high frequency of *BRAF* mutation in PTMC indicates a role in the initiation of PTC tumorigenesis, and the higher frequency of *BRAF* mutation in clinically evident PTMC than in incidental PTMC suggests the need for more careful management of patients with incidental PTMC harboring $BRAF^{V600E}$ [55].

Ionizing radiation is known to be a relevant cause of genetic alteration and carcinogenesis. The role of ionizing radiation in thyroid carcinogenesis has been studied extensively in Belarus, Ukraine and parts of the Russian Federation, which have been affected by the Chernobyl accident. These studies showed that the Chernobyl accident resulted in a dramatic increase in the number of thyroid cancers of PTC type with a frequency of rearrangements of the *RET* proto-oncogene higher than in sporadic PTC [56,57]. These data are consistent with the notion that ionizing radiation is particularly effective at inducing DNA double-strand breaks. Unlike *RET* rearrangements, the percentage of *BRAF* mutations in post-Chernobyl PTC was significantly lower than in sporadic adult PTC [58-60]. These data indicate that *BRAF* mutation is a rare event in thyroid cancer that has developed in subjects exposed to radiation. The involvement of different etiologic/pathogenetic mechanisms in the development of *BRAF* mutations and RET rearrangements is also suggested by the observation that these two oncogenes are mutually exclusive [61].

Table 2. Prevalence of *BRAF* mutation in PTC subtypes; positive/total (%).

Study	cPTC	fvPTC	tcPTC
Nikiforova *et al.* [63]	28/53, (53)	2/30, (7)	6/6, (100)
Cohen *et al.* [75]	28/42, (67)	6/51, (12)	
Kim *et al.* [102]	58/70, (83)		
Trovisco *et al.* [41]	28/53, (53)	0/32, (0)	1/3, (33)
Frattini *et al.* [103]			11/14, (77)
Fugazzola *et al.* [104]	18/47, (38)	0/6, (0)	
Puxeddu *et al.* [105]	19/35, (54)		
Salvatore *et al.* [106]	16/35, (45)	3/22, (14)	5/9, (55)
Sapio *et al.* [62]	14/31, (45)	5/12, (41)	
Fugazzola *et al.* [107]	85/176, (48)	9/51, (17)	
Frasca *et al.* [82]	116/223, (52)		
Girlando *et al.* [108]	34/44, (77)	9/16, (56)	
Kebebew *et al.* [84]	126/245, (51)	7/29, (24)	
Lupi *et al.* [85]	56/82, (68)	21/112, (18)	32/40, (80)
Riesco-Eizaguirre *et al.* [109]	18/35, (51)	5/25, (20)	4/5, (80)
Giannini *et al.* [110]	10/18, (55)	9/17, (53)	12/15, (80)
Oler and Cerutti [111]	48/73, (66)	10/47, (21)	
Wang *et al.* [83]	45/94, (48)	1/3, (33)	8/11, (73)
Abubaker *et al.* [112]			8/13, (61)
Ito *et al.* [86]	230/583, (39)	4/20, (20)	6/12, (50)
Overall	977/1939, (50)	91/473, (19)	93/128, (73)

cPTC: Conventional papillary thyroid cancer; fvPTC: Follicular variant papillary thyroid cancer; PTC: Papillary thyroid cancer; tcPTC: Tall cell variant papillary thyroid cancer.

