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Rapid and Efficient Detection of *EGFR* Mutations in Problematic Cytologic Specimens by High-Resolution Melting Analysis

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Abstract

Background and Objective: Chemotherapy for advanced non-small-cell lung cancer (NSCLC) remains marginally effective, with a 5-year overall survival rate of approximately 5%. Recently, the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib was approved in Slovakia for the treatment of metastatic NSCLC. Gefitinib is a selective EGFR inhibitor that binds to the adenosine triphosphate binding pocket of the kinase domain and blocks downstream signaling pathways. Mutations of the *EGFR* gene, particularly an in-frame 15 bp deletion (delE746_A750) in exon 19 and the L858R mutation in exon 21, correlate with enhanced clinical responsiveness to EGFR tyrosine kinase inhibitors. However, the detection of these mutations and thereby prediction of the therapy outcome is sometimes unreliable due to the low sensitivity of direct sequencing if the proportion of tumor cells in the tissue is less than 25%. Therefore we decided to test the applicability of other methods, particularly high-resolution melting analysis (HRMA), for detection of these mutations in clinical samples.

Methods: We analyzed 53 archival cytologic specimens for the presence of *EGFR* mutations, using the HRMA method. Results were verified by direct sequencing. For samples containing less than 25% tumor cells, we used mutant-enriched PCR before sequencing. We also performed a titration assay to establish the lower limit of the proportion of tumor cells for detection of *EGFR* mutations.

Results: *EGFR* mutations were detected in 13 cases (24%). In-frame deletions in exon 19 were detected in eight cases (15%) and the L858R mutation in exon 21 was detected in five cases (9%). The positive results of the HRMA were confirmed by direct sequencing only in five of 13 cases. In the remaining eight positive samples, HRMA results were confirmed by sequencing analysis after mutant-DNA enrichment. The titration assay established that the lower limit for detection of *EGFR* mutations by HMRA was 1% tumor cells in the clinical sample.

Conclusion: Our results indicated that HRMA in combination with mutant-enriched PCR represents a sensitive method for detection of *EGFR* mutations from cytologic specimens. When properly executed, this protocol allows identification of *EGFR* mutations in specimens containing a minimal percentage of tumor cells.

Introduction

Lung cancer is one of the most common forms of cancer in Slovakia, with an incidence of more than 2500 new cases per year. Lung cancer is also the leading cause of cancer deaths in males and the second leading cause of cancer deaths in females in Slovakia.[1] Lung cancer is classified as either small-cell lung cancer (SCLC) or non-small-cell lung cancer (NSCLC). Approximately 80% of lung cancers are NSCLC.[2] Most of the patients with NSCLC have advanced or metastatic disease at

the time of diagnosis, thus surgery with tumor resection is performed only in about 15% of NSCLC cases; adjuvant chemotherapy is administered for most of these patients. Conventional chemotherapy has little effect on the outcome of patients with advanced, metastatic NSCLC, for whom the median survival is about 8–10 months.[3]

Over the last decade, extensive genetic research has provided a lot of useful information about molecular genetic abnormalities, including chromosomal aberrations, overexpression of oncogenes, and deletions or mutations in tumor-suppressor genes. These results have been applied to early detection, classification, and prognosis of NSCLC.[4,5] To date, several molecular markers of novel target therapy in NSCLC have been detected.

Receptor tyrosine kinases, especially the epidermal growth factor receptor (EGFR), play a key role in lung cancer tumorigenesis and progression.[6] EGFR is a transmembrane receptor protein with a ligand-binding extracellular domain, transmembrane domain, and cytoplasmic tyrosine kinase domain. Binding of specific ligands to the extracellular domain leads to EGFR dimerization. Dimerization induces the activation of the tyrosine kinase domain, which leads to autophosphorylation of the receptor on multiple tyrosine residues. This activates a series of intracellular signaling pathways, which in turn results in cancer proliferation, reduced apoptosis, invasion, and metastasis, and stimulates tumor-induced angiogenesis.[7]

Several selective tyrosine kinase inhibitors of the EGFR have been introduced to treat NSCLC.[8] One of them, the small-molecule tyrosine kinase inhibitor gefitinib, was recently approved for treatment of patients with NSCLC in Slovakia. Response to gefitinib is significantly enhanced by the presence of activating EGFR mutations.[9] Extensive studies of the activating mechanism and effects on gefitinib binding have demonstrated that mutations activate the kinase and accelerate catalysis. This in turn results in tighter binding of gefitinib to the active conformation of the kinase.[10] These mutations are localized in exons 18–21 of the EGFR gene, most frequently in two hotspots: an in-frame deletion of codons 746–750 in exon 19 and a missense mutation at codon 858 in exon 21.[9,11,12] The presence of these mutations strongly correlates with sensitivity to gefitinib and their detection is used to predict the response to therapy.[12]

