Pyrosequencing Analysis of *EGFR* and *KRAS* Mutations in EUS and EBUS-Derived Cytologic Samples of Adenocarcinomas of the Lung

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Introduction: Patients with stage IV non–small-cell lung cancer harboring an activating epidermal growth factor receptor (*EGFR*) mutation are eligible for treatment with *EGFR* tyrosine kinase inhibitors. With pyrosequencing, low-frequency mutations may be detected more easily even in small diagnostic samples like endoscopic ultrasound-guided fine needle aspirations (EUS-FNA) and endobronchial ultrasound-guided transbronchial needle aspirations (EBUS-TBNA). The diagnostic performance of pyrosequencing in analyzing cytological specimens is compared with the routinely used high-resolution melting (HRM) and Sanger sequencing.

Methods: Patients diagnosed with adenocarcinoma of the lung were selected from a fine needle aspiration and transbronchial needle aspiration specimen database. If formalin-fixed paraffin-embedded tumor blocks were available, mutation analysis was performed for *EGFR* and *V-Ki-ras2* Kirsten rat sarcoma viral oncogene homolog genes using both pyrosequencing and HRM. When HRM showed abnormalities, Sanger sequencing was used.

Results: A total of 126 samples were available for mutation analysis. The analysis success rate for pyrosequencing and HRM were 97% and 93%, respectively. HRM failures were observed in fragmented DNA showing chains of 100 to 200 bp. A significant correlation between length of DNA fragments (100–300 bp versus 300–400 bp) and mean sample age (797 versus 317 days) was found ($p < 0.0001$), suggesting an influence of sample age on DNA quality.

Conclusion: Pyrosequencing on cytological blocks, especially older tumor blocks, is feasible with a high diagnostic success rate. Failures in HRM were observed in DNA samples with short fragments related to longer storage times.

Key Words: Deoxyribonucleic acid mutational analysis, Endoscopic ultrasonography, Cytology, Non–small-cell lung cancer, DNA sequencing.

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A subset of the pulmonary adenocarcinomas (ACs) harboring mutated epidermal growth factor receptor (*EGFR*) genes has a prolonged survival irrespective of treatment.[1] Mutations cause alterations in the intracellular part of the transmembrane *EGFR*. This results in a stronger binding of tyrosine kinase inhibitors (TKIs) than adenosine triphosphate (ATP) with subsequent inhibition of *EGFR*.[2] When compared with chemotherapy, treatment with *EGFR*-TKIs showed higher response rates and significant better progression-free survival with mild toxicity.[1,3–6]

This benefit in patients with activating *EGFR* mutations requires mutation analysis as a standard diagnostic procedure in patients with stage IV AC of the lung.

Another even more frequently encountered mutation in ACs of the lung occurs in the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene.

Recommendations on tissue and test characteristics for *EGFR* mutation analysis were recently published in an European consensus report.[7] The authors prefer the use of histology and mention the possible mutation analysis in cytological specimens. In reported large clinical studies, mutation analysis is almost invariably performed on tissue samples.

In routine clinical practice tissue samples are required, however, a diagnosis is often made on cytological specimens. Tissue biopsies are not always available. Therefore, mutation analysis of small tumor samples is necessary. Mutation analysis on cytological specimen, including transbronchial and transesophageal aspirates, has been previously reported in several studies.[8–20] This is particularly relevant for the majority of stage III patients progressing to stage IV, enabling archived mediastinal aspiration cell blocks to be used for molecular processing. Those aspirates can be performed in the same lymph node in different directions gathering multiple aspirates from different parts of the same node metastasis thereby increasing the yield of tumor cells.

Although it is not clear which method is the best for mutation analysis in non–small-cell lung cancer, a combination of DNA amplification and direct sequencing is the most practiced.

After DNA extraction, amplification of target sequences with the polymerase chain reaction (PCR) is the next step. Prescreening for abnormal target alleles is possible with high-resolution melting (HRM).[21] The sensitivity of detecting mutations can be increased by amplification refractory

mutation system polymerase chain (ARMS-PCR),[22] allele-specific PCR,[23,24] peptide nucleic acid clamping methods,[25,26] or preferential amplification of mutant alleles with coamplification at lower denaturation temperature–PCR technique.[20]

When genomic abnormalities are detected, further characterization with DNA sequencing follows. Various techniques for sequencing have been developed. Test characteristics have improved over time, and more recently developed tests require less tumor cells.