5. Methods to detect *BRAF* mutation

Detection of the *BRAF* mutation has been performed successfully in tissues and in cells obtained from FNB. Direct sequencing is the gold standard to detect a single nucleotide mutation. However, a sensitive technique is required when a low level of mutation is mixed with abundant wild-type gene copies. Dideoxy sequencing of polymerase chain reaction products can detect $BRAF^{T1799A}$ mutation in heterozygosity only when present in most of the cells in a cell mixture [62]. Thus, this method is unable to detect the mutation in thyroid aspirates when largely contaminated by normal surrounding or intranodular cells. Contamination with wild-type *BRAF* from non-tumor cells occurs regularly in FNB samples, especially when the nodule is located in the posterior thyroid and the needle track through the gland is long. Moreover, some authors suggested that *BRAF* mutation can be restricted to the papillary component of thyroid tumors, further reducing the mutated/wild-type *BRAF* ratio [63]. Several methods have been developed and applied to search for *BRAF* mutations in thyroid specimens. The sensitivity of *BRAF* mutation detection is markedly increased by polymerase chain reaction (PCR) amplification followed by restriction fragment length polymorphism (RFLP), mutant allele-specific polymerase chain reaction amplification (MASA), real-time LightCycler PCR (LC-PCR), also called Mutector assay, and real-time quantitative gap ligase PCR (GLCR) [64-68]. The first two methods are analytical assays based on observation of an electrophoresis gel band, whereas the other two are quantitative assays based on DNA probe elongation. Dual priming-based (DPO) multiplex PCR analysis is a very sensitive technique that is able to detect the presence of $BRAF^{T1799A}$ in as few as 2% of cells in a wild-type population [69]. However, DPO-based multiplex PCR analysis diagnosed 5 false positives over 693 nodules [70]. Although this is a very low percentage (0.7%), it must be taken into account because a malignant diagnosis switches a medical treatment to a surgical one. Pyrosequencing is a recently developed method of nucleotide sequencing based on real-time pyrophosphate measurement [71]. This is a sequencing-by-synthesis method that measures the incorporation of each of the four nucleotides at each template position in an automated process involving a pyrosequencer device. This method has been shown to be more sensitive than the dideoxy sequencing method, allowing detection of low amounts of the mutant allele in thyroid FNB [72]. The prevalence of *BRAF* mutation in tissue samples is not dramatically affected by the detection method applied, as occurs for *RET* rearrangements [73]. However, the choice of the most appropriate detection method for FNB specimens must take into account its sensitivity, sensibility and reproducibility (**Table 3**). Real-time LightCycler PCR and pyrosequencing fit these requirements.

6. Application of *BRAF* mutations in thyroid cancer diagnosis

6.1 Testing for *BRAF* mutations in FNB

The high frequency of *BRAF* mutations in thyroid cancer and the availability of several accurate methods of detection offered new perspectives for the classification, diagnosis and

Table 3. More frequently used methods for detection of *BRAF* mutation.

Method	Main features	Accuracy	Sensitivity	Ref.
Chain-terminator sequencing (Sanger method)	Determines the consecutive order of the nucleotide sequence	+++	+	[66,75]
PCR amplification followed by restriction fragment length polymorphism (RFLP)	Amplicons are digested with specific restriction enzymes	++	++	[64,69]
Single stranded conformation polymorphism (SSCP)	Single mutant and wild-type DNA strands migrate differently in acrylamide gel. Useful as screening assay	+	++	[41]
Mutant allele-specific polymerase chain reaction amplification (MASA)	Amplification of mutant and wild-type DNA by two distinct primers	++	++	[62,75]
LightCycler PCR (LC-PCR) (Mutector assay)	Primer extension occurs only if matching mutant DNA. Color reaction is observed if nucleotides are incorporated	++	+++	[67,75]
LightCycler fluorescence melting curve analysis PCR	Real-time PCR and fluorescence melting curve analysis analyzed by the LightCycler instrument	++	+++	[63]
Real-time quantitative gap ligase PCR (GLCR)	Real-time PCR with adjacent primers labeled with a reporter and a quencher dye	++	+++	[68]
Dual priming-based multiplex PCR analysis (DPO-PCR)	PCR products obtained using allele-specific primers modified by deoxyinosine linkers are electrophoresed in acrylamide gel	++	+++	[69,70]
Pyrosequencing	Sequencing-by-synthesis method. Determines the order of the nucleotide sequence. Requires a pyrosequencer device	+++	+++	[72,113]

