Impact of Different Methodologies on the Detection of Point Mutations in Routine Air-dried Fine Needle Aspiration (FNA) Smears

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Key words
- thyroid cancer
- diagnostics
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- fine-needle aspiration
- thyroid cytology

Abstract

Currently the best method to select suspicious thyroid nodules for surgery is fine needle aspiration (FNA) cytology. However, FNA cytology has some inherent limitations, which can partly be overcome by molecular analysis. Therefore, molecular testing for somatic mutations has emerged as the most promising approach for molecular FNA diagnostics. The objective of this methodological study was to evaluate the feasibility of detecting *BRAF*, *NRAS*, *HRAS*, and *KRAS* mutations from routine air-dried thyroid FNA smears, and to find an optimal method for detecting these mutations in FNA samples. DNA was extracted from 110 routine air-dried FNA smears and the corresponding surgically obtained formalin-fixed paraffin-embedded tissues. The presence of *BRAF*, *NRAS*, *HRAS*, and *KRAS* mutations was assessed by real-time PCRs and high resolution melting analysis, and/or pyrosequencing in comparison to real-time PCRs using hybridization probes and fluorescence melting curve analysis. The high-resolution melting-PCRs revealed a significantly lower number of PCR failures and questionable results, and detected more mutations than the PCRs using hybridization probes. The number of PCR failures ranging from 14–16% by high-resolution melting-PCRs could be further reduced to 5–14% by adding pyrosequencing assays. Moreover, pyrosequencing increased the specificity of the assays, up to 98–100%, while the sensitivity ranged between 32–63%. In summary, the mutation detection, especially in air-dried FNA samples, improves when using PCR assays in combination with high resolution melting analysis. Additional improvement can be obtained by subsequent pyrosequencing in comparison to previously described real-time PCRs using hybridization probes and fluorescence melting curve analysis.

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Introduction

Thyroid cancer comprises 94.5% of all endocrine malignancies, and ranks 13th in frequency of occurrence among all cancers, accounting for 2.6% of all cases [1]. The majority of thyroid cancers are well-differentiated malignancies originating from thyroid follicular cells. The most frequent histotypes are papillary thyroid carcinomas (PTCs), followed by follicular thyroid carcinomas (FTCs). Currently the best method to select suspicious nodules for surgery is ultrasound-guided fine needle aspiration (FNA) cytology [2, 3]. However, despite high specificity and sensitivity, FNA cytology has some inherent limitations, which can partly be overcome by molecular analysis, since *RET*/*PTC* rearrangements and *BRAF* point mutations have been detected in 13–43% and 29–69% of PTCs, respectively [4]. In FTCs, *PAX8*/*PPARG* rearrangements and *RAS* mutations have been detected in 25–63% and 40–53%, respectively [4]. Therefore, molecular testing for somatic mutations has emerged as the most promising approach for molecular FNA diagnosis [5–7]. It allows improved discrimination of the "follicular proliferation/indeterminate" and "suspicious" FNA categories, leading to reduced numbers of diagnostic thyroidectomies and false negative FNAs [8].

Hitherto a variety of molecular techniques have been used to detect these mutations. These comprise LightCycler PCR using hybridization probes, quantitative (q)PCR and post-PCR melting curve analysis, allele specific PCR, direct sequencing, restriction fragment polymorphism analysis, colorimetric assays, and nested PCR [9–15]. In archived FNA smears a similar sensitivity in the

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Table 1 Primers for the detection of *BRAF* (V600E), *HRAS* (codon 61), *KRAS* (codon 12/13), and *NRAS* (codon 61) point mutations by HRM-PCRs and pyrosequencing.