The question remains whether screening should precede sequencing or whether sequencing should be performed upfront. The diagnostic accuracy of tests in relation to the quality of samples as well as the costs and diagnostic speed are important factors in designing the optimal testing strategy.

Pyrosequencing is one of the latest assays using luminometric instead of electrophoretic detection.[27] This technique enables characterization of mutations and quantification of mutated alleles in samples with low tumor cell density and detection with high accuracy rates.[28]

The objective of this study is to compare the diagnostic performance of pyrosequencing with the comprehensive strategy of HRM (followed by Sanger sequencing[29] in case of abnormalities) for *EGFR* and *KRAS* mutation analysis in paraffin-embedded cytological specimens of AC patients obtained with endoscopic-ultrasound– guided needle aspiration (EUS) and endobronchial-ultrasound–guided needle aspiration (EBUS). The analysis success rates and concordance rates for both techniques are determined.

PATIENTS AND METHODS

Patients

Patients with a cytological diagnosis of AC established on EUS- or EBUS-derived samples were selected from our local patient database. The diagnosis was based on morphologic and immunohistochemical characteristics for all tumors.

All samples were coded and managed independently. The study was approved by the Medical Ethical Committee of the Isala Clinics in Zwolle, The Netherlands.

EUS and EBUS

EUS and EBUS was performed with Pentax ultrasound endoscopes (EUS FG-36UX respectively EBUS EB-1970UK; Pentax, Tokyo, Japan) with a Hitachi EUB-5500 processor (Hitachi, Tokyo, Japan). The fine-needle aspirations were performed under conscious sedation with midazolam and with local anesthesia sprayed in the oropharynx (lidocain 1%) and lidocain gel 20 mg/ml. Needles of 22 gauge were used for sampling and at least two aspirates were smeared on slides initially. Aspirations per site (3–4 passes in different directions of the tumor or enlarged mediastinal lymph node) were performed and deposited in carbowax 2% fixative. Cell blocks were made using cell pellets embedded in AGAR 10%, followed by formalin fixation, dehydration, and paraffinization.

Tumor Cell Density Estimation

Sections were cut from formalin-fixed paraffin-embedded (FFPE) tissue blocks. The first and last sections were stained with hematoxylin and eosin for histopathological examination. The percentage of tumor cells is estimated using the first and last hematoxylin and eosin sections. The estimated ratio is based on the tumor cell amount compared with stromal cells and lymphocytic background.

DNA Extraction

Genomic DNA was extracted from the remaining sections. The sections used for DNA extraction were deparaffinated and genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instruction. The extracted DNA was eluted in 100 µl ATE buffer.

DNA quality was checked by multiplex ladder PCR,[30] using 3 µl Mastermix (500 µl 10× Gold buffer, 40 µl 100 mM Gene Amp dNTP Blend, 250 µl Glycerol 87%, 50 µl Cresol Red, 300 µl 25 mM MgCl₂, 360 µl double-distilled H2O [ddH₂O]); 5 µl primermix; 0,08 µl Amplitaq Gold (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands); 200 nM of each primer; 1,0 µl ddH₂O and 1 µl sample per test. Primers used are listed in e-Table 1 (Supplemental Digital Content 1, http://links.lww.com/JTO/A445). PCR cycling was performed on a Veriti 96-well Thermal Cycler (Applied Biosystems) using the following conditions, one 12-minute cycle at 94.4°C was followed by 30 cycles of, respectively, 30 seconds at 95°C, 30 seconds at 59°C, 45 seconds at 72°C, and finally one cycle of 5 minutes at 72°C. The produced DNA amplicons were separated using agarose gel electrophoresis (3% agarose gel; 200 volt for 1 hour) and assessed with ultraviolet light.

Mutation Analysis

Exons 18, 19, 20, and 21 of the *EGFR* gene and exons 2 and 3 of the *KRAS* gene were examined with HRM as previously described.[21,31] HRM was performed in a total volume of 10 µl, containing 4 µl LightScanner mastermix (Idaho Technology Inc., Salt Lake City, UT), 2 µl genomic DNA, 2 µl 100 nM of each primer, and 2 µl ddH₂O. PCR cycling and HRM analysis were performed on a LightCycler 480II (Roche Diagnostics, Almere, The Netherlands) according to conditions previously described.[32] When the HRM plots were abnormal HRM amplicons were checked with Sanger sequencing. HRM products were purified using ExoSAP-IT (GE Healthcare, Hoevelaken, The Netherlands) according to the manufacturer's instruction. Primers used are listed in e-Table 2 (Supplemental Digital Content 2, http://links.lww.com/JTO/A446).