risk stratification of thyroid tumors. The diagnostic potential of *BRAF* mutation has been investigated extensively on clinical grounds in an attempt to provide the pathologist with a further tool to refine the inconclusive cytology. Xing *et al.* investigated whether detection of $BRAF^{V600E}$ in FNB specimens was technically possible and compared the accuracy of DNA sequencing and Mutector assay on 45 FNB specimens, finding a *BRAF* mutation in 8/16 PTC and demonstrating that it can be readily and reliably detected in thyroid cytological specimens [67]. The possibility of amplifying a very low amount of DNA and its molecular stability make it possible to analyze material scratched from glass slides, left behind in the needle, or processed for liquid-based cytology. Each of these sources has advantages (recovery from archival glass slides, repetition of DNA extraction from ThinPrep) or disadvantages (less or insufficient material from glass slide or needle washout after smear preparation for cytology) that must be taken into account when choosing the most appropriate one.

BRAF mutation can potentially be helpful in different clinical situations: i) to identify malignant nodules with indeterminate or inadequate FNBC; ii) to confirm the malignancy in suspicious FNBC; iii) to reduce false negative results in benign FNBC; and iv) to select micro-PTCs requiring a more aggressive therapeutic approach

Retrospective analyses of surgical series have suggested a malignancy rate of 20 – 30% in indeterminate cytology, a percentage largely dependent on the pathologist's experience [19,23-24]. This cytological category, accounting for 11 – 25% of FNBC, was proposed for all FNB characterized by a high number of follicular cells, microfollicular arrangement and scanty or absent colloid, and as such it includes follicular adenoma (FA) and FTC together with Hashimoto's thyroiditis and benign nodular goiter with regressive changes [23,74]. However, PTCs are a frequent finding in indeterminate cytology, accounting for more than half of the malignancies [24]. Thus, whereas testing for *BRAF* mutation is useless for distinguishing FTC from FA, it is effective in recognizing PTC in this cytological category. Follicular, trabecular and solid-patterned PTC account for most of the indeterminate cytological findings because these variants are characterized by a prevalent follicular structure. As *BRAF* mutation positivity in PTC correlates with the presence of the papillary structure, the presence of these PTC variants reduces the overall sensitivity of *BRAF* mutation analysis in indeterminate FNBC. In a series of 55 indeterminate FNBC, Cohen *et al.* found 5 samples (3 cPTC and 2 fvPTC) harboring $BRAF^{V600E}$ out of a total of 32 malignant specimens (17%), 29 of which were PTC (21 fvPTC,

8 cPTC) [75]. Zatelli *et al.* searched *BRAF* mutations by dye terminator cycle sequencing in 89 indeterminate aspirates, finding positive 1/11 PTC [66]. More successful were Nikiforov *et al.*, who found 17 PTC in 52 indeterminate aspirates, 7 of which were bearing $BRAF^{V600E}$, and Kim *et al.*, who identified 13/18 PTC bearing $BRAF^{V600E}$ [76]. The utility of the test depends on the prevalence of *BRAF* mutation in PTC, so that in geographical areas such as Korea where it is > 80%, searching for $BRAF^{V600E}$ was demonstrated to be a highly sensitive test [42]. Overall, considering the studies reported in **Table 4**, a diagnostic test based on detection of $BRAF^{V600E}$ shows 27% sensitivity with only 1 false positive over 318 tests. PTC accounts for the vast majority of FNBC suspicious for malignancy. Hence, as expected, *BRAF* mutation testing is useful for identifying PTC in this cytological category, showing a sensitivity of 65% in 211 aspirates (**Table 4**).

