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A High-Resolution Melting Protocol for Rapid and Accurate Differential Diagnosis of Thyroid Nodules

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A large majority of thyroid nodules are benign, and only 5% have malignant features on cytological examination. Unfortunately, fine-needle aspiration is inconclusive in approximately 30% of all thyroid biopsies, because the cytological features are indeterminate (suspicious for malignancy but not completely diagnostic or nondiagnostic). Wide panels of somatic mutations have been identified in thyroid cancers, and detection of genetic alterations in fine-needle aspirate has been demonstrated to improve diagnostic accuracy. Nevertheless, the relatively high number of genetic targets to be investigated, in comparison with the low percentage of malignant samples, makes the usual diagnostic protocol both time-consuming and expensive. We developed a reliable and sensitive protocol based on high-resolution melting analysis for the rapid screening of mutations of *KRAS, HRAS, NRAS*, and *BRAF* oncogenes in thyroid fine-needle aspirations. The entire procedure can be completed in approximately 48 hours, with a dramatic reduction in costs. The proposed protocol was applied to the analysis of 260 consecutive fine-needle aspiration biopsy (FNAB) samples. In 35 of 252 samples, 36 sequence variants were detected for *BRAF* (17 samples), *NRAS* (6 samples), *HRAS* (3 samples), *KRAS* codon 12 (9 samples), and *KRAS* codon 61 (1 sample). ***(J Mol Diagn 2012, 14: 501–509; http://dx.doi.org/10.1016/j.jmoldx.2012.03.003)***

Thyroid cancer is the most common malignancy of the endocrine system, accounting for approximately 1% of all malignancies in Western countries.[1] The incidence of thyroid cancer has increased 2.6-fold in the last 30 years. This change is attributed not only to an increment in papillary thyroid carcinoma,[2] but also to more wisely conducted medical surveillance and improvements in diagnostic tools.[3] The large majority of thyroid nodules, as discovered with the use of new diagnostic imaging techniques, are asymptomatic and benign. In this context, diagnostic studies are becoming essential to identify the small fraction of thyroid nodules that harbor malignant disease and to predict when surgery is indicated. Fine-needle aspiration biopsy (FNAB) has emerged over the past 30 years as an accurate and cost-effective procedure for the preoperative screening of thyroid nodules, representing the gold standard for differential diagnosis of benign and malignant nodules.[4] Under recent guidelines,[5,6] cytological smears are classified in five categories for the diagnostic report: Thy 1, nondiagnostic; Thy 2, benign or negative for malignant cells; Thy 3, all follicular lesions (including atypia/follicular lesion of undetermined significance and follicular neoplasm or suspicious for follicular neoplasm); Thy 4, suspicious; and Thy 5, diagnostic for malignancy.

Although the overall accuracy of FNAB is considered excellent, approximately 30% of cytological aspirates do not allow definitive diagnosis of malignancy, because of intrinsic and unavoidable characteristics of samples.[7] The major limitations of FNAB procedures are linked to inadequate and indeterminate specimens and, in that sense, are also linked respectively to the nondiagnostic or follicular lesions categories.[8,9] Thus, a clinical need emerges for the characterization of aspirates with suspicious features but with unsatisfactory cellularity, to allow accurate distinction of benign from malignant forms of follicular lesions.

Several somatic mutations have been identified in thyroid cancer, stimulating the search for genetic alterations in FNAB that could increase the diagnostic accuracy of traditional cytology. Numerous studies have demonstrated that identification of specific mutations in cytological specimens can assist in the diagnosis by FNAB and in the clinical decision to excise the nodule and to intensify the follow-up.[10–16] In most cases, genetic alterations are represented by activating mutations of oncogenes that are mutually exclusive and linked to distinct histological subtypes, with a demonstrated pathogenic role in thyroid cell transformation as effectors of the RAS/RAF/MAPK signaling cascade.

