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High-Resolution Melting Is a Sensitive, Cost-Effective, Time-Saving Technique for *BRAF* V600E Detection in Thyroid FNAB Washing Liquid: A Prospective Cohort Study

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Key Words

BRAF gene · High-resolution melting · Washing liquids of fine needle aspiration biopsy · Papillary thyroid cancer · Thyroid

Abstract

Objective: The diagnostic accuracy of thyroid fine needle aspiration biopsy (FNAB) can be improved by the combination of cytological and molecular analysis. In this study, washing liquids of FNAB (wFNAB) were tested for *the BRAF* V600E mutation, using the sensitive and cost-effective technique called high-resolution melting (HRM). The aim was to demonstrate the feasibility of *BRAF* analysis in wFNAB and its diagnostic utility, combined with cytology. ***Design:*** Prospective cohort study. ***Methods:*** 481 patients, corresponding to 648 FNAB samples, were subjected to both cytological (on cells smeared onto a glass slide) and molecular analysis (on fluids obtained washing the FNAB needle with 1 ml of saline) of the same aspiration. *BRAF* V600E analysis was performed by HRM after methodological validation for application to wFNAB (technique sensitivity: 5.4%). ***Results:*** The cytological results of the FNAB were: 136 (21%) nondiagnostic (THY1); 415 (64%) benign (THY2); 80 (12.4%) indeterminate (THY3); 9 (1.4%) suspicious for malignancy (THY4); 8 (1.2%) diagnostic of malignancy (THY5). The *BRAF* V600E mutation was found in 5 THY2, 2 THY3, 6 THY4 and 6 THY5 samples. Papillary carcinoma diagnosis was histologically confirmed in all *BRAF*+ thyroidectomized patients. *BRAF* combined with cytology improved the diagnostic value compared to cytology alone in a subgroup of 74 operated patients. ***Conclusions:*** HRM was demonstrated to be a feasible method for *BRAF* analysis in wFNAB. Thanks to its sensitivity and cost-effectiveness, it might be routinely used on a large scale in clinical practice. In perspective, standby wFNAB samples could be analyzed a posteriori in case of indeterminate cytology and/or suspicious findings on ultrasound.

M.M. and M.L.M. contributed equally to the study. This work has been awarded with a poster price at the 16th European Congress of Endocrinology, Wrocklaw, May 3–7, 2014.

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Introduction

BRAF point mutations are common in papillary thyroid cancer (PTC) [1]. The most frequent missense mutation *BRAF* V600E is due to the replacement of valine by glutamic acid (NM_004333.4), resulting in a constitutive MEK/ERK activation, with consequent promotion of cell proliferation and tumor growth [1–3].

In the thyroid, *BRAF* mutations exclusively occur in PTC and PTC-derived anaplastic cancer, but not in follicular neoplasms [4]. Thus, *BRAF* V600E can be considered a diagnostic marker of PTC [1].

The wide clinical use of ultrasound (US) [5] allows detecting an increasing number of nodules that often require fine needle aspiration biopsy (FNAB) for ruling out thyroid cancer [6]. As the diagnostic value of FNAB is limited by the occurrence of false-negative (FN) results and diagnosis belongs to the indeterminate cytological category (15–25%) [7, 8], ancillary tests are required to improve diagnostic accuracy and patient treatment outcomes [7, 8]. For this purpose, the analysis of FNAB specimens for *BRAF* V600E was demonstrated to be feasible [9], increasing the diagnostic value of FNAB compared with cytology alone [7–10].

Formerly, the molecular marker *BRAF* V600E was tested on FNAB specimens [11] or on cells scraped from slides [12], and only recently on washing liquids of FNAB (wFNAB), obtained by washing the FNAB needle with sterile saline solution [13–15]. Until now, wFNAB has been analyzed by PCR [13], dual priming oligonucleotide [15] and amplification refractory mutation system PCR [14]. Nikiforov et al. [16] tested samples stored in an acid preservative solution for *BRAF* mutation by fluorescence melting curve analysis.

In the literature, the *BRAF* V600E mutation was found in 3–5% of benign nodules at cytology and demonstrated a high specificity for PTC [13, 15], thus supporting a role in the rule-in approach to FNAB [7].

In the present study, we specifically searched for the *BRAF* V600E mutation in wFNAB samples, independently from cytological results, using an accurate and sensitive method such as the high-resolution melting (HRM) technology, validated ad hoc. Our first target was to set up HRM, applied to wFNAB, for the detection of *BRAF* V600E and to assess the method sensitivity. The second aim was to validate it on a prospective cohort of patients undergoing FNAB, in order to demonstrate whether *BRAF* mutation analysis in wFNAB is a convenient, additional diagnostic tool, useful in clinical practice, allowing the simultaneous assessment of molecular and cytological features of the same FNABs.