The utility of adjunctive methods of cancer identification in inadequate and benign FNBC is consistent with the risk of malignancy and the rate of false negatives, respectively. According to the findings reported in **Table 4**, whereas in inadequate FNBC testing for *BRAF* mutation disclosed 15 PTC in 115 aspirates (13%), in benign FNBC only 11 in 1155 aspirates (0.9%) were positive for *BRAF* mutation. Also, in a very recent study in the Korean population, where BRAF mutation is very frequent, none of the 504 benign FNBC examined displayed $BRAF^{V600E}$ [42]. The low rate of false negative FNBC limits the utility and cost-effectiveness of *BRAF* mutation testing or other diagnostic tests for thyroid cancer in benign FNBC. Evaluation of the actual benefit and cost-effectiveness of *BRAF* mutation testing in inadequate and benign FNBC should consider overall the cytology and molecular diagnosis yields. Indeed, the utility of *BRAF* mutation testing increases with the risk of malignancy in these FNBC categories. This risk is very dependent on the cytopathologist's expertise.

Searching for mutated *BRAF* might have a great impact in decision-making of micro-PTCs. According to the recommendations developed recently by the American Thyroid Association [11], the treatment for PTC < 1 cm, low risk, unifocal and with intrathyroidal extension should be lobectomy. Multifocality and thyroid capsule invasion are histological findings and can be ascertained only postoperatively. A recent study performed by Basolo *et al.* [54] demonstrated that the presence of mutated *BRAF* in micro-PTCs strongly correlates with extrathyroidal extension and thyroid capsule invasion; because of this, the detection of *BRAF* mutation in micro-PTCs indicates the possibility of an aggressive behavior, thus suggesting that clinicians choose a more aggressive therapeutic approach.

6.2 Testing for *BRAF* mutation in blood

Thyroglobulin is the current primary marker for thyroid cancer persistence or recurrence after surgical resection and radioactive iodine therapy. However, its utility is hampered by the presence of large thyroid remnants or by thyroglobulin antibodies in the serum [77]. Melanoma and other cancer types at advanced disease stages shed sufficient cells to allow blood-based detection of specific cancer markers, including mutated *BRAF* [78,79]. Real-time quantitative gap ligase PCR was used by Chuang *et al.* in a small series of patients, demonstrating that 3/5 (60%) of cases positive for $BRAF^{V600E}$ in primary tumors also had detectable *BRAF* mutation in serum [68]. Cradic *et al.* investigated whether $BRAF^{V600E}$ could be detected in the blood of PTC patients with residual or metastatic disease and whether it might provide diagnostic information [80]. They searched for $BRAF^{V600E}$ by an allele-specific real-time PCR method in blood samples of 173 PTC patients with different disease status, finding 8/38 PTC patients positive with persistent or recurrent disease. Although circulating measurement of mutated *BRAF* does not appear to offer substantial advantages over serum thyroglobulin measurement, it might be useful in patients with a large remnant or detectable thyroglobulin antibodies, where thyroglobulin measurement is of limited value.

7. Application of *BRAF* mutation in prognosis

In recent years several studies have been performed to assess the impact of *BRAF* status on clinical outcome in PTC patients. So far the presence of an association between *BRAF* mutation and clinicopathological features related with a poor prognosis has been reported by several authors. A study performed by Xing *et al.* including 219 patients from different geographic areas showed a clear correlation between $BRAF^{V600E}$ and extrathyroidal extension, lymph node metastasis and advanced stages III/IV [39]. A large meta-analysis performed by Lee *et al.* including 1168 patients from 12 selected studies revealed a strong correlation between *BRAF* mutation and extrathyroidal extension and advanced disease stage [81]. The effectiveness of *BRAF* status as an independent predictor of such high-risk clinical indicators was demonstrated further by using a multivariate regression analysis in three more recent studies including, respectively, 323 Italian patients, 108 Chinese patients and 314 American patients [82-84]. Some other studies failed to confirm the prognostic value of *BRAF* mutation in PTC. Recently, two multivariate analyses including, respectively, 500 and 631 patients, showed no correlation between *BRAF* mutation and clinical characteristics and prognosis [85,86].