The presence of *BRAF* mutations, a frequent alteration in papillary carcinoma (PTC), evolves into an unregulated activation of the intracellular MAPK pathway that can promote tumorigenesis and tumor progression. The main mutation of *BRAF*, identified exclusively in PTC, affects

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nucleotide 1799 in exon 15 and results in thymine-to-adenine transversion, which translates into valine-to-glutamate substitution at residue 600 (p.V600E).[17] The adjacent p.K601E mutation has rarely been identified,[18] and no mutation in exon 11 has been described in thyroid cancers. *BRAF* mutations are associated with poor clinical prognosis due to extrathyroid invasion and higher risk of relapse and metastasis.[19]

Mutations in the family of *RAS* oncogenes, which encode for G-proteins that also convey signals to the MAPK pathway, are more common in follicular carcinomas (FTC)[20] and in follicular variant of papillary carcinoma (fvPTC).[21] Point mutations in the *HRAS*, *KRAS*, and *NRAS* genes are associated with specific domains of the protein and are able either to increase its affinity for substrate (substitution in residues 12 and 13) or to inactivate the autocatalytic GTPase function (residue 61). *RAS* mutations seem to be related to benign as well as malignant growth of nodules; however, it is becoming evident that alterations in this family of oncogenes are competent to lead toward anomalous cellular transformation, through mechanisms of genomic instability[22,23] and promotion of additional mutations.[24]

Because of the frequency and clinical relevance of *BRAF* mutations in thyroid papillary carcinomas (~45% of all cases) and *RAS* mutations in follicular subtypes (40% to 50% of these tumors),[25] the detection of these genetic alterations in FNABs has been widely adopted, to increase the specificity of testing. Development of rapid and accurate molecular methods could therefore be important for the screening of the large number of samples routinely collected by FNAB, to obtain a molecular diagnosis in a time frame compatible with clinical decision-making.

High-resolution melting (HRM) analysis is a technique recently developed for mutation scanning of PCR products.[26–29] The discrimination between wild-type and variant sequences is obtained by the comparison of the dissociation shape of amplicons when exposed to increasing temperature. A change in melting profile, generated by signal of fluorescent dyes intercalating only double-stranded DNA, is caused by a variation in the sequence, relative to the reference sample.[30]

Our aim in the present study was to assess an inexpensive HRM analysis platform for the accurate analysis of a consistent number of cytological samples. The principal objectives were the optimization of an accurate test for a rapid screening of mutation-positive thyroid nodules and the evaluation of a molecular marker panel to refine the diagnostic accuracy among categories of cytological specimens.

Materials and Methods

FNAB Collection

Nodule biopsies were obtained from 260 consecutive patients [53 men and 207 women; mean age, 55.1 years (range, 21–80 years)] undergoing FNAB for suspicious thyroid nodules. Eight samples out of the series, resulted negative at thyroglobulin assay, were excluded from subsequent molecular analysis.

FNABs were performed under ultrasound guidance using a 21- to 23-gauge needle by performing five or six passes. FNAB was performed on all single nodules with a diameter >5 mm. In 13 patients with multiple nodules, FNAB was performed on a maximum of two dominant nodules for each patient. One biopsy was performed for each nodule. All samples to be submitted to cytopathology analysis were processed according to thin-layer cytology technique, as described previously.[31] The widest diameter of all nodules ranged from 6 to 75 mm (23.8 ± 11.0, mean ± SD).

The sample obtained from biopsies was used for classical cytology and the needle washing was used for molecular assay. Fifty patients randomly selected underwent a second FNAB, which was used for molecular analysis (on both tissue and needle washing). All FNAB samples were collected in a single tube containing 500 μL of RNAlater stabilizing reagent (Qiagen, Milan, Italy). After one night at 4°C, samples were stored at −80°C until extraction of nucleic acids. The study was approved by the local ethics committee. Informed consent had been obtained previously from all patients.

DNA and RNA Purification from FNAB and Control Cell Line Selection

Cytological material was centrifuged at 15,890 × *g* for 15 minutes, and then the buffer was removed without disturbing the pellet. Immediately, 700 μL of RLT lysis buffer from an RNeasy micro kit (Qiagen) was added to the samples. Resuspended samples were divided into two 350-μL aliquots, one for DNA and one for RNA extraction. DNA was extracted using a Qiagen QIAamp DNA micro kit according to manufacturer's protocol for isolation of genomic DNA from tissue. Similarly, RNA was extracted using a Qiagen RNeasy micro kit according to the manufacturer's protocol for purification of total RNA from animal and human tissues. The entire RNA sample was reverse-transcribed using MuLV Reverse Transcriptase (Applied Biosystems-Life Technologies, Foster City, CA) and random hexamer primers in a final volume of 40 μL. Before testing for chromosomal rearrangements, 2.5 μL of cDNA was evaluated for *GAPDH*, using Applied Biosystems TaqMan control reagents, and for thyroglobulin gene expression (TaqMan gene expression assays; Hs00794359_m1, NM_003235.4). RNA from the human papillary thyroid carcinoma cell line TPC1 and from the human prostatic carcinoma cell line PC3 was used as positive and negative control, respectively, for thyroglobulin expression.