Subjects and Methods

Patients

With a prospective cohort study design, in 16 months we enrolled 485 patients, undergoing diagnostic examination at the Endocrinology Unit of the University of Modena and Reggio Emilia (fig. 1). The Local Ethic Committee approved this study (Protocol No.: 122/08), and each subject provided written informed consent.

Four patients were excluded because of the lack of cytological classification data. Finally, 481 patients were considered: 117 men (24%) and 364 women (76%). Since some patients presented more than 1 nodule, a total of 648 thyroid biopsies were analyzed (fig. 1).

FNAB Procedure

Seven experienced operators performed US-assisted (Siemens Acuson Antares®, Philadelphia, Pa., USA; 10 MHz-linear scanner, B mode) FNAB with a 22- to 23-gauge needle. FNAB samples were expelled onto glass slides, smeared, fixed and stained according to standard procedures.

wFNAB samples were obtained washing out the needle with 1 ml of sterile saline solution and collecting the remaining material into a 2-ml sterile tube. This material was stored at –20°C for future molecular analyses.

Cytological Analysis

According to the American Thyroid Association guidelines [8], the cytological report resulted in the following five different classes: THY1, nondiagnostic; THY2, benign; THY3, indeterminate; THY4, suspicious for malignancy; THY5, diagnostic of malignancy. The cytological samples associated with *BRAF* positivity in wFNAB were re-reviewed by the same pathologist (A.M.).

Cell Lines

For HRM validation, we used both negative (C–) and positive controls (C+) for *BRAF* V600E mutation. C– was represented by germline DNA, extracted from whole peripheral blood of patients with documented normal thyroid US. DNA extracted from two cell lines of metastatic BRAF mutated melanoma, WM266-4 and Lu1205, represented C+.

DNA Isolation

Somatic DNA was extracted from wFNAB using a lysis buffer and proteinase K (Roche Diagnostics, Mannheim, Germany). Genomic DNA of negative controls was extracted using the Nucleon BACC1 Genomic DNA Extraction Kit (GE Healthcare, Life Sciences, Little Chalfont, UK). DNA quality and quantity were assessed by a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, NanoDrop, Wilmington, Del., USA).

HRM Analysis

Primer pairs, flanking the site of *BRAF* c.1799 (forward 5′-AGG TGATTTTGGTCTAGC-3′ and reverse 5′-ATCCAGACAACT GTTCAA-3′), were designed with Beacon Designer 7.9 (Premier Biosoft International, Palo Alto, Calif., USA). 20 ng of DNA was enough to discriminate the samples V600E+, wild-type and V600D+ (fig. 2).

HRM analysis correctly classified all samples and was randomly confirmed by direct sequencing in 23/648 (3.5%) samples, both *BRAF*+ and *BRAF*–, previously screened by HRM. Additionally, randomly chosen 30/648 samples, both *BRAF*+ and *BRAF*–, were