Primer	GenBank ID	Sequence
BRAF-HRM-F	NM_004333	GGTGATTTTGGTCTAGCTACAG
BRAF-HRM-R	NM_004333	GGCCAAAAATTTAATCAGTGGA
KRAS-HRM-F	NM_033360	AGGCCTGCTGAAAATGACTG
KRAS-HRM-R	NM_033360	GCTGTATCGTCAAGGCACTCT
NRAS-HRM-F	NM_002524	TGGTGAAACCTGTTTGTTGG
NRAS-HRM-R	NM_002524	TCGCCTGTCCTCATGTATTG
HRAS-Pyro-F	NM_005343	GGTCATTGATGGGGAGACGT
HRAS-Pyro-R	NM_005343	Biotin-GCATGGCGCTGTACTCCT
HRAS-Pyro	NM_005343	TCCTGGATACCGCCG

detection of *BRAF* mutations has been shown for allele specific PCR, direct sequencing, colorimetric assay, and LightCycler PCR using hybridization probes [8, 11].

The objective of this methodological study was 1) to evaluate the feasibility of detecting *BRAF*, *NRAS*, *HRAS*, and *KRAS* point mutations from routine air-dried FNA smears from thyroid nodules, and 2) to find the optimal method for detecting these point mutations in FNA samples, starting with a well-described LightCycler PCR using hybridization probes (hybridization probe-PCR) [16]. We demonstrate that the mutation detection, especially in our air-dried FNA samples, improves when using a qPCR assay in combination with high-resolution melting (HRM-PCR) analysis, and show a further improvement by subsequent pyrosequencing in comparison to the previously described hybridization probe-PCR [16].

Materials and Methods

Patients and samples

In total, 110 routine air-dried FNA slides from patients who subsequently underwent surgery for thyroid nodules at the Odense University Hospital (Odense, Denmark) were retrospectively included into this study. Additionally, 110 corresponding formalin-fixed paraffin embedded (FFPE) slices were analyzed. All FNA samples were graded according to the ATA 2006 guidelines [17] by an experienced pathologist (A.K.). Overall, cytological evaluation of the FNA slides revealed 27 malignant, 67 indeterminate, 14 benign, and 2 nondiagnostic samples. Histological evaluation of the corresponding FFPE samples revealed 66 follicular adenomas (FAs), 13 FTCs, 24 PTCs, and 7 goiters. The study was approved by, and conducted according to the regulations of the Danish scientific ethics committees.

Nucleic acid extraction from FNA smears

DNA was extracted from the routine air-dried FNA smears by extending the recently published RNA extraction protocol [18]. Three hundred µl of 96% EtOH was added to the lower phase of the phenol/chloroform extraction and the tube was gently mixed and centrifuged at 8000×*g* for 3 min. Afterwards, the supernatant was removed and the pellet was incubated with sodium citrate solution for 30 min. The tube was centrifuged at 8000×*g* for 3 min and again incubated with sodium citrate solution for an additional 30 min. After further centrifugation at 8000×*g* for 3 min at room temperature, the pellet was washed with 70% EtOH. Following a further centrifugation, the pellet was dried at room temperature for 15 min and then resuspended in 50 µl of TE buffer. After freezing the DNA for 24 h it was thawed, vortexed, and centrifuged at 12000×*g* for 1 min. The supernatant containing the DNA was then transferred to a new tube.

Nucleic acid extraction from FFPE slides

DNA was extracted from the FFPE slides by extending the RNA extraction protocol [18]. While the flow through of the gDNA elimination spin column was used for RNA extraction according to the miRNeasy FFPE kit protocol, 200 µl TE buffer was added to the column and incubated at room temperature for 5 min. Subsequently, the column was centrifuged at 8000×*g* for 1 min. Six hundred µl of 96% EtOH and 30 µl of aqueous sodium acetate were added, mixed, and incubated for 10 min at room temperature. After centrifugation for 10 min at room temperature the DNA pellet was washed with 70% EtOH and eluted with 50 µl TE buffer.