For investigation of DNA point mutations, positive controls were obtained from cell lines harboring sequence variation in the target genes. DNA from the human T-cell lymphoblast-like cell line CCRF-CEM and the human colorectal adenocarcinoma cell line SW948 was selected as reference for the *KRAS* codon 12 (p.G12D, heterozygous) and 61 (p.Q61R, heterozygous) variants, respectively. CCRF-CEM was also used as a control for *HRAS* (p.A59A, heterozygous). DNA

Table 1. Primer Sets and Conditions for Hot Spots of Interest in HRM Analysis of Thyroid Nodules

Gene	Codon	Sequence	Product size (bp)	Melting temperature* (°C)
BRAF	600–601	Fwd: 5′-TGCTTGCTCTGATAGGAAAATG-3′ Rev: 5′-CCACAAAATGGATCCAGACA-3′	173	75–86
HRAS	61	Fwd: 5′-ATGGCAAACACACACAGGAA-3′ Rev: 5′-GATTCCTACCGGAAGCAGGT-3′	140	79–95
NRAS	61	Fwd: 5′-CCCCTTACCCTCCACACC-3′ Rev: 5′-TGGCAAATACACAGAGGAAGC-3′	162	77–88
KRAS	12–13	Fwd: 5′-GTCACATTTTCATTATTTTTATTATAAGG-3′ Rev: 5′-TTTACCTCTATTGTTGGATCATATTC-3′	155	75–86
KRAS	61	Fwd: 5′-ACTGTGTTTCTCCCTTCTCAGG-3′ Rev: 5′-ATGGCAAATACACAAAGAAAGC-3′	161	77–88

*Annealing temperature was consistently 58°C.

from the human bladder carcinoma cell line HT1197 was used as mutated reference for the *NRAS* codon 61 variant (p.Q61R, heterozygous). Finally, a reconstituted sample of human skin melanoma cell line SK-MEL-28 (*BRAF* p.V600E, homozygous) mixed with human breast cancer cell line MCF-7 DNA (wild-type *BRAF*) was used as heterozygous reference for exon 15 *BRAF* gene. HT1197, MCF-7, SK-MEL28 cell lines were supplied by Banca Biologica e Cell Factory (IRCCS Azienda Ospedaliera Universitaria San Martino – IST Istituto Nazionale per la Ricerca sul Cancro). Moreover, CCRF-CEM and SW948 cell lines were provided by ATCC-LGC Standards Partnership. DNA was extracted from all cell lines using a QIAamp DNA mini kit (Qiagen).

Amplification Conditions for DNA Mutation Screening

The primer sets covering the hot-spot sites of genes were as listed in Table 1. During primer design (Primer3Plus software; *http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi*), the acceptable annealing temperature was set to be identical among different pairs and the product size was considered optimal within a range of 150 to 200 bp. Each amplicon was tested to exclude amplification of sequence homolog regions [UCSC Genome Browser applications In-Silico PCR (*http://genome.ucsc.edu/cgi-bin/hgPcr?org=Human&db=hg19&hgsid=285213467*) and BLAT Search (*http://www.genome.ucsc.edu/cgi-bin/hgBlat?command=start*)]. PCR reactions were performed in an ABI 2720 thermal cycler (Applied Biosystems-Life Technologies) using 10 ng DNA in a total volume of 20 μL containing a final concentration of 1× PCR buffer II (10 mmol/L Tris-HCl, pH 8.3; 50 mmol/L KCl) (Applied Biosystems-Life Technologies), 1.5 mmol/L $MgCl_2$ solution, 0.2 mmol/L each dNTP, 0.5 μmol/L each primer, 0.5 μmol/L SYTO 9 green fluorescent nucleic acid stain (Invitrogen-Life Technologies, Carlsbad, CA), and 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems-Life Technologies). Cycling conditions entailed an initial denaturation at 95°C for 10 minutes, followed by 45 cycles at 95°C for 20 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, with a final elongation step at 72°C for 20 minutes.