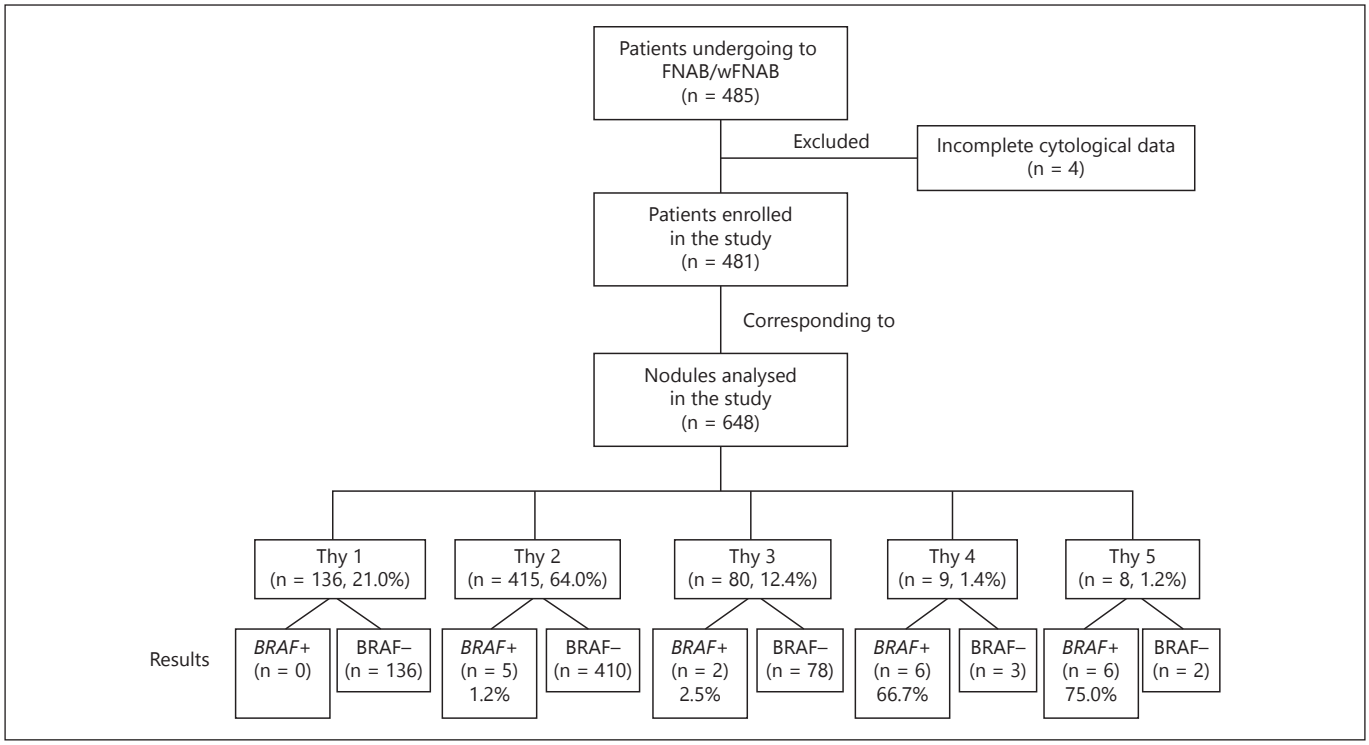

Fig. 1. Study design. *BRAF*+ = Positivity of *BRAF* mutation by HRM; *BRAF*– = wild-type sample by HRM; THY1 = nondiagnostic; THY2 = benign; THY3 = indeterminate; THY4 = suspicious for malignancy; THY5 = diagnostic of malignancy.

run 3 times in HRM, using independent reaction plates, to confirm the method reproducibility.

The sensitivity threshold of the HRM protocol was assessed analyzing through HRM serial DNA dilutions, obtained mixing the DNA of the WM266-4 cell line (V600D+) with the DNA of a *BRAF*– control. The HRM analysis detected correctly the *BRAF* mutation in very diluted samples down to samples with 5.4% of positive DNA. Therefore the validated technique is able to detect a point mutation when the mutated DNA represents at least 5.4% of the sample. These data were confirmed by pyrosequencing.

HRM raw data (preliminary melting curves) were analyzed using the specific software CFX Manager and Precision Melt Analysis (Bio-Rad Laboratories, Hercules, Calif., USA).

Pyrosequencing

Pyrosequencing with a Therascreen *BRAF* Pyro Kit on the PyroMark Q96 ID instrument (Qiagen GmbH, Hilden, Germany) was used to confirm HRM results in a subset of specimens.

Direct Sequencing

Direct sequencing was used to assess the mutational status of *BRAF* in negative and positive controls (protocol not shown).

Assessment of Sample Stability

Once thawed, the somatic DNA was extracted from wFNAB samples, immediately analyzed by HRM and refrozen at –20°C. The stability over time of the refrozen DNA was tested in HRM after years, obtaining the same excellent results (fig. 3).

Results

At cytological analysis, the 648 FNAB samples were classified as follows: THY1, 136 (21%); THY2, 415 (64%); THY3, 80 (12.4%); THY4, 9 (1.4%); THY5, 8 (1.2%) (fig. 1).

Overall, 629 (97%) wFNAB samples showed a wild-type melting curve, while 19 (3%) samples were *BRAF*+. None of the 136 THY1 samples was *BRAF*+, while 5 THY2 (1.2%), 2 THY3 (2.5 %), 6 THY4 (66.7%) and 6 THY5 (75%) samples were found to be *BRAF*+, respectively (fig. 1). The distribution of the 19 *BRAF*+ in the cytological categories was: THY2, 26.3%; THY3, 10.5%; THY4, 31.6%, and THY5, 31.6%.

Among THY2 nodules, 5 wFNAB samples (identified as s1B, s2, s3, s4 and s5) of 5 different patients (respectively patients 1, 2, 3, 4, 5) were *BRAF*+, and 4 of them were verified by pyrosequencing on scraped cells from glass slides (sFNAB; table 1).