Detection of point mutations by hybridization probe-PCRs

DNA extracted from the FNA samples was screened for point mutations in *BRAF* codons 600/601 and for point mutations in *HRAS* codon 61, *KRAS* codons 12/13, and *NRAS* codon 61 by real-time PCRs using hybridization probes and fluorescence melting curve analysis on a Lightcycler 480 according to Nikiforov et al. [16]. The PCRs for the detection of these point mutations (with exception of the PCR detecting point mutations in *HRAS*) were applicable to our DNA samples, which are (due to the extraction from routine FNA samples) of lower quality than the DNAs extracted from fresh FNA material.

Samples tested positive were subsequently analyzed by pyrosequencing on a PyroMark Q24 (QIAGEN).

Detection of point mutations by PCRs using high resolution melting (HRM) analysis

BRAF, *KRAS*, and *NRAS* point mutations were detected by real-time PCR and HRM (HRM-PCR) using primers flanking the mutation hotspots (**Table 1**) and the LightCycler 480 High Resolution Melting Master chemistry (Roche, Mannheim, Germany) on a LightCycler 480 (Roche, Mannheim, Germany) amplifying DNA fragments of less than 200 bp. Although several primer pairs were tested to establish a HRM-PCR to detect point mutations in codon 61 of *HRAS*, none of the PCRs gave reliable results. PCRs were processed through an initial denaturation at 95 °C for 10 min followed by 55 cycles of a 3-step PCR, including 3 s of denaturation at 95 °C, a 12-s annealing phase at 58 °C (*NRAS*, *KRAS*)/60 °C (*BRAF*), and an elongation phase at 72 °C for 10 s. Subsequently, a high-resolution melting curve was assessed from 75–95 °C with an increase of 0.02 °C/s and 25 acquisitions per degree. DNA from patient specimens known to carry *BRAF*, *KRAS*, and *NRAS* point mutations were used as positive controls in each analysis. Samples tested positive were hereafter analyzed by pyrosequencing on a PyroMark Q24 (QIAGEN).

Detection of point mutations by pyrosequencing

Point mutations in *BRAF*, *KRAS*, and *NRAS* were detected by pyrosequencing using the therascreen BRAF Pyro Kit, therascreen NRAS Pyro Kit, and PyroMark KRAS Kit (all from QIAGEN, Hilden, Germany) according to the manufacturer's instructions. In brief, 5 µl of genomic DNA was amplified using template specific PCR primers (including one biotin-labeled primer) and template specific PCR conditions. Afterwards, the PCR products were immobilized to streptavidin sepharose beads and single

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	FNA Hybridization probe-PCR	HRM-PCR	FFPE Hybridization probe-PCR	HRM-PCR
BRAF				
Positive in mutation screening	5	9	9	12
Wild-type in mutation screening	14	40	32	37
Questionable result	10	0	5	1
No PCR product/low efficiency PCR	21	1	4	0
Chi-square p-value		4.33E-09		0.049
KRAS				
Positive in mutation screening	0	0	0	0
Wild-type in mutation screening	38	49	50	50
Questionable result	6	0	0	0
No PCR product/low efficiency PCR	6	1	0	0
Chi-square p-value		0.001499		1
NRAS				
Positive in mutation screening	1	10	14	10
Wild-type in mutation screening	43	38	35	39
Questionable result	1	0	1	1
No PCR product/low efficiency PCR	5	2	0	0
Chi-square p-value		0.009495		0.7416

The number of samples positive in mutation screening, wild-type in mutation screening, the number of questionable results, and the number of samples that could not be analyzed (due to no PCR product/low efficiency PCR) were compared by Chi-square tests between the 2 methods applied (hybridization probe-PCR and HRM-PCR)

Table 2 Comparison of detecting *BRAF*, *KRAS*, and *NRAS* point mutations by hybridization probe-PCRs and by HRM-PCRs in a subset of 50 FNA and the corresponding FFPE samples.

stranded DNA was prepared allowing subsequent annealing of the sequencing primer to the template DNA. Then, the primed single stranded DNA was released from the streptavidin surface and transferred to a PyroMark Q24 (QIAGEN, Hilden, Germany) for pyrosequencing. *HRAS* point mutations were detected by pyrosequencing using self-designed primers (**Table 1**) and the following PCR conditions: PCRs were processed through an initial denaturation at 95 °C for 15 min followed by 45 cycles of a 3-step PCR, including 20-s of denaturation at 95 °C, a 30-s annealing phase at 64 °C, and a 30-s elongation phase at 72 °C, followed by a final 5 min extension phase at 72 °C. Then, the PCR products were treated as described above.