HRM Analysis

The DNA from FNAB samples was screened by HRM analysis in a RotorGene 6000 system (Qiagen, Hilden, Germany). The denatured samples, obtained by an initial hold of 3 minutes at 95°C and 3 minutes at 40°C, were analyzed by the acquisition of fluorescence signal in a temperature range experimentally determined for each tested gene (Table 1). The melting ramp was set at 0.08 degrees/each step for each assay. All samples were run in duplicate.

DNA Sequencing

To confirm HRM analysis results, sequencing analysis was also performed in all samples. After HRM, samples were purified with a PCR purification kit (Qiagen) and submitted to cycle sequencing with 2 μL of BigDye Terminator ready reaction mix (Applied Biosystems-Life Technologies) and the same primers used in PCR at a final concentration of 0.16 μmol/L in a volume of 20 μL. After purification with a DyeEx 2.0 spin kit (Qiagen), samples were analyzed with an ABI Prism 310 genetic analyzer (Applied Biosystems-Life Technologies).

Amplification Conditions for Chromosomal Rearrangement Screening

The screening of chromosomal rearrangements (*RET/PTC1*, *RET/PTC3*, and *PAX8/PPARG*) was developed using primer sets reported by Nikiforov et al.[16] Amplification reactions were performed in simplex assay on a 7900HT fast real-time PCR system (Applied Biosystems-Life Technologies) using 2.5 μL of cDNA in a total volume of 12.5 μL containing a final concentration of 1× QuantiTect SYBR Green from a PCR kit (Qiagen) and 300 nmol/L of each primer. PCR was performed as follows: an initial hold at 95°C for 15 minutes, followed by 45 two-step cycles at 95°C for 15 seconds and 60°C for 60 seconds. The amplification products of chromosomal rearrangements assays were submitted to melting analysis to confirm the specificity of the fluorescence signal obtained during the amplification of cDNA. The thermal profile consisted of a denaturing/annealing stage (95°C for 15 seconds, 60°C for 15 seconds), followed by a dissociation stage from 60°C to 95°C (ramp rate, 2%).

Results

Quality of Nucleic Acids

The quality of nucleic acids purified from 260 cytological biopsies indicated that the collection procedure and the extraction systems were suitable for development of mutation screening. In fact, it was possible to amplify by PCR all the DNA samples, as well as the RNA reverse-transcribed into cDNA extracted from the related biopsy. The performance of RNA extraction was verified by the amplification of the control gene, *GAPDH*; thyroglobulin expression was evaluated with a thyroid-specific marker to confirm the presence of thyroid cells in the FNAB specimens. Only eight samples out of the series (3%) were negative for thyroglobulin assay and therefore unreliable for molecular testing. Five of the eight samples had been classified as inadequate biopsies during the previous cytological diagnosis.

Mutation Scanning by HRM Analysis and Sequencing

The HRM analysis, set up by using DNAs from cell lines, was useful in the recognition of DNA mutations along all hot-spot regions investigated (Figure 1). Screening the FNAB samples provided a satisfactory resolution of melting for all of the DNA sites of interest and allowed the amplification of all genes simultaneously.

To evaluate the theoretical sensitivity of our method, a detection limit was calculated, as described previously,[29] by using serial dilution of positive controls (cell lines CCRF-CEM, SW948, HT1197, SK-MEL28) in wild-type DNA (from MCF-7 cell line) for each gene under study. We were able to detect the presence of mutated DNA up to 5% in a background of wild-type DNA (data not shown).

Examples of HRM profiles for mutated samples and corresponding sequencing results, obtained after HRM analysis, are shown in Figure 2. The dideoxy-sequencing always confirmed HRM genotyping of mutated samples, including those cases in which the electropherogram demonstrated a faint peak corresponding to a mutated allele.