Considering patient 1, 2 different nodules (s1A and s1B) underwent FNAB. At cytological examination, s1A was THY3 (presence of nests of epithelial cells with microfollicular architecture), while s1B was THY2 (no

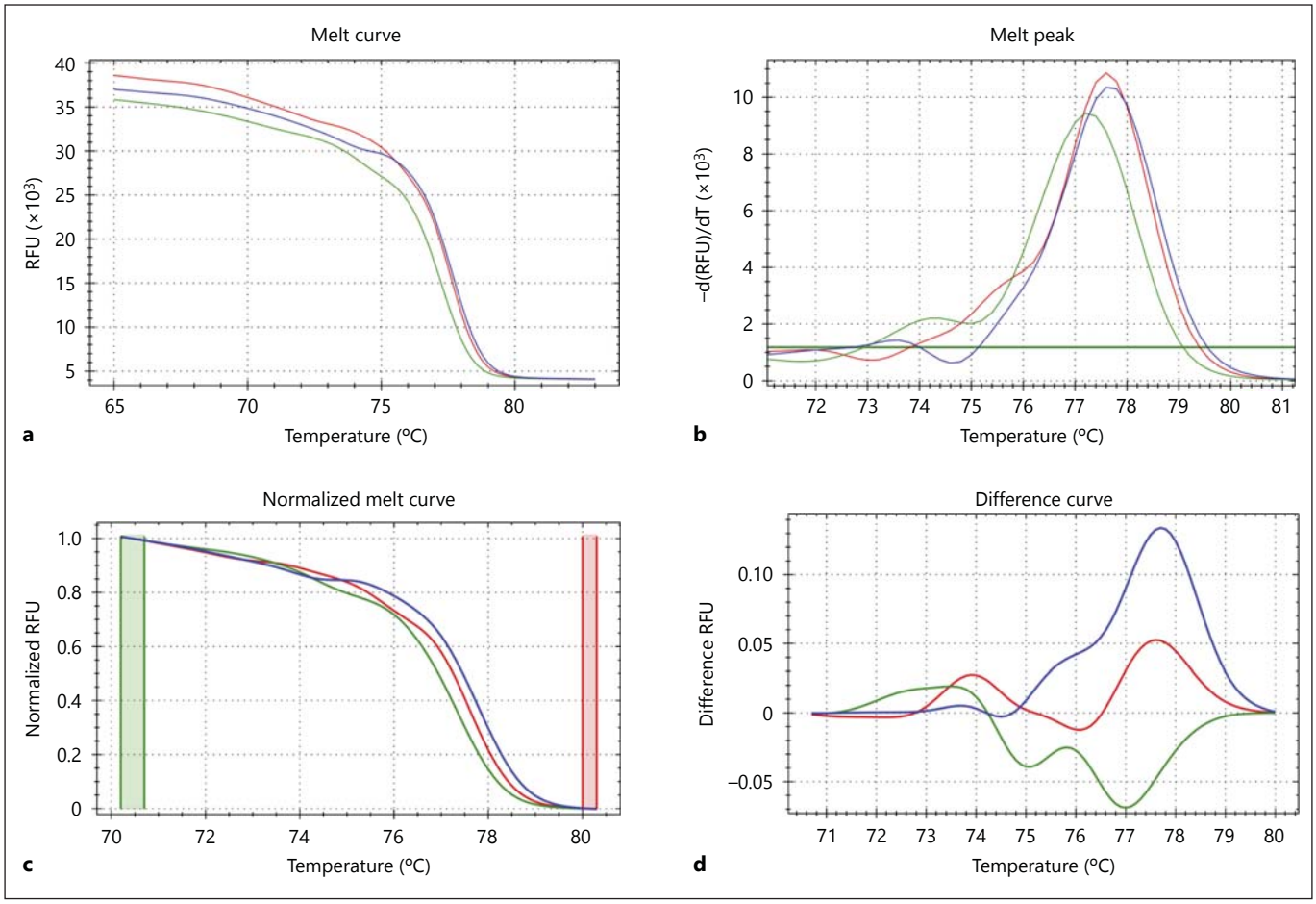

Fig. 2. Different clusters of HRM melting curves. The three different clusters, present in the picture, are obtained by the amplification and HRM analysis of exon 15 of the *BRAF* gene. While the DNA of V600D+ and V600E+ controls (green and red curves in **a–d**, respectively) were extracted by a commercial kit, the somatic DNA from wFNAB was obtained by pelletting the cells and by using 60 µl of lysis buffer (50 mM Tris-HCl at pH 8.5, 1 mM EDTA, 0.5% Tween 20 and sterile water) with 20 µl of proteinase K (10 mg/ml; Roche Diagnostics), and by incubating the samples at 56°C overnight and then at 95°C for 20 min to have a high yield from few cells also. The cluster of *BRAF*– samples is represented by a blue curve. All HRM analyses were performed using the SSO Fast Eva Green Supermix 2× (Bio-Rad Laboratories) and the same following protocol: 98°C for 2 min, 44 cycles of 3 s at 98°C and 30 s at 56.1°C, 1 cycle at 98°C for 30 s and 65°C for 1 min and 30 s, a progressive denaturation from 65 to 83°C, increasing the temperature by 0.2°C every 10 s and recording the fluorescence intensity for each increment. –d(RFU)/dT = Negative derivative (–d) of relative fluorescence units (RFU) over temperature (dT). **a** Original melting curves of V600D+, V600E+ and wild-type samples in green, red and blue, respectively. **b** Melting peaks showing the three characteristic shapes of V600D+ (green), V600E+ (red) and wild-type (blue) samples. **c** Normalization of V600D+ (green), V600E+ (red) and wild-type (blue) melting curves. **d** The differences between the three normalized curves, belonging to V600D+ (green), V600E+ (red) and wild-type (blue) samples.

cellular atypia). Surprisingly, s1B, i.e. the cytologically benign sample, was *BRAF*+ (table 1) accordingly with its US features (microcalcifications and ill-defined margins). In consideration of the US features, cytological finding and *BRAF* positivity, the patient underwent total thyroidectomy resulting in a papillary thyroid microcarcinoma in the THY2 nodule (s1B) and the presence of follicular hyperplastic architecture in the other nodule (s1A) at histology.

In patient 2, the presence of the *BRAF* V600E mutation at HRM analysis was repeatedly (3 times) verified in wFNAB and also confirmed by pyrosequencing on sFNAB. Despite THY2, US features were (hypoechoic nodule) suspicious for malignancy, and the patient underwent total thyroidectomy resulting in a partially cystic PTC at histology (table 1).

In patient 3, *BRAF* V600E positivity, at HRM on wFNAB, was confirmed by pyrosequencing on sFNAB