Such controversial results may be related to different issues, such as different staging of the disease at the time of the initial diagnosis, variability in the diagnostic criteria, different methodologies of clinical data collection and application of different methods for detecting *BRAF* mutation. Also, geographic, genetic and environmental factors can account for these apparently conflicting results.

Also, the age composition of the different series may hide the association between *BRAF* mutation and clinical characteristics and prognosis. Indeed, *BRAF* mutations are

Table 4. Sensitivity of *BRAF* mutation testing in different FNB categories.

Study, assay	Total samples, total PTC, *BRAF* mutation/total cancer (sensitivity %)			
	Inadequate	Indeterminate	Suspicious	Benign
Cohen *et al.*, Mutector [75]		55, 29, 5/32 (17)		11, 2, 0/2 (0)
Sapio *et al.*, MASA [100]	46, 0, 0/0	21, 0, 0/2 (0)	16, 6, 4/6 (67)	15, 0, 0/0
Sapio *et al.*, MASA [99]		25, 1, 1/2 (50)	47, 20, 9/24 (37)	18, 0, 0/0
Kim *et al.*, pyrosequencing [113]		27, 18, 13/21 (62)		16, 0, 0/0
Zatelli *et al.*, DNA sequencing [66]	1, 0, 0/0	89, 11, 1/18 (6)	22, 18, 10/22[‡] (45)	308, 6[§], 6/6[§] (100)
Nikiforov *et al.*, LC-PCR [76]		52, 13, 7/21 (33)		12, 2, 1/5 (20)
Cantara *et al.*, DNA sequencing [94]	53, 13, 8/16 (50)	41, 7, 2/7 (29)	54, 46, 37/46 (80)	87, 8, 2/9 (22)
Kim *et al.*, DPO-PCR [70]	12, 11, 7/11 (64)	8, 0, 1*/4 (0)	72, 50, 50/70 (71)	688, 2, 5[¶]/2 (100)
Overall	112, 24, 15/27 (55)	318, 79, 29/107 (27)	211, 140, 110/168 (65)	1155, 20, 11/24 (49)

*False positive.

[‡]Two breast cancer metastases.

[§]Presumed as based on follow-up because the patients with negative tests were not operated on.

[¶]Three false positives.

DPO-PCR: Dual priming-based multiplex PCR analysis; FNB: Fine-needle biopsy; LC-PCR: Real-time LightCycler PCR; MASA: Mutant-allele specific amplification.

rare in childhood PTC and significantly more frequent in old than in young patients with conventional PTC [50,63]. The association between *BRAF* mutation and greater age could reflect important biological features of this oncogene.

An overall assessment of the clinical impact of *BRAF* status on PTC can be obtained solely with an accurate evaluation of disease recurrence during a sufficiently long follow-up. Several studies focused on the relationship between *BRAF* mutation and long-term outcome. The first report concerning this field was by Xing in 2005 [43]. The author found a positive correlation between *BRAF* mutation and disease recurrence over a median clinical follow-up of ~ 15 months. Such results were obtained by multivariate analyses with adjustment for all the recognized clinicopathological prognostic factors, including a history of radioiodine treatment. A recent study performed by Elisei *et al.* reported for the first time the overall survival of 102 patients over a mean follow-up of 15 years [87]. The authors demonstrated by multivariate analysis a significantly higher mortality in the *BRAF*-positive group than in the *BRAF*-negative group. These reports strongly support the negative prognostic impact of oncogenic *BRAF* on PTC outcome.