Mutated FNABs, Cytological Categories, and Histopathology

Using a simple and rapid application of HRM analysis, we identified 38 mutations in a total of 37 nodules. Number and type of the genetic variants divided on the basis of

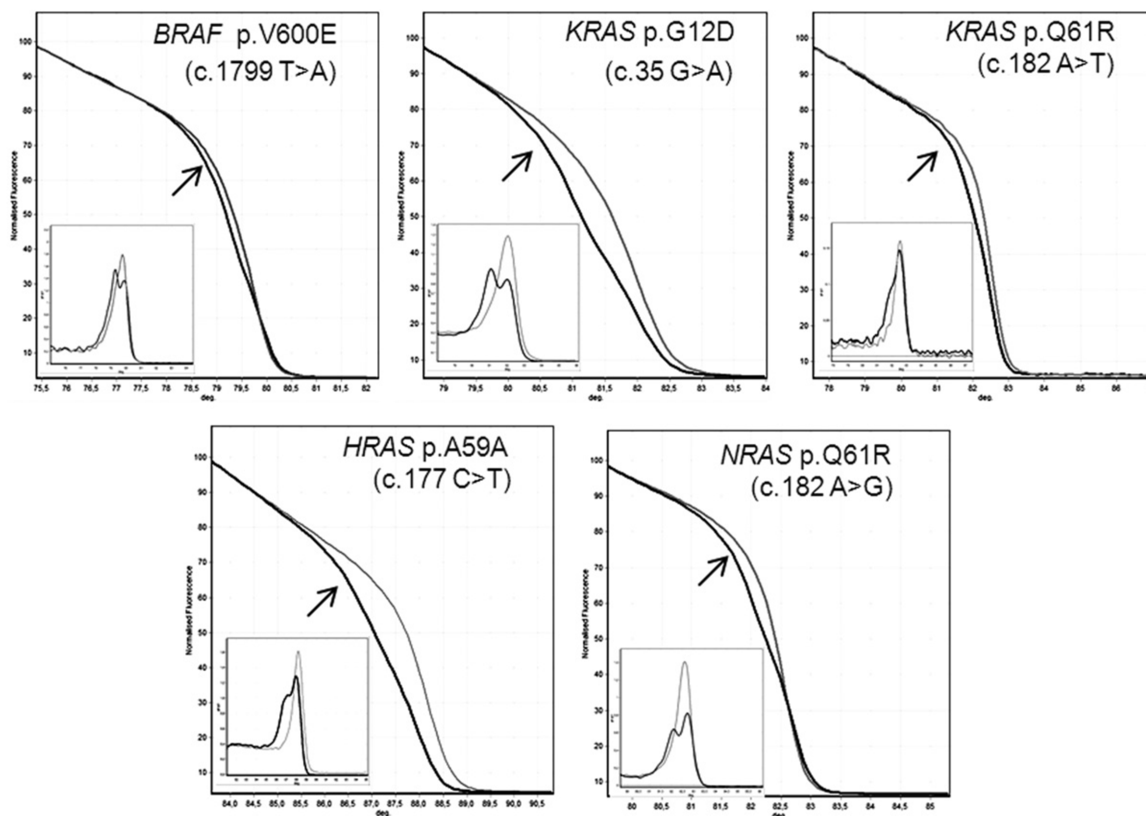

Figure 1. HRM analysis of cell lines harboring mutations in *BRAF*, *KRAS* (codons 12 and 61), *HRAS*, and *NRAS*, with HRM profiles and melting curves of control cell lines used as reference during prescreening of FNAB sample. Results are shown for a sample reconstituted from DNA of SK-MEL-28 (*BRAF* p.V600E, homozygous) mixed with MCF-7 (*BRAF* wild type) (**top left**); DNA from CCRF-CEM analyzed for *KRAS* codon 12 (p.G12D, heterozygous) (**top middle**) and SW948 for *KRAS* codon 61 (p.Q61R, heterozygous) (**top right**); and DNA from CCRF-CEM used as heterozygous control for *HRAS* gene (p.A59A) (**bottom left**) and from HT1197 as mutated reference for *NRAS* codon 61 (p.Q61R, heterozygous) (**bottom right**). The mutated reference sample for the gene of interest is indicated by an **arrow**.

Figure 2. Examples of hot-spot mutations detected by HRM in FNAB samples. The mutated sample identified by HRM assay is indicated by an **arrow**. Electropherograms (**top right** in each panel) confirm the presence of a mutation in the sample with different melting behavior; the nucleotide affected by mutation is bordered by a **dotted box**. For *HRAS* and *KRAS*, mutations caused by different nucleotide substitutions that are clearly detectable by HRM.