The purified HRM products were used for Sanger sequencing using the Big Dye Terminator v3.1 Kit (Applied Biosystems). The reaction mix consisted of 2 µl Sequencing RR-100, 3 µl 5x Sequencing buffer, 4 µl ddH₂O, 1 µl purified PCR product, and 10 µl 2.5 µM M13 primer in a final volume of 20 µl.

The sequence reaction was run on a Veriti 96-well Thermal Cycler (Applied Biosystems) using the following conditions, one cycle of 96°C for 1 minute followed by 25 cycles of 96°C for 10 seconds, 60°C for 125 seconds and at 4°C subsequently. The products were purified using the DyeEx 2.0 Spin Kit (Qiagen) according to the manufacturer's instructions and run on a 3130 Genetic Analyzer (Applied

Biosystems). Afterward, the sequences were analyzed using the Sequencer 3.0 software (Applied Biosystems).

Pyrosequencing was performed using the PyroMark Q24 (Qiagen). The therascreen *EGFR* and *KRAS* Pyro Kits (Qiagen) were used according to the manufacturer's instruction. The targeted sequences of *EGFR* and *KRAS* were amplified using PCR (Veriti 96-well Thermal Cycler; Applied Biosystems) using the following conditions, one 15-minute cycle at 95°C followed by 42 cycles of, respectively, 20 seconds at 95°C, 30 seconds at 53°C, 20 seconds at 72°C, and finally one cycle of 5 minutes at 72°C. Each PCR product was used as a template. The sequencing primer hybridizes close to the sequence of interest. Pyrosequencing was performed using PyroMark Gold Q96 reagents (Qiagen) containing enzyme and substrate mixture, dATP-S, dCTP, dGTP, and dTTP. Nucleotide incorporation is followed by release of ATP. Luciferin and ATP generates light emission after a reaction catalyzed by luciferase. The unique dispensing order described by the manufacturer is used to detect possible mutations in the targeted sequence. The pyrogram is analyzed using the pyrosequencing data analysis software (Qiagen).

Costs of Materials and Handling Time

The costs of materials to run a single sample for HRM, Sanger sequencing, and pyrosequencing are $100, $355, and $700, respectively. If possible, costs can be reduced by analyzing up to two patient samples per run for *EGFR* exon 18 to 21 and *KRAS* exon 2 to 3 ($520 per patient for 2 cases including blanc controls). The time required for a laboratory technician to perform a single HRM analysis is 50 minutes. When Sanger sequencing follows HRM the total handling time is 90 minutes. Pyrosequencing takes 100 minutes, and the total time required for HRM followed by pyrosequencing is 150 minutes.

Statistical Analysis

Patient characteristics were examined with descriptive statistics. A Pearson's correlation coefficient was used to assess the association between estimated tumor density and allele frequency. The difference in mean sample age between base pairs categories 100 to 300 and 300 to 400 was analyzed using an unpaired Student's *t* test.

RESULTS

Patient Samples

Between June 2008 and September 2011, pulmonary AC was diagnosed in 169 patients by different pathologists using EUS-fine needle aspiration (*n* = 90) or EBUS-transbronchial needle aspiration (*n* = 79).

After reviewing the slides for study purposes the diagnoses changed to a different pathologic classification in five patients. For all other patients (*n* =164) there was an enquiry for adequate tumor material in deposit. From 24 patients no agar-embedded material was stored, and from 13 other patients no DNA was available (see also Fig. 1).

In six patients DNA had been isolated previously and was not available anymore. FFPE tissue blocks did not contain

material to redo the DNA extraction after the first extraction and immunohistochemical analysis. HRM had been performed in the samples of all of these patients followed by Sanger sequencing in three cases. There were five *EGFR* and *KRAS* wild type and one *EGFR* exon 19 deletion.

Tumor samples of 126 patients were processed for DNA analysis. Patient characteristics, including disease stages, are described in Table 1. Amplification was not successful in 4 of 126 patients, resulting in 122 samples that were further analyzed with both HRM and pyrosequencing.