Statistical analysis

The PCR results obtained by real-time PCR, using hybridization probes, and by real-time PCR and HRM were compared by the Chi square test. Cases with a wild type result in FNA and FFPE were considered "true negatives", and cases with a positive mutation screening in both, FNA and FFPE were considered "true positives". Cases with a positive mutation screening in the FNA sample and a wild type result in the FFPE were considered "false positive" and cases with a wild-type screening in the FNA and a positive mutations screening in the FFPE were considered "false negatives".

Results

The HRM-PCR assays were developed and optimized using pGEM-T vectors carrying wild-type or mutated sequences of *BRAF*, *KRAS*, and *NRAS*, respectively. Normal and mutant sequences were mixed in proportions to give the following levels of mutation: 0, 12.5, 25, 50, and 100 %. These samples were run together with wild-type FFPE samples in one HRM-PCR run to determine the limit of detection for the different HRM assays. In all HRM-PCRs the limit of detection was approximately 12.5 % (data not shown). In pyrosequencing, samples showing a mutation level greater than 5 % were deemed mutation positive.

Comparison of detecting point mutations by hybridization probe-PCRs and by HRM-PCRs

Point mutations in *BRAF*, *KRAS*, and *NRAS* were detected by hybridization probe-PCRs and by HRM-PCRs in a subset of 50 FNA smears and corresponding FFPE samples, using DNA obtained from the same extractions for both methods. The results of hybridization probe-PCR and HRM-PCR for the FNAs were rated as "positive in mutation screening", "wild-type in mutation screening", "questionable result", or "no PCR product/low efficiency PCR" (**Table 2**). While there are few or no significant differences between the hybridization probe-PCR and HRM-PCR for FFPE samples, the comparison of the 2 methods gave significant differences for the FNA samples (**Table 2**). For all 3 PCRs, HRM-PCR revealed a significantly lower number of questionable results, PCRs with no product, or low PCR efficiency, than the hybridization probe-PCR. For example, while the BRAF-hybridization probe-PCR revealed 31 questionable results/PCRs with no product or low PCR efficiency, only one BRAF-HRM-PCR revealed no product or low PCR efficiency (**Table 2**). Moreover, more mutations were detected by HRM than by hybridization probe-PCR. While 9 samples were tested mutation positive in the BRAF-HRM screening only 5 positive results were obtained by the hybridization probe-PCR (**Table 2**). While no *KRAS* mutation positive FNA sample could be identified by either hybridization probe based PCR or by HRM-PCR, 10 *NRAS* mutation positive samples were identified by HRM-PCR, in comparison to 1 mutation positive sample by hybridization probe-PCR. Based on these results, the remaining 60 FNA and FFPE samples were analyzed by HRM-PCR only.

Detection of point mutations by HRM-PCRs, HRM-PCRs and/or pyrosequencing in the total sample set of 110 FNA smears and corresponding FFPE samples

First, all FFPE samples were screened for *BRAF*, *KRAS*, and *NRAS* mutations by HRM-PCRs. Positive and questionable HRM-PCR results were verified by pyrosequencing as were samples giving no PCR product in the HRM-PCR. Screening for *HRAS* mutations

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Table 3 Loss rate, sensitivity, specificity, and accuracy for detecting *BRAF*, *HRAS*, *KRAS*, and *NRAS* point mutations in 110 FNA smears compared to the corresponding FFPE samples.