Table 2. Genetic Variants Identified by HRM Analysis of Thyroid Nodules, by Cytological Category

	Thy 1 [no. (%)]	Thy 2 [no. (%)]	Thy 3 [no. (%)]	Thy 4 [no. (%)]	Thy 5 [no. (%)]	All samples [no. (%)]
Total sample	31 (12.3)	154 (61.1)	48 (19.0)	9 (3.6)	10 (4.0)	252 (100)
Samples with mutation	4 (12.9)	8 (5.2)	11 (22.9)	5 (55.6)	10 (100.0)	38 (15.1)
BRAF (codon 600–601)	0	1	2	4	10	17 (6.7)
NRAS (codon 61)	0	3	3	0	0	6 (2.4)
HRAS (codon 61)	0	1	1	1	0	3 (1.2)
KRAS (codon 12–13)	3	2	3	0	1	9 (3.6)
KRAS (codon 61)	0	0	1	0	0	1 (0.4)
RET/PTC	1	1	1	0	0	3 (1.2)

One sample carried two mutations simultaneously (*BRAF* p.V600E and *KRAS* p.G12D).

the cytological category and histological findings are reported in Tables 2 and 3, respectively. Only one sample simultaneously carried mutations in two different genes (*BRAF* p.V600E and *KRAS* p.G12D). The *BRAF*^{V600E} mutation was confirmed as the most frequent alteration in FNAB samples, particularly in the cytological categories of suspicious (Thy 4) and malignant nodules (Thy 5), with a frequency of 44.4% and 100%, respectively. In *NRAS*, only the most recurrent p.Q61R was identified, in a total of six samples (three Thy 2 and three Thy 3) (Table 2). Conversely, mutations caused by different nucleotide substitutions were detected by HRM analysis in the *HRAS* and *KRAS* genes (*HRAS* p.Q61R and p.Q61K; *KRAS* p.G12R and p.G12D) (Figure 2). Nodules positive for a mutation in *RAS* genes varied in distribution across cytological categories and were more frequently found in indeterminate specimens (Thy 3, with a frequency of 16.7%). Within the Thy 3 category, the *BRAF* p.V600E mutation was detected in two samples (Table 2), both of which were classified histologically as PTC.

In two cases the analysis by HRM analysis and sequencing identified unusual in tandem mutations in *KRAS*, caused by the substitution of adjacent nucleotides (Figure 3) and resulting in the presence of both a synonymous and a missense mutation: *KRAS* p.[G60G(+)Q61K] and p.[G12G(+)G13R]. Similar mutations have been described previously in colorectal cancer,[32] but never in thyroid FNAB samples, nor in thyroid carcinoma.

Last, during chromosomal rearrangement investigation, we found three samples characterized by the presence of *RET/PTC* rearrangement (2 *RET/PTC1* and 1 *RET/PTC3*; Table 2).

Histopathology results were available for a total of 56 patients (Table 3). In addition to the expected concordance in the Thy 4 and Thy 5 groups, molecular diagnosis in the Thy 3 category identified 5/9 (55%) patients with mutations among the histologically positive samples and 26/29 (90%) patients without mutations (wild-type DNA) among histologically negative samples.

Discussion

In recent years, both research and clinical management of thyroid cancer have been deeply modified and revised on the basis of genetic features of the tumor. Involvement of independent pathways in the carcinogenesis of thyroid cancer is supported by the great variability in the pattern of somatic mutations.[33] In a parallel fashion, the number of patients requiring clinical evaluation of thyroid nodules has dramatically increased. Even if FNAB is considered an accurate preoperative test to distinguish benign from malignant thyroid nodules, cytological examination is inconclusive in approximately 30% of all thyroid nodule biopsies[7,34] classed as indeterminate (Thy 3, follicular lesion of undetermined significance or samples suspected for follicular neoplasm) or nondiagnostic (Thy 1, insufficient cells). These patients are typically subjected to surgery, to exclude a thyroid cancer diagnosis. Approximately 20% of patients with FNAB biopsy showing indeterminate cytological features have thyroid cancer revealed on histological examination and may require a complete thyroidectomy.[12] Typical genetic alterations are described for most differentiated thyroid cancers, which constitute approximately 90% of all cases.[25] In clinical management, given the importance of a correct classification of cytologically undefined FNAB, rapid and accurate methods are needed to screen the most common somatic mutations in a series of known oncogenes.