Considering that in the European Union, gefitinib has been recently approved for treatment of patients with NSCLC, a suitable method for detection of *EGFR* mutations should be established. Detection of activating *EGFR* mutations through direct sequencing is currently considered the 'gold standard'. However, this approach is not suitable for practical clinical usage, since many patients with unresectable or metastatic disease are not candidates for surgery. Therefore, the only materials available for mutation detection in these patients are cytologic specimens obtained by bronchoscopy. Taking into account that this is a heterogeneous material, frequently with a small number of tumor cells accompanied by an abundance of normal cells without mutations, a suitable methodology should be chosen to prevent false negative or false positive results.

In this report, we established and validated an easy, inexpensive, and rapid method for the detection of activating *EGFR* mutations from cytologic specimens by the use of high-resolution melting analysis (HRMA).[13] HRMA is based on a PCR amplification of genomic DNA with target-specific primers in the presence of a fluorescent intercalating DNA dye. Subsequent analysis of changes in fluorescence during the melting of PCR amplicons enables us to discriminate between wild-type and mutated DNA.[14] This method does not require labeling of primers with dye. PCR amplification and melting analysis can be performed in the same tube, minimizing the sample handling and reducing the possibility of error and sample contamination. HRMA is easy, rapid, and inexpensive to perform.

The HRMA assay is a pre-screening test and requires confirmation of the positive results by direct sequencing. The sensitivity of direct sequencing depends on the quantity of tumor cells in the examined materials. The criterion of more than 25% tumor cells was defined by clinical laboratory validation studies showing the sensitivity of sequencing analysis.[15,16] In samples containing less than 25% tumor cells, we were able to confirm positive HRMA results by direct sequencing after the enrichment of tumor-associated DNA. We used a mutant-enriched PCR assay that has been proven to enrich one mutant gene among as many as 10^3 to 10^4 copies of wild-type gene.[17]

In the present study, we compared the results of *EGFR* mutation detection from archival cytologic preparations, using the following methods: HRMA, mutant-enriched PCR, and sequencing analysis. We determined their sensitivity and proposed an algorithm of molecular-genetic examination of the *EGFR* mutations, depending on the quality of the investigated material.

Materials and Methods

Samples

We analyzed 53 archival cytologic specimens, fixed with methanol and stained with Giemsa or Papanicolaou staining. The histologic subtypes of NSCLC of the analyzed samples were as follows: 25 were adenocarcinomas and 28 were squamous cell carcinomas. Specimens were obtained by standard

fiber-optic bronchoscopic procedure of endobronchial brushing. In co-operation with pathologists we obtained information about the total number of nucleated cells and the percentage of representative tumor cells on each slide.

DNA Extraction

DNA was extracted from the cells on the slides using the QIAamp DNA Micro Kit (Valencia, CA, USA). Coverslips were removed by immersion in xylene for 72 hours, and the slides were rinsed three times in 95% ethanol, followed by three rinses in distilled water. Tumor cells were scraped off the slide with a scalpel, put into a tube, and suspended in ATL buffer (Qiagen, Hilden, Germany) and proteinase K in 1.5 mL tubes. Subsequent procedures were performed according to the manufacturer's protocol.

Cell Lines and Dilution Assay

We performed dilution assays for the assessment of HRMA sensitivity and to determine the limiting proportion of tumor cells. Three lung adenocarcinoma cell lines – NCI-H1650, NCI-H1975, and NCI-A549 – were obtained from the American Type Culture Collection (Manassas, VA, USA). The NCI-H1650 cell line contains the mutation in exon 19 (delE746_A750), the NCI-H1950 cell line contains the mutation in exon 21 (L858R), and the NCI-A549 cell line contains wild-type *EGFR*. Dilutions for sensitivity studies were performed by mixing the DNA extracted from positive control lung cancer lines with the DNA extracted from cell lines containing wild-type *EGFR*. Mixtures with the following proportions of DNA from mutant cells were prepared: 100%, 50%, 25%, 10%, 5%, 2%, 1%, 0.5%, and 0% (no mutant cells).