	HRM-PCR (percent)	HRM-PCR combined with PS (percent)
BRAF		
Loss rate	16	5
Sensitivity	54	63
Specificity	97	98
Accuracy	91	92
KRAS		
Loss rate	14	6
Sensitivity	NA	NA
Specificity	96	100
Accuracy	96	100
NRAS		
Loss rate	14	12
Sensitivity	47	32
Specificity	95	99
Accuracy	86	85
HRAS	**PS (percent)**	
Loss rate	14	
Sensitivity	40	
Specificity	100	
Accuracy	97	

BRAF, *KRAS*, and *NRAS* point mutations were detected by HRM-PCRs alone, and by HRM-PCRs in combination with pyrosequencing (PS). *HRAS* point mutations were detected by pyrosequencing alone. NA: Not analyzable

was done by pyrosequencing alone. The results of these analyses were deemed as the gold standard for the comparison of the results obtained from the FNA smear screening.

As done for the FFPE samples, all FNA samples were screened initially by HRM-PCRs (*BRAF*, *KRAS*, *NRAS*) or pyrosequencing (*HRAS*). For all mutation screenings, the loss rates (percentage of samples, which could not be analyzed due to the fact that the PCR revealed no PCR product or due to a low efficient PCR) were comparable. While 16% of FNA samples could not be analyzed in the BRAF-HRM-PCR, 14% of FNA samples could not be analyzed by either KRAS- or NRAS-HRM-PCR (**Table 3**). To reduce these loss rates all samples characterized by a less efficient PCR or no PCR product during the HRM-PCR screening were subsequently analyzed by pyrosequencing. In FNA smears, this procedure allowed a reduction of the loss rate to 5% in case of *BRAF* mutation screening, 12% in case of *NRAS* mutation screening, and to 6% in *KRAS* mutation screening.

Although the HRM-PCR-screening of all mutations tested is characterized by a high specificity, ranging from 95% (*KRAS*) to 100% (*HRAS*), the combination with pyrosequencing further increased the specificity (**Table 3**). In contrast to the high specificity of the assays, the sensitivity of the FNA smear mutation screening was rather low, ranging from 32% (*NRAS*, HRM-PCR with pyrosequencing) to 63% (*BRAF*, HRM-PCR with pyrosequencing). Since no *KRAS* mutations could be detected a sensitivity for this assay cannot be calculated.

Discussion

With the discovery of rearrangements (*RET*/*PTC* and *PAX8*/*PPARG*) and point mutations in the *BRAF* and several *RAS* genes in PTC and FTC, respectively, much knowledge has been added in relation to their frequency, detection method, and their applicability in medical practice. Although, at present, ultrasound-guided FNA is the most sensitive method to diagnose nodules suspected of malignancy [2,3], molecular analysis has been demonstrated to be an important additional technique in raising the sensitivity of this method, especially in case of indeterminate samples [5,6,8,9].