Table 3. Genetic Variants Identified by HRM Analysis of Thyroid Nodules, by Histological Findings

Category	Total samples (no.)	Histology available (no.)	Histology findings (no.)	Mutations in related cytological sample (no.)
Thy 2	154	4	1 positive	1 *BRAF*
			3 negative	1 *RAS*; 2 wild type
Thy 3	48	38	9 positive	2 *BRAF*; 2 *RAS*; 1 *RET/PTC*; 4 wild type
			29 negative	3 *RAS*; 26 wild type
Thy 4	9	9	8 positive	4 *BRAF*; 1 *RAS*
			1 negative	1 wild type
Thy 5	10	5	5 positive	5 *BRAF*
			0 negative	

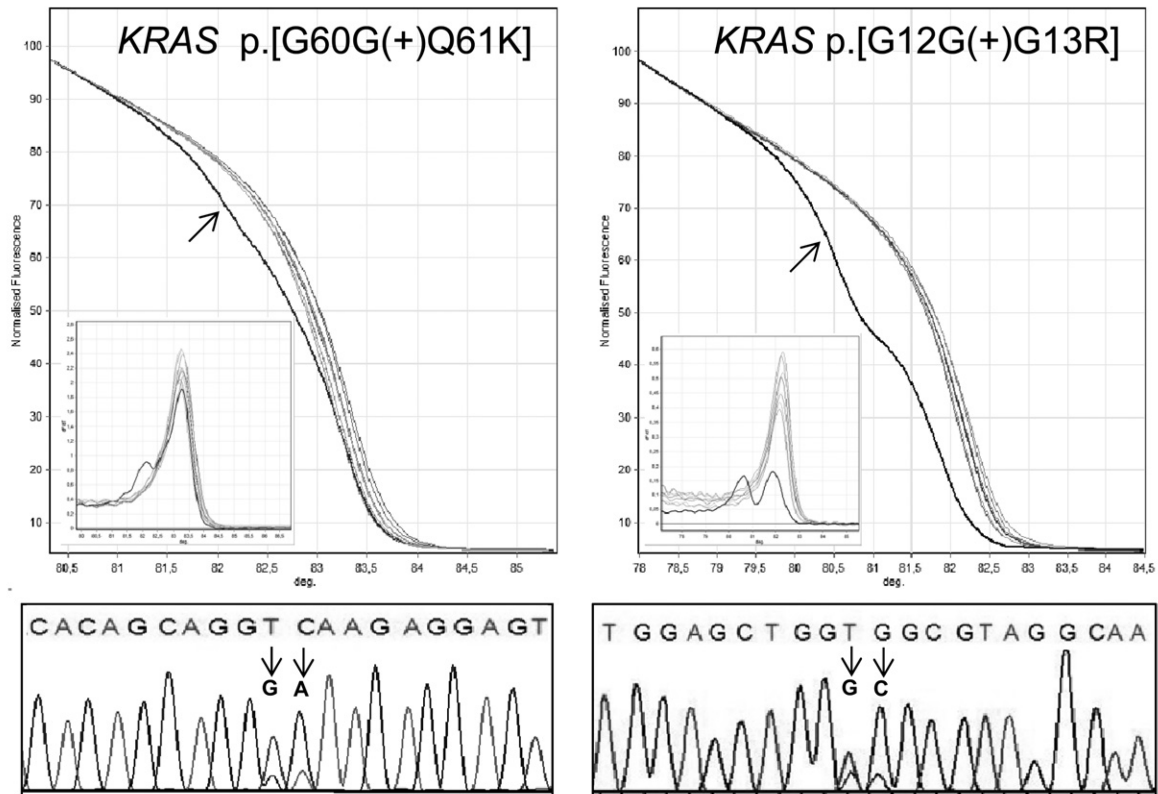

Figure 3. Examples of rare variants detected by HRM in FNAB samples. The presence of a double mutation in *KRAS* is easily identified from the abnormal melting profiles of two samples (indicated by **arrows**), compared with wild-type samples. In electropherograms, **arrows** indicate the nucleotides affected by adjacent substitutions.