The hypothesis of a negative prognostic significance of *BRAF* mutation in PTC is further empowered by its association with the tall cell variant that represents the most aggressive histological subtype of PTC [88,89]. Indeed, among the various subtypes tcPTC, cPTC and fvPTC, the average prevalence of *BRAF* mutation is, respectively, 73, 50 and 19%, echoing the order of aggressiveness of PTC subtypes (**Table 2**). A negative prognostic significance of *BRAF* mutation was also demonstrated in micro-PTC in a large study where $BRAF^{V600E}$ mutation was associated with multifocality, absence of tumor capsule, extrathyroidal extension, lymph node metastasis and advanced stage [54]. Owing to this large body of literature, many authors propose preoperative *BRAF* mutation testing of FNBC for predicting the extent of the initial disease and subsequent clinical outcomes in both micro- and large PTC [54,90-92].

8. Conclusions

Thyroid cancer, the most common endocrine malignancy, is a rare disease, accounting for only 1% of all new cancers worldwide, and when correctly diagnosed its prognosis is very favorable. The most relevant clinical problem with thyroid cancer is the difficulty in distinguishing it from the much more frequent benign nodular goiter. At present, a significant percentage of FNBC, the most accurate and cost-effective method for evaluating thyroid nodules, yields inconclusive results that call for other diagnostic tools. $BRAF^{V600E}$ mutation represents an ideal thyroid cancer marker as it is a genetic alteration present in a significant percentage of PTC, the most prevalent thyroid cancer type, and it is absent in benign thyroid diseases. Detection of the *BRAF* mutation has been successfully performed by different methods in cellular material obtained by FNB. Contamination with wild-type *BRAF* from non-tumor infiltrating or perinodular cells, a frequent occurrence in FNBC, has been overcome by applying sensitive methods. A *BRAF* mutation detection method will always be less sensitive than conventional cytology because this genetic alteration is present only in a fraction of PTC, which in turn is only the major fraction of total thyroid cancer. The excellent specificity but limited sensitivity of *BRAF* mutation as a malignancy marker limits its application, so that a positive test is diagnostic of cancer but a negative one is not conclusive for benignity. Thus, a *BRAF* mutation-based assay is a good means to diagnosing the malignant nature of a thyroid nodule but it

is useless to exclude it. In view of this limit, searching for *BRAF* mutation in nodules with inconclusive FNBC has a relevant impact in decision-making only in positive cases. Indeed, the presence of *BRAF* mutation allows clinicians to make a definitive diagnosis of malignancy and to advise patients to have radical surgery, reducing the need for a second intervention for completion. According to the authors' meta-analysis (**Table 4**), testing for *BRAF* mutations significantly improves the preoperative diagnosis of malignancies in the case of suspicious FNBC (~ 65% sensitivity). Less accurate is testing for *BRAF* mutation in nodules with indeterminate cytology (~ 27% sensitivity), where most thyroid cancers do not harbor the mutation (FTC) or the latter is infrequent (trabecular and solid-patterned PTC). A promising molecular approach in this field could be represented by searching for a combination of specific markers, including other oncogenes and microRNAs (miRNAs) [83,93].

The detection of *BRAF* mutation might help in decision-making for micro-PTCs, where this oncogene correlates with clinicopathological features predictive of a more aggressive disease. The presence of *BRAF* mutation in such cases might help endocrinologists to choose a more effective treatment.

9. Expert opinion

So far, cytologic evaluation of FNB is the most accurate and cost-effective method for evaluating thyroid nodules. This technique is easy to perform, cheap and highly accurate, with rare false positive and false negative results. Unfortunately, inconclusive results represent a large gray area that does not provide firm indications on the therapy to adopt.

The discovery of the molecular pathogenesis of thyroid cancer has provided the basis for further improvements of presurgical diagnostic tools. At least theoretically, a molecule with a specific pathogenetic role is the best candidate as a hallmark of that specific disease. For this reason, since the role of *BRAF* in thyroid carcinogenesis has unfolded, numerous studies have investigated its possible applications as a diagnostic marker, prognostic indicator and as a site for targeted therapy. $BRAF^{V600E}$ mutation is a 100% specific thyroid cancer marker and its detection in a biopsy specimen classifies its nature as malignant.