Because of insufficient DNA quality in five samples, HRM was unsuccessful (HRM for both *EGFR* and *KRAS* failed in 3 patients and HRM for *EGFR* alone failed in 2 patients). In 39 of 117 (33%) patients, HRM showed abnormalities in the *EGFR* exons and in 50 of 119 (42%) patients, HRM showed abnormalities in the *KRAS* exons. In these patients direct sequencing was performed to confirm or exclude mutations. In 24 patients, abnormalities in more than one gene were detected and required multiple Sanger sequencing tests.

Mutations

Table 2 shows the results of pyrosequencing for *EGFR* and *KRAS* in EUS and EBUS samples. In 122 samples of AC, 15 EGFR mutations (12%; 12 activating and 3 inhibiting mutations) and 51 KRAS mutations (42%) were detected.

Detection Rate and Tumor Analysis Success Rate

The detection rate of pyrosequencing for *EGFR* and *KRAS* mutations was 122 of 122 (100%), and the tumor analysis success rate was 97% (122 of 126) for both genes. The detection rate for *EGFR* mutations in all endosonography-guided samples with HRM in combination with Sanger sequencing was 117 of 122 (96%), and the tumor analysis success rate 93% (117 of 126).

Sanger sequencing for *EGFR* and/or *KRAS* genes after HRM was performed in 76 of 119 samples showing single-nucleotide polymorphisms (SNPs) in 19 samples when HRM was abnormal for exons 18 (*n* = 11) and 21 (*n* = 8). Detection of SNPs is relatively high because of the chosen primer sets, which include a common SNP18 hotspot. The SNP rate for exon 18 could be reduced by using different primer sets.

For *KRAS* the analysis success rate was 94% (119 of 126). Abnormalities detected with HRM in codons 12, 13, and 61 were all based on mutations.

Concordance Rates

For *EGFR* mutation analysis, HRM with Sanger sequencing in case of HRM abnormalities and pyrosequencing showed a concordance rate of 100% (117 of 117). For *KRAS* mutation analysis the concordance rate was 98% (117 of 119). In two patients pyrosequencing revealed a G12C point mutation that was not discovered with HRM.