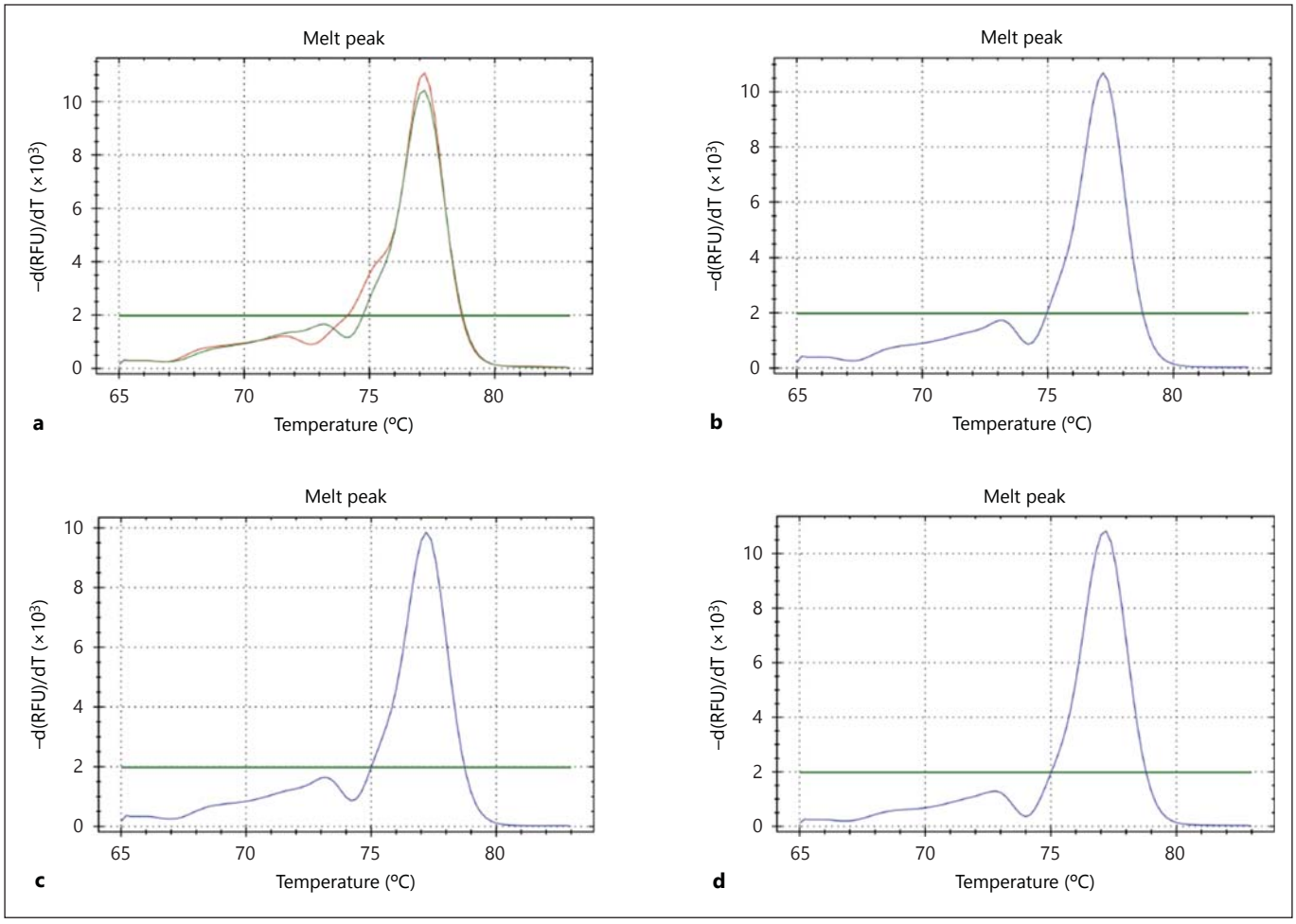

Fig. 3. Test of sample stability over time and reliability of the HRM results. We decided to test the stability of the refrozen DNA after a lapse of time by HRM analysis. Thus, 3 groups of samples, thawed and tested by HRM for the first time in April 2012, 2013 and 2014 were rethawed in May 2014 and reanalyzed by HRM, obtaining for all samples good amplification curves and excellent melting profiles, comparable to the control profiles, also confirming the same results of their first HRM analyses. –d(RFU)/dT = Negative derivative (–d) of relative fluorescence units (RFU) over temperature (dT). **a** The green and the red curves refer to the negative (wild-type) and to the *BRAF* V600E+ control, respectively. **b–d** Melting profiles obtained in May 2014, of *BRAF*– samples that were analyzed by HRM for the first time in April 2012 (**b**), April 2013 (**c**) and April 2014 (**d**), respectively.

(table 1). On US, this nodule appeared calcified. Because of the *BRAF* positivity, the patient underwent total thyroidectomy, and a classical variant of PTC was histologically documented.

Patient 4 had a multinodular goiter, with a nodule hypoechoic with halo. *The BRAF* V600E mutation was detected by HRM analysis on the wFNAB sample, but pyrosequencing did not confirm this result on the wFNAB sample (table 1). The discrepancy between HRM and pyrosequencing on wFNAB prompted us to test also the sFNAB sample that showed the wild type in HRM, pyrosequencing and direct sequencing analysis (table 1). Since the patient had previously undergone surgery for cutaneous melanoma, a possible confounding effect of a *BRAF*-mutated cutaneous neoplasia was excluded testing the melanoma histological specimen by pyrosequencing. The melanoma showed *BRAF* negativity. Considering these results and US features, a wait-and-see approach with US follow-up was considered appropriate for this patient.