Different methods have been applied successfully to detect *BRAF* mutation in thyroid biopsy specimens. An ideal detection method is one that is reliable and sufficiently sensitive to detect the *BRAF* mutation even when it is mixed with abundant wild-type gene copies. Direct sequencing fits the first requirement, and in this respect it is more indicated than other analytical methods where the presence of the oncogene is detected by viewing an electrophoretic gel band. However, dideoxy sequencing of polymerase chain reaction products can detect $BRAF^{V600E}$ mutation in heterozygosity only when present in most of the cells in a cell mixture. This can produce a certain number of false negative results because contamination with wild-type *BRAF* from non-tumor cells occurs regularly in FNB samples, especially when a large immune reaction is present. Real-time LightCycler PCR, a quantitative assay based on DNA probe elongation, is much more sensitive than direct sequencing and sufficiently reliable, representing a suitable method to be applied to material obtained by FNB. Recently, pyrosequencing, a quantitative nucleotide extension sequencing, has emerged as a new methodology suitable for detecting mutated among more abundant wild-type genes. Pyrosequencing is a simple, fast, low-cost and sensitive method. In this regard, it seems to be one of the most appropriate methods for detecting *BRAF* mutation in a clinical setting.

Whereas the finding of *BRAF* mutation in a thyroid nodule biopsy has a clear diagnostic value and is of great clinical impact for patients with a nodule with inconclusive cytology, its absence leaves the nodule undiagnosed and the clinician in the difficult situation of choosing between medical or surgical therapy. The negative predictive value (NFV) for malignancy (true negative results/true negative results + false negative results) of *BRAF* mutation is ~ 0.45 in unselected FNB. As such this information is of modest clinical utility. To be useful on clinical grounds, the NPV should be calculated for each cytological category for an individual pathologist. As shown in **Table 4**, the NPV for each cytological category is largely variable between different institutions, depending on the incidence of malignancy, on the incidence of PTC variants, on the prevalence of *BRAF* mutation in PTC in that geographical/ethnical area and, to a lesser extent, on the method used to detect the mutation. For example, in the study by Zatelli *et al.* [66] in indeterminate FNBC the risk of malignancy was 20% and the incidence of PTC was 12.4%, and this determined a NPV for *BRAF* mutation detected by direct sequencing of only 0.06. In the study by Cantara *et al.* [94] in the same geographical/ethnical area, the risk of malignancy in indeterminate FNBC was 17% and the incidence of PTC was 17%, and this determined a NPV for *BRAF* mutation detected by the same method of 0.29. In different geographical/ethnical areas, by a different method, the NPV can be greatly different, as shown in the study by Kim *et al.*, where NPV was 0.62 [70]. The NPV is a parameter that can be considered by the clinician to decide the more appropriate therapy, but because this genetic alteration is present only in a fraction of PTC, which in turn is only the major fraction of total thyroid cancer, a *BRAF* mutation-based assay will never exclude totally the malignant nature of a thyroid nodule. In view of this limit, a negative test for *BRAF* mutation in nodules with inconclusive FNBC cannot be used to choose between medical and surgical treatment, but it is rather helpful to guide the extension of the surgery.

Preoperative knowledge of the malignant nature of a thyroid nodule is of indisputable value for the surgeon for deciding on the type and extension of the surgery. Total thyroidectomy without lymphadenectomy is the operation of choice for multinodular goiter [95,96]. For single benign nodular goiter, the extension of surgery is a controversial