Tumor Cell Density

The mean estimated tumor density in samples with mutations was 35.5% (range, 5–90%). The mean frequency of
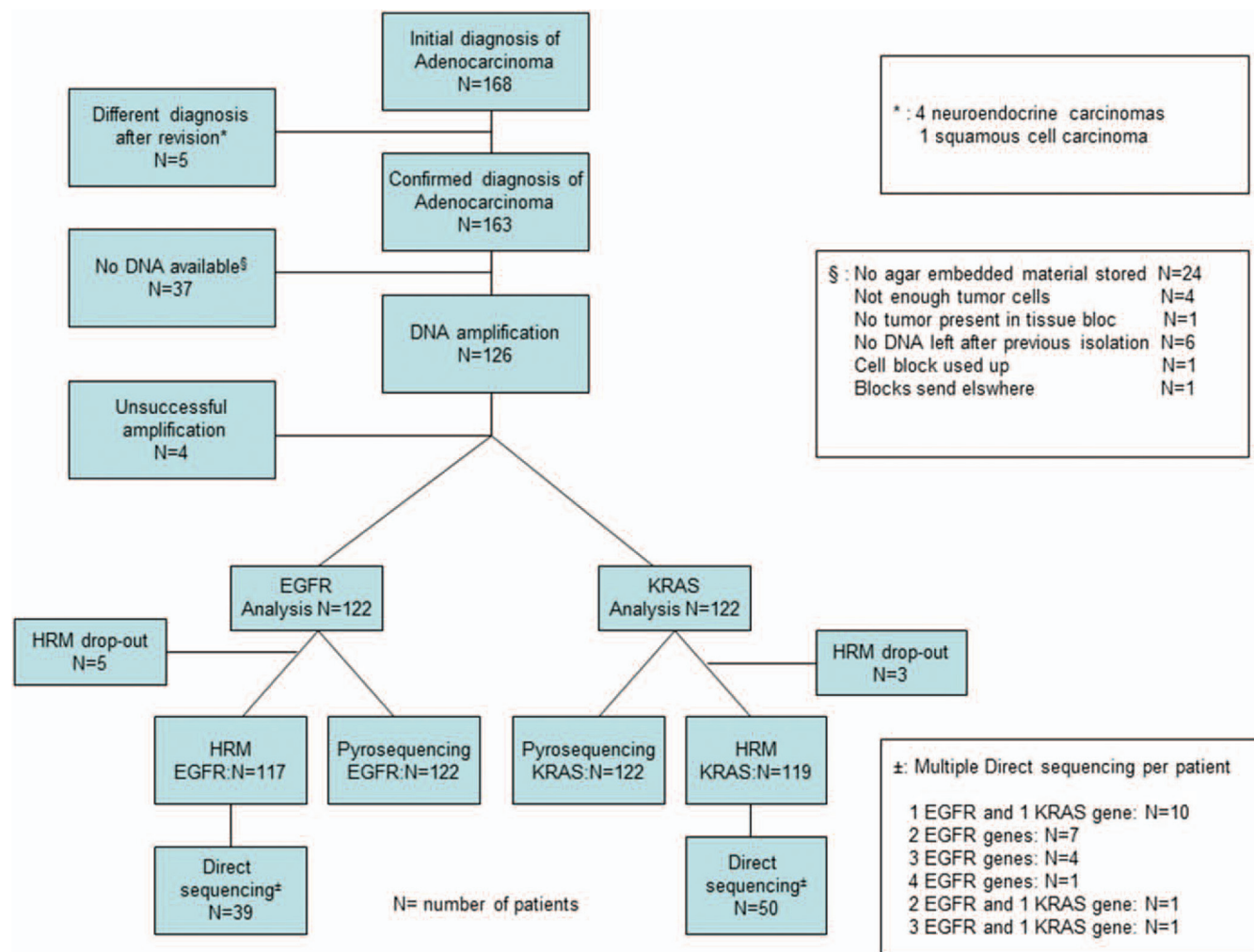
FIGURE 1. Flow chart showing the selection of patient samples and the successive stages of processing and analysis performed on them. EGFR, epidermal growth factor receptor; HRM, high-resolution melting; KRAS, V-Ki-ras2 Kirsten rat sarcoma viral onco-gene homolog gene.

TABLE 1. Characteristics of 126 Patients Eligible for DNA Processing

Age, Average (Range), yr	**66 (26–85)**
Sex	
Male	65
Female	61
EUS: EBUS	63:63
Tumor stage	
1b	2
2a	4
2b	2
3a	63
3b	12
4	43
Mean sample age in days (range)	585 (2–1323)
Mean tumor cell content (range)	30% (5–90)

EBUS, endobronchial ultrasound; EUS, endoscopic ultrasound.

the mutated alleles provided by the pyrosequencing software was 38.7% (range, 5.7–89.3%). The estimated tumor density and the frequency of mutated alleles were well associated (Pearson's correlation coefficient of 0.57 [$p < 0.001$]).

Factors Influencing Test Results

The quality of DNA, expressed as the length of base pairs was studied with agarose gel electrophoresis of PCR products. Shorter DNA chains indicate more fragmentation. All patients were classified into two subgroups with DNA of 100–300 bp and 300–400 bp showing mean sample ages of 784 days and 354 days, respectively ($p < 0.0001$). All five HRM failures showed short DNA fragments (100–200 bp).

The estimated tumor cell density in five samples, which proved to be inadequate for HRM analysis, was 5% ($n = 1$), 10% ($n = 1$), 30% ($n = 2$), and 50% ($n = 1$). In the four samples with an amplification failure, the estimated tumor cell densities were 5% ($n = 1$), 10% ($n = 2$), and 60% ($n = 1$).

TABLE 2.   Mutations Found in EUS- and EBUS-Guided Fine-Needle Aspirations of 122 Patients with Pyrosequencing Technique

EGFR Mutations			KRAS Mutations		
		n			*n*
Exon 19	delE746-A750	4 (27%)	Exon 2	G12C	24 (47%)
Exon 19	delL747-P753insP	1 (7%)	Exon 2	G12A	8 (16%)
Exon 20	T790M	3 (20%)	Exon 2	G12V	7 (14%)
Exon 21	L858R	7 (47%)	Exon 2	G12D	6 (12%)
			Exon 2	G12R	1 (2%)
			Exon 3	G13C	1 (2%)
			Exon 3	G13D	3 (6%)
			Exon 3	Q61H	1 (2%)
Total		15			51

EGFR, epidermal growth factor receptor; KRAS, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog gene.