Finally, sample s5 showed a hypoechoic nodule with ill-defined margins and intranodular vascularization. The FNAB sample was shown to be positive for *BRAF* V600E by HRM, but not by pyrosequencing. Direct sequencing revealed that *BRAF* positivity was due to K601E rather than V600E mutation (table 1). Conversely, wFNAB was positive with all three methods. In particular,

Table 1. Characteristics of THY2 and THY3 cytological samples with *BRAF* mutation positivity at HRM on wFNAB compared to HRM on FNAB, pyrosequencing and direct sequencing on wFNAB and FNAB

ID	Size, mm	Suspicious on US	Cyt.	*BRAF* by HRM on wFNAB/sFNAB	*BRAF* by pyrosequencing on wFNAB/sFNAB	*BRAF* by direct sequencing on wFNAB/sFNAB	Histology	Stage
s1B	8	Yes	THY2	V600E+/n.a.	n.a.	n.a.	mPTC	pT1aNxMx
s2	21	Yes	THY2	V600E+/n.a.	n.a./V600E+	n.a.	Partially cystic PTC	pT2NxMx
s3	6	No	THY2	V600E+/n.a.	n.a./V600E+	n.a.	Classical variant of PTC	pT3mN1bMx
s4	11	No	THY2	V600E+/WT	WT/WT	WT/WT	No surgery	n.a.
s5	11	Yes	THY2	V600E+/V600E+	WT/K601E+	K601E+/K601E+	Follicular variant of PTC	pT1NxMx
s6	7	Yes	THY3	V600E+	n.a.	n.a.	Classical variant of PTC	pT3NxMx
s7	13	Yes	THY3	V600E+	n.a.	n.a.	Classical and follicular variant of PTC	pT1bNxMx

Cyt. = Cytology; ID = identification number; mPTC = micropapillary thyroid carcinoma; THY2 = benign; THY3 = indeterminate; sFNAB = scraped cell DNA from FNAB slides; WT = wild-type; + = positive for the mutation; n.a. = not assessed.

pyrosequencing and direct sequencing attributed *BRAF* positivity to K601E rather than the V600E mutation. The positive *BRAF* mutational status, together with the US characteristics, recommended a surgical treatment. The molecular analysis, performed on the surgical sample by pyrosequencing, confirmed the presence of the *BRAF* K601E mutation, and the histological analysis documented a follicular variant of PTC.