HRM analysis can identify, with high sensitivity, samples harboring mutations, irrespective of the substitution type. Thus, HRM could be considered a valid prescreening method to analyze, through a single methodological approach, a large sample in a short time and to make an accurate characterization of several molecular markers that are affected by many types of mutations. This capability is clearly confirmed for different types of nucleotide changes, both in the case of first-class substitutions such as G/A and C/T, which are easily detectable, and in the case of the fourth-class A/T change, which is generally more difficult to discriminate. In addition, the use of an amplicon-based melting method with saturating dyes allows the detection of non-hot-spot sequence variants[30] with potential clinical value that would not be identifiable with allele-specific techniques. In the present study, we identified two samples carrying rare *KRAS* variants previously described in colorectal cancer but not in thyroid cancer. Thus, HRM analysis provides a reliable, cost-limited, and accurate approach to rapid genetic screening. Because specific mutated allele detection is not the crucial point in FNAB thyroid samples, comparison among different methods should be based mainly on the basis of test practicability, costs, and turnaround time of sample evaluation.

Most of the available assay methods used for FNAB molecular characterization produce similar results in terms of sensitivity and specificity. As reported by Jin et al[35] for *BRAF* p.V600E mutation, the choice of four different approaches (direct sequencing, the colorimetric TrimGen Mutector assay, PCR with FRET probes, and PCR with SYBR Green) does not significantly affect the molecular diagnosis, and the choice of the best test depends mainly on the laboratory expertise and available financial and technical resources.

In addition, the present HRM-based technique provides levels of sensitivity higher than direct sequencing. In a previous study[29] on colorectal cancer, we demonstrated the high sensitivity of HRM analysis, which allowed identifying at least 5% of mutated alleles in a background of wild-type DNA. Even though HRM analysis has been widely applied for the screening of somatic variants in biopsies of solid cancers, only a few previous applications of HRM analysis have been reported in the screening of cytological material, as fixed fresh cells[36] or scraped cells from archival slides[37] obtained from needle aspiration.

The main objective in the present study was to develop a reliable and sensitive test for the rapid screening of mutations in *KRAS*, *HRAS*, *NRAS*, and *BRAF* oncogenes in thyroid FNAB. Within the proposed protocol, we can provide results of the complete screening of FNAB samples in approximately 48 hours. This result was obtained by optimization of PCR protocols, based on the simultaneous amplification of the different targets at the same primer annealing temperature. After amplification, each tube is immediately submitted to HRM. In the presence of an abnormal melting profile, the same amplification product can be immediately submitted to direct sequencing,

without further amplifications. FNAB samples that are negative for any of the oncogene mutations included in our panel are immediately submitted to quantitative real-time PCR and for detection of thyroglobulin mRNA and chromosomal rearrangements. The presence of mRNA for thyroglobulin indicates the effective presence of thyroid-derived nucleic acids in the sample and precludes false negative results.

As already noted, the entire procedure can be completed in a reduced time interval without affecting reliability of the results. The other relevant consequence of our protocol is a dramatic reduction of costs. First of all, HRM is a post-PCR step that does not require significant further costs (the addition of intercalating dye, for example, can be quantified in €0.006 per tube). Furthermore, the execution of direct sequencing limited only to samples that yielded abnormal melting profiles reduced by more than 80% the costs connected to the entire process for sequence analysis.

The optimization of the proposed procedure guarantees high levels of sensitivity, because we obtained correct amplification in all FNAB samples, as well as in those samples for which only needle washing was available. In this particular application, the low amount of starting biological sample available from FNAB washing and the consequent need for a highly sensitive method to obtain accurate results, especially in the case of multiple assays, has to be taken into account. On the other hand, the definition of test reliability and sensitivity as percentage of mutated allele is highly relevant for the evaluation of the analytical performance of a diagnostic assay method. This sensitivity precludes taking a second biopsy only to collect tissue for molecular testing.

In the present study, we detected sequence variants of the proposed oncogenes in approximately 15% of all samples, a percentage similar to that reported by others.[10,12] Moreover, we detected 10/48 mutated samples (21%) with a higher incidence of *RAS* variants in comparison with previous reports in Thy 3 patients.[10,16]

In addition to establishing the evident advantages of HRM prescreening in terms of time and economic improvement, this pilot study confirmed the accuracy of the proposed method. Complete concordance was found between the standard protocol, based on sequencing of all samples, and the HRM-based screening.

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