DISCUSSION

In this study, the diagnostic performances of two sequencing strategies were compared on cytological samples from mediastinal lymph node metastases in patients with AC. Pyrosequencing was compared with HRM followed by Sanger sequencing for *EGFR* and *KRAS* mutations in specimens obtained by EUS and EBUS. Pyrosequencing on cytological blocks, especially older tumor blocks, is feasible. The diagnostic performance of both tests was good. HRM failed in a few samples in which the DNA was degraded as a result of longer sample storage times.

Diagnostic Success Rates

The high diagnostic success rates of both pyrosequencing (97%) and HRM (93%) in this study confirm that cytological aspirates from EUS and EBUS are suitable for molecular analysis. In a few samples we were unable to yield DNA with sufficient quality for HRM and pyrosequencing. After DNA amplification, HRM failed in a few more samples as well. In contrast to HRM analysis, pyrosequencing was successful in all samples, indicating a better sensitivity.

EGFR mutation analysis has been performed previously on various kinds of cytological samples such as pleural effusion cell blocks, percutaneous aspirates,[8,15,33] pericardial effusion and bronchoalveolar lavage,[9] and also EBUS-[8–10,12,14–16,19,33,34] and EUS-guided aspirates.[14,16,33] Reported success rates of mutation analysis on EUS-guided or EBUS-guided fine-needle aspirates ranged from 72% to 99% in previous studies.[8,9,11,12,14,16,17,20]

A comparison of the diagnostic performance of pyrosequencing with other sequencing techniques is difficult. In contrast to our study, samples in previous studies were often selected on estimated tumor density, and most studies were prospective studies and consequently used relatively younger specimens.

Concordance Rates

In all but five patients with amplifiable DNA, HRM could be performed. The concordance between pyrosequencing and HRM was 100% for *EGFR* and 98% for *KRAS*. Two samples showed wild-type *KRAS* using HRM analysis, but turned out to be *KRAS* mutated after pyrosequencing. This finding is likely related to the higher test sensitivity.

Two previous reports compared different sequencing methods but none described pyrosequencing. A recent study of 49 cytological samples of bronchial brushings and pleural effusions compared five different sequencing tests (PCR-Invader, peptide nucleic acid-locked nucleic acid PCR clamp, direct sequencing, Cycleave, and Scorpion ARMS).[18] Concordance rates among different methods ranged from 93.1% to 100% (bronchial brushings) and 85% to 100% (pleural effusions). In a report on 94 patients, Sanger sequencing was less sensitive for detecting *EGFR* mutations than (ARMS).[10]

Tumor Cell Density

In this study a significant, although modest, correlation was found between the estimated tumor cell density and the allele frequency of different *EGFR* and *KRAS* mutations.

Previous studies used predefined cutoff values of tumor cell density to consider samples suitable for molecular analysis.[8,13,16,33] The cutoff values ranged from 25%[33] to 70%.[13] In one report, authors claimed that as little as eight tumor cells from paraffin-embedded or fresh specimens obtained after microdissection were considered sufficient for mutation analysis in various cytological specimens.[9] Other studies on molecular analysis of cytological samples described no cutoff values at all.[10–12,15,18] Estimation of tumor cell percentages is not as relevant as previously stated. Moreover, tumor content is a subjective measure with interobserver variation. Finding a mutation in a sample with very low tumor density is considered a true-positive finding. However, the finding of a wild type in a very low tumor-density sample could result in a false-negative interpretation of a present mutation. In addition to this technical issue the question remains whether these very low-frequency mutations are clinically significant. Are they drivers or bystander mutations?

Sample Age

A relationship was demonstrated between sample age and DNA chain lengths after PCR. All HRM failures showed short DNA fragments in the ladder PCR, suggesting a relationship between sample age and successful molecular analysis. Nevertheless, we found only a few samples unsuitable for

molecular analysis, despite long storage times for up to 3.5 years.

This observation is relevant in case of using archival cytological specimen. Subtyping and staging is frequently performed at once in stage III non–small-cell lung cancer by EUS or EBUS. However, there is no clinical reason to analyze the mutation status in stage III disease because treatment with TKIs is not indicated according to present insights. Most patients with stage III disease, however, progress to stage IV during follow-up. When adequate material is available for these patients, samples can easily be reprocessed for molecular analysis without new invasive tests.