Among THY3, HRM detected the *BRAF* V600E mutation in 2 wFNAB samples (s6 and s7; table 1). Both nodules (s6 and s7) had also US features suspicious for malignancy (markedly hypoechoic), and both patients underwent surgery resulting in a classical variant of PTC (s6) and follicular and classic variants of PTC (s7). The a posteriori review of sample s7 by the cytopathologist resulted in a reclassification into THY4 (suspicious for PTC).

Finally, HRM detected the *BRAF* V600E mutation in 6/9 THY4 and in 6/8 THY5 samples. In all these patients the histology confirmed the diagnosis of PTC.

Comparing the three different techniques, direct sequencing, pyrosequencing and HRM, and comparing the positive results of HRM analysis with the histological results, we can assert that we did not have false-positive results.

However, we do not have data regarding the FN rate of HRM analysis. Nevertheless, considering that pyrosequencing and HRM sensitivity is similar, we suppose that their FN rate is approximately the same.

Diagnostic Value of BRAF HRM Analysis on wFNAB

Based on history, clinical evaluation, US features and biochemical analyses, only 74 of the 481 patients underwent thyroid surgery providing histology outcome on a total of 94 nodules for which also the cytological analysis was available (table 2). In this subgroup, the addition of the *BRAF* analysis on wFNAB (obtained by HRM) to the cytological analysis improved sensitivity (from 43.6 to 59.0%) and the positive predictive value (from 43.6 to 100%) of the presurgical diagnostic procedures. The accuracy of cytology alone and cytology plus *BRAF* on wFNAB was 76.6 and 81.8%, respectively. Cytology combined with BRAF resulted in a better performance of US-assisted FNAB as far as the 'rule-in' approach is concerned, without any improvement of the 'rule-out' approach.

Discussion

The novelty of this study lies in the application of the powerful, highly sensitive, low-cost and easy-to-perform molecular analysis technique of HRM on wFNAB samples collected during routine clinical practice.

The validation of this technique allows collecting left-over cells in the needle without a further ad hoc FNAB. Compared to traditional procedures requiring 2 different FNABs for molecular and cytological analyses [16–18], the advantages of wFNAB are: (i) the certainty that both the material for molecular and cytological analyses derives from the same nodule; (ii) reduction of the patient's discomfort; (iii) procedural saving time (only 1 neck puncture); (iv) a more accurate diagnosis; (v) a posteriori analysis only in case of indeterminate cytology and/or other clinical indications (i.e. suspicious US features) after wFNAB storage. Besides, HRM on wFNAB seems to be further cost-saving. Differently from other wFNAB techniques [14, 19, 20], in fact, the use of saline for storing

Table 2. Diagnostic value of cytology alone and cytology combined with HRM/*BRAF* analysis on wFNAB compared with histology in the subgroup of patients (n = 74) who underwent thyroid surgery

a Cytology

Cytological category	Nodules with histology/total, n (%)	Benign histology, n	Malignant histology, n	wFNAB *BRAF*+ by HRM, n
THY1	5/136 (3.7)	4	1	0
THY2	25/415 (6.0)	17	8	4
THY3	47/80 (58.7)	34	13	2
THY4	9/9 (100)	0	9	6
THY5	8/8 (100)	0	8	6

b Overall diagnostic value of US-FNAB procedures

	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Cytology alone	43.6	100	43.6	71.4
Cytology + *BRAF* on wFNAB	59.0	100	100	75.4

BRAF+ = Positivity of BRAF mutation by HRM; THY1 = nondiagnostic; THY2 = benign; THY3 = indeterminate; THY4 = suspicious for malignancy; THY5 = diagnostic of malignancy; US-FNAB = ultrasound-assisted fine needle aspiration biopsy; sensitivity = number of true positives divided by the number of true positives plus the number of false negatives; specificity = number of true negatives divided by the number of true negatives plus the number of false positives; PPV = positive predictive value (number of true positives divided by the number of true positives plus the number of false positives); NPV = negative predictive value (number of true negatives divided by the number of true negatives plus the number of false negatives).