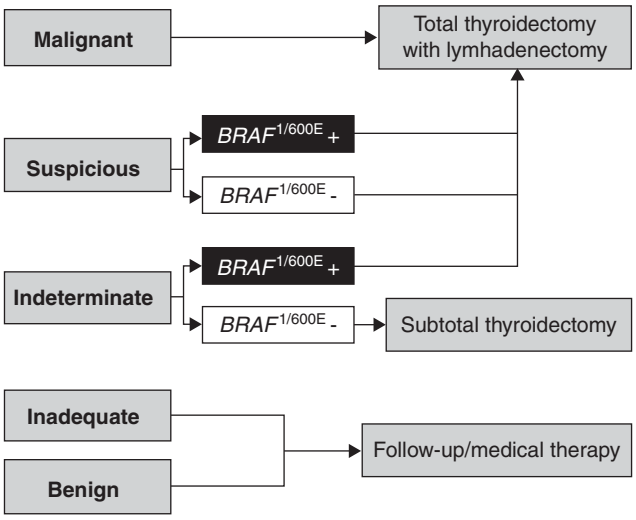

Figure 1. Testing for *BRAF* mutation in the management of thyroid nodules with different fine-needle biopsy cytology yields.

issue. Conservative surgery is an alternative choice to total thyroidectomy to reduce the risk of operative complications. Patients with single nodules with indeterminate FNBC should be referred to diagnostic thyroidectomy. Then, histological diagnosis of malignancy will impose a second intervention for completion accompanied by central lymph node dissection. The risk of malignancy for the 318 patients with indeterminate FNBC reported in **Table 4** was 33.6%; this was the percentage of patients who would need a second intervention for completion. Testing for *BRAF* mutation would address 29 patients directly to total thyroidectomy with lymphadenectomy, reducing to 24.5% the patients who would need a re-intervention.

The detection of *BRAF* mutation in routine cytology evaluation is not cost-effective in all patients undergoing FNB. Patients with FNB that is suspicious for malignancy should be referred to total thyroidectomy with lymphadenectomy because the risk for cancer is ~ 80%. A positive test for *BRAF* mutation would give the surgeon a greater awareness of the type of operation to be performed, but a negative test would not change the type of surgery. In benign and unsatisfactory cytology, *BRAF* mutation analysis would be helpful to reduce false negative or non-diagnostic results. However, owing to the very large number of FNB performed and the low risk of malignancy in these FNB categories, *BRAF* mutation analysis is not advised because of its low clinical utility and high economic impact on the public health system. This conclusion must take into account the actual cytology accuracy, which is operator dependent. A benign FNBC with a *BRAF* mutation-positive result poses the dilemma of which is the correct assessment. Mutational analysis is less subjective than cytologic smear assessment and can also detect malignant cells in suboptimal sampling that hampers correct cytological assessment. Therefore, the unequivocal presence of *BRAF* mutation (i.e., ascertained by sequencing) changes the presurgical diagnosis of nodules with benign FNBC. In the studies by Cameselle-Teijeiro *et al.* and Musholt *et al.*, PTC or atypical hyperplasia was the final histological diagnosis of benign FNBC carrying *BRAF* mutation [44,97].

Although *BRAF* mutation has been shown to be a specific PTC marker, this genetic alteration is present only in a fraction of thyroid cancer. This important limit can be overcome, as proposed in several studies, by searching for multiple thyroid cancer markers. Besides *BRAF* mutation, a panel of molecules aberrantly expressed or genetic alterations associated with thyroid cancer would include galectin-3, *RET/PTC*, *Trk*, *PAX8/PPR*γ, *RAS* mutations and miRNAs [76,98-100,94]. These studies indicate that molecular testing for a panel of mutations enhances the accuracy of FNB. Only a few years ago the use of specific molecular markers to improve the diagnostic accuracy of inconclusive nodules was not recommended by The American Thyroid Association Guidelines Taskforce (recommendation 8) [101]. Now, after a large body of evidence provided by many laboratories worldwide, that recommendation has been revised and the use of molecular markers is considered to refine indeterminate cytology on FNB to help critical situations in thyroid nodule management (**Figure 1**) [11].

In the near future, screening for genetic alterations will enter the clinical routine as an adjunctive tool to conventional cytology. Larger studies will lead to a more precise definition of the best cost-effective combinations of markers and methods to apply.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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