Pyrosequencing in Relation to Other Assays

The high concordance rate between HRM and pyrosequencing demonstrates the value of both tests for DNA analysis of EUS- and EBUS-derived samples. Because previous reports selected samples based on tumor cell percentages and were obtained from different sites with various sampling techniques, comparison with other methods is difficult. In contrast to previous studies, this study used samples derived from archival tissue with suboptimal (fragmented) DNA.

Comparable diagnostic performances for *EGFR* and *KRAS* mutation analysis, however, were described with coamplification at lower denaturation temperature–PCR in combination with Sanger sequencing in EBUS samples, enabling the detection of mutation frequencies as low as 5% to 10%.[20]

Tumor percentages in the same range of 5% to 10% allowed for mutation detection in our study as well although there is some doubt to call a sample a wild type when no mutations are detected in these samples. With pyrosequencing, analysis in samples with tumor percentages below 5% is feasible and can be used to confirm HRM analysis.

Other features, particularly cost aspects, are important to accomplish the comparison between pyrosequencing and other methods.

Financial Considerations

The least expensive method is HRM combined with Sanger sequencing. Pyrosequencing is too expensive to be used as a routine method in daily practice. The financial gap between HRM followed by Sanger sequencing and pyrosequencing is irreconcilable, even when more patient samples are analyzed in one run. More relevant is the comparison of costs between HRM prescreening followed by Sanger sequencing and HRM prescreening followed by pyrosequencing. A 38% increase in expenses was calculated for the latter option.

The difference in costs when Sanger sequencing is replaced by pyrosequencing for samples with an abnormal HRM, is substantial; nevertheless, replacement of Sanger sequencing by pyrosequencing does have considerable advantages (described below) that compensate the surplus of costs to some extent.

The Position of Pyrosequencing in Mutation Analysis

Sanger sequencing is performed on amplified DNA after HRM analysis. Failure of the HRM, because of poor DNA quality, will consequently result in a failure of Sanger sequencing as well.

Identification of mutations with pyrosequencing, if the HRM melting curve and difference plots show deviations from the wild-type curves, could serve as an alternative for Sanger sequencing. An important advantage of such a combined approach is that the molecular analysis is based on two independent techniques, allowing for a more confident molecular diagnosis.

The high sensitivity of pyrosequencing enables detection of low-frequency mutations and analysis in samples with a low tumor content or fragmented DNA. Sample characteristics such as tumor cell percentage, older sample age, and short DNA fragments in the ladder PCR (e.g., <200 bp), could serve as criteria to choose pyrosequencing over Sanger sequencing.

RESULTS OF MUTATION ANALYSIS

The *EGFR* mutation incidence was 12%, and is somewhat lower when compared with large European series (14%–16.6%).[3,35] In contrast, the incidence of *KRAS* mutations in this study (42%) is considerably higher compared with a large French study (14%). The difference in mutation incidence is likely the result of patient selection. The French study was performed nationwide, and consisted of stage IV patients. Our study population was derived from a relatively small rural area and includes stage III and IV patients. In a previous study from The Netherlands a comparable incidence for *KRAS* mutations (37%) and an even lower incidence for *EGFR* mutations (7%) has been described.[14]

LIMITATIONS

Important for the patient is a fast diagnostic track to allow a treatment start as soon as possible. Performing HRM as an initial screening step is a swift method to separate wild-type samples from mutated samples. The disadvantage is that this method requires a few days more when HRM abnormalities have to be determined by sequencing (in this study 65% of patients) in contrast to the use of pyrosequencing as the initial screening method.

CONCLUSION

Mutation analysis in EUS- and EBUS-guided needle aspirates using pyrosequencing is feasible and showed a high diagnostic success rate. The use of cytological specimens did not lead to analytical difficulties, and mutation frequencies were similar to known *EGFR* and *KRAS* mutation frequencies in our population. When comparing pyrosequencing with HRM, a high concordance rate was found. All HRM failures were observed in samples with fragmented DNA associated with longer storage times of the FFPE cell blocks. Mutation analysis by pyrosequencing enables the use of shorter DNA fragments, increasing the yield of molecular analysis on older and less optimal tissue samples.

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