FNAB leftover cells is less expensive than preservative solutions [16, 18, 19] and allows storing samples for long. Moreover, HRM is more sensitive and faster than DNA sequencing and both less expensive and time saving than direct sequencing and pyrosequencing, respectively. Accordingly, the entire procedure costs about EUR 7.50 and requires half and one third of the time compared to pyrosequencing and direct sequencing, respectively.

Differently from previous studies involving subjects with documented malignant [21] or indeterminate cytological diagnosis [16], in this study all patients were prospectively enrolled regardless of their cytological categories (THY1–THY5). Even the analysis of the small subgroup of patients who underwent surgery covers all the cytological categories and suggests that the diagnostic value of FNAB is improved by the addition of *BRAF* analysis on HRM, as previously suggested by using different molecular techniques [7, 22]. However, larger prospective studies, aimed at defining the 'number needed to diagnose', are required to provide information on the real effectiveness of this molecular diagnosis in thyroid cancer patients.

Among THY2 samples, the *BRAF*+ rate (1.2%) was higher than in previous studies [20, 23], but concordant with the known estimated risk of malignancy of 0–3% [24]. Cytological FN rates are underestimated in the THY2 category since histological verification is not always available since patients rarely undergo thyroidectomy. FN results in the THY2 category might depend on the cytopathologist's experience, sampling errors (e.g. FNAB within healthy thyroid rather than nodular tissue) or microcarcinoma [25]. In thyroid nodules greater than 4 cm, FNAB might be not representative of the entire lesion resulting in an increased FN rate (about 13%) [26]. Thus, the overall FN rate in THY2 ranges from 3 to 13% in different settings [22, 27] and accounts for a relevant number of misdiagnoses in consideration of the considerable prevalence of THY2 nodules [23]. Again, HRM molecular analysis on wFNAB reduced the FN rate in 4 THY2 nodules that were BRAF+ and improved the diagnostic value of cytology alone (table 2).

In addition, HRM seems to work even in detecting mutations in DNA positions close to the classic V600E mutation since, correctly identified as *BRAF*+, patient 5 (table 1) carried the rare *BRAF* mutation K601E in a follicular variant of PTC; the latter is known to be associated with the K601E mutation [28–30].

The *BRAF* V600E mutation was detected only in 2 out of 80 THY3 nodules (2.5%), a rate similar to that (about

1.5%) of previous studies [16]. Thus, HRM on wFNAB coupled to cytology might be useful for better selecting patients deserving surgery among those with indeterminate nodules. As THY3 nodules were shown to be benign at histology in 70–80% of cases [31], unnecessary surgery should be avoided.

No *BRAF* mutation was found in inadequate samples even though the possibility of *BRAF* positivity cannot be completely ruled out in THY1 [20, 22]. Differently from previous reports [32], in our hands HRM is a valid methodology also in samples characterized by poor cellularity (<6 clusters of 10 cells/group).

In clinical practice, several characteristics make HRM on wFNAB an ideal procedure to be introduced in a routine diagnostic setting as an adjunct to cytology [17, 22, 33, 34]. In particular, HRM on wFNAB is feasible and allows saving of time, financial resources and human efforts especially when used in an a posteriori approach. This sort of '*BRAF* reflex' could be useful especially in case of suspicious nodules on US, cytologically classified as benign or indeterminate since it improves the diagnostic value of cytology alone, resulting in early cancer diagnosis and a more appropriate indication for surgery (e.g. central neck compartment) in FN nodules. Further costs due to inappropriate surgery in indeterminate nodules and the reoperation after lobectomy for thyroid cancer might also be avoided. This cost-effectiveness of *BRAF* analysis has already been demonstrated also by using *BRAF* assays which are more expensive than HRM [7, 34].

Furthermore, the HRM approach could include analysis of other discrete point mutations related to thyroid cancer (e.g. *RAS*), extending its diagnostic performance. A possible interesting molecular analysis regards the mutations in the telomerase reverse transcriptase. The inclusion of telomerase reverse transcriptase promoter mutations in the molecular analysis of FNABs allows a better preoperative risk stratification, especially when coupled with *BRAF* analysis [35].

In conclusion, the development of less expensive, highly sensitive methods for *BRAF* assays allows improving the cost-effectiveness of molecular analyses coupled to FNAB, a prerequisite for introducing these tests in the routine workout for the diagnosis of thyroid nodules, especially in geographic areas where the frequency of *BRAF* mutations is not high [36].

HRM analysis on wFNAB represents a valid and sustainable procedure for large-scale *BRAF* analysis useful to help the clinician's decision-making in THY2 and THY3 nodules, especially when suspicious US features are present.

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Disclosure Statement

The authors declare that there is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

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