Recently, we could demonstrate, for the first time, the possibility of detecting *RET*/*PTC* and *PAX8*/*PPARG* rearrangements in routine air-dried FNA smears and in the corresponding FFPE [18]. This progress is associated with a number of advantages for the patients and society alike [8]. The aim of the present study was to evaluate the previously described hybridization probe-PCR [16] for the detection of *BRAF*, *NRAS*, *KRAS*, and *HRAS* point mutations in DNA from routine air-dried FNA smears. This DNA is degraded and of lower quality than the DNA from fine needle wash-outs, used by Nikiforov et al. [16]. Jin et al. [11] previously compared the detection of *BRAF* mutations by evaluating 4 different methods, including direct sequencing, Colorimetric Assay Mutector, LightCycler PCR, using hybridization probes, and an allele-specific PCR, using LightCycler SYBR Green showed similar sensitivities for these 4 detection methods. However, a multitude of other variables, such as sample source, number of samples, method of extraction, and conservation of the material, are also of importance. Indeed, there was no significant difference in the initial screening results of 50 FFPE samples of our set, analyzed by the hybridization probe-PCR [16] in comparison to a HRM-PCR (**Table 2**). However, the screening of the initial 50 FNA samples revealed completely different results. The screening of these revealed a loss rate of 10% for *NRAS* and 42% for *BRAF* when analyzing the FNA samples by the hybridization probe-PCR (**Table 3**) [16]. In contrast, analyzing these FNA samples by the HRM-PCR reduced the loss rate to 0% for *NRAS* and *KRAS*, and to 2% for *BRAF*. Moreover, the use of HRM-PCR allowed the detection of more mutations than did the hybridization probe-PCR, in case of *BRAF* and *NRAS* (**Table 2**). These results clearly show that 1) the source of material, and the method of extraction and conservation of the material influence the outcome of the screening assay, and 2) that assays showing a similar performance in one type of material, can perform very differently using another type of material. Therefore, with respect to our samples, all further air-dried FNA smear analyses are based on the HRM-PCR, which is superior to the hybridization probe-PCR in analyzing routine air-dried FNA samples.

Although we observed low loss rates in the initial sample set by HRM-PCR, the analysis of the total sample set (n = 110) resulted in loss rates of 14 (*KRAS*, *NRAS*) to 16% (*BRAF*). Therefore, to further reduce the loss rates and to verify the results of the HRM screening, we performed pyrosequencing of all samples, which showed a positive, a questionable, or no result in HRM-PCRs. This algorithm resulted in a reduction of the loss rates in case of *BRAF* and *KRAS* down to 5% and 6%, respectively, while the loss rate for the *NRAS* assay decreased only slightly from 14 to 12%. On top of improving the loss rates in 2 screening assays, adding pyrosequencing increased specificity in all screening assays (**Table 3**). However, while the specificity of our mutation screening is very high, the sensitivity is rather low. One reason for these low sensitivities might be the assay itself. This can be seen especially for the *NRAS* screening, where the HRM-PCR alone has a sensitivity of 47%, which decreased to 32% after adding the pyrosequencing assay. This means that in particular the commercial *NRAS* pyrosequencing assay lacks sensitivity when

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analyzing degraded DNA samples. On the other hand we were able to improve the sensitivity from 54% to 63% in the *BRAF* screening by adding pyrosequencing. Another reason for the low sensitivities might be tumor heterogeneity. Recently a pyrosequencing approach has reported percentages of mutant *BRAF* alleles from 5.1–44.7% [19]. Tumor heterogeneity has been reported for *RET*/*PTC* rearrangements by fluorescence in situ hybridization [20,21]. This implies that the mutation screening of the FFPE sample may reveal a positive result, while the mutation screening of the FNA sample is negative. We speculate that potential reasons for this discrepancy could be aspiration of a mutation negative subpopulation or dilution of mutation positive cells by mutation negative cells during the aspiration process, resulting in a lower sensitivity. Further reasons for the rather low sensitivities might be a higher DNA degradation in older slides, a small number of tumor specific cells carrying the mutation in the FNA smear or a high number of contaminating cells (which might dilute a positive result from the tumor cells). Interestingly, the negative screening results were not significantly correlated with age of the samples, low cellularity of the FNA smears, or the presence of other cells. Therefore, the focus of optimizing the point mutation detection in DNA samples from routine air-dried FNA smears should be on improving the assays, in particular the *NRAS* pyrosequencing assay, aiming for a higher sensitivity.

In conclusion, we have shown that the previously described hybridization probe-PCRs [16], and the novel HRM-PCRs gave similar results, when analyzing FFPE samples. However, when analyzing degraded DNA from routine air-dried FNA smears, the HRM-PCR (in combination with pyrosequencing) outperformed the hybridization probe-PCR. Therefore, for the mutation analysis of routine air-dried FNA smears, HRM-PCR analysis should be the method of choice.

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Conflict of Interest

The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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