PCR

The sequences of the primers for HRMA are shown in table I. The primers were designed to span entire exons 19 and 21 of the *EGFR* gene, with product sizes of 194 bp and 235 bp, respectively. PCR for HRMA was performed in a 0.2 mL tube in the presence of the intercalating dye LC Green Plus (Idaho Technology Inc., Salt Lake City, UT, USA). The PCR was performed in 15 μL volume containing 20 ng genomic DNA, 2 pmol of each primer, 7.5 μL Thermo-Start PCR master mix with 2.5 mM $MgCl_2$ (Thermo Fisher Scientific, Waltham, MA, USA), 2 μL nuclease-free water, and 1.5 μL LC Green Plus. PCR conditions included preactivation of the enzyme for 15 minutes at 95°C, followed by 30 cycles at 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and a final extension of 10 minutes at 72°C.

High-Resolution Melting Analysis (HRMA)

The 10 μL of PCR product was transferred to a Framestart 96 black plate (4titude Ltd, Surrey, UK) and mixed with a drop of light mineral oil (Sigma-Aldrich Chemie GmbH, Munich, Germany). The plate was covered with adhesive PCR film (Thermo Fisher Scientific) and briefly centrifuged. The PCR products were denatured at 95°C for 30 seconds and cooled to 4°C in the MultiGene Cycler (Labnet International, Inc., Edison, NJ, USA) to form heteroduplexes. HRMA was performed on the Light Scanner (Idaho Technology Inc.) and the acquired data were analyzed using the provided software (Idaho Technology Inc.). The HRMA instrument was heated at a transition rate of 0.3°C per second. The normalized graph shows the degree of the reduction in fluorescence over a temperature range of 75–98°C. Melting profiles of each sample were compared with those of the control samples. Genomic DNA isolated from NCI-A549 cell line containing the wild-type *EGFR* was used as the negative control. DNA isolated from tumor cell lines NCI-H1650 and NCI-H1975 were used as positive controls for mutations in exons 19 and 21, respectively. Samples were considered positive (carrying mutation) if the shape of their difference melting plot differed significantly from that of the wild-type sample (represented by the x-axis).

Mutant-Enriched PCR

The PCR was performed to amplify exons 19 and 21 of the *EGFR* gene, using the primers described previously. The first round

Table I. Primers used for high-resolution melting analysis (HRMA) of exon 19 (ex19) and exon 21 (ex21) of the epidermal growth factor receptor (*EGFR*) gene

Exon	Primer name	Sequence	Amplicon size (bp)
19	EGFR_ex19_F	5′-GCACCATCTCACAATTGCCAGTTA-3′	194
	EGFR_ex19_R	5′-GAGGTTCAGAGCCATGGACCC-3′	
21	EGFR_ex21_F	5′- CCATGATGATCTGTCCCTCACA-3′	235
	EGFR_ex21_R	5′-AGGAAAATGCTGGCTGACCTAAAG-3′	

F = forward; **R** = reverse.

of PCR was performed in 20 μL volume containing 20–100 ng genomic DNA, 2 pmol of each primer, 10 μL of Thermo-Start PCR master mix with 2 mM $MgCl_2$ (Thermo Fisher Scientific), and 6 μL of nuclease-free water. PCR conditions included preactivation of the enzyme for 15 minutes at 95°C, followed by 40 cycles at 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and a final extension of 10 minutes at 72°C. The principle of the restriction assay for enrichment of mutated genes in exons 19 and 21 of the *EGFR* gene was described previously.[17] The restriction digestion was performed by using FastDigest restriction endonucleases (Fermentas International Inc., Burlington, ON, Canada). Five microliters of the restricted products was used as a template for the second round of PCR amplification under the same conditions as the first round of PCR. Products of the second amplification were analyzed by sequencing analysis. The results of the mutant-enriched PCR were simultaneously indirectly detected on a 12% PAGE.

DNA Sequencing

We performed the sequencing analysis for the detection of *EGFR* mutations in exons 19 and 21, using the same primers and PCR conditions that were used for HRMA or mutant-enriched PCR. The 15 μL of PCR products were purified with Exo-Sap (Fermentas International Inc.), followed by a sequencing reaction with Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The sequencing products were purified through an Ex-Terminator kit (A&A Biotechnology, Gdynia, Poland) before being run on a 3130 Genetic Analyzer (Applied Biosystems). The sequencing data were analyzed using Sequencing Analysis Software v5.2 Patch 2 (Applied Biosystems).

Results

The sensitivity of the HRMA method was estimated by applying the method to mixtures of DNA with varying ratios of mutant and wild-type DNA. The mutated DNA, isolated from tumor cell lines containing activating mutations of the *EGFR* gene, was mixed with various amounts of the wild-type DNA from cell lines containing wild-type *EGFR*. Using the HRMA method we were able to detect mutations in exons 19 and 21 of the *EGFR* gene in the mixture with as low as 1% of mutant DNA, which suggested high sensitivity of this method (figure 1).

We analyzed the activating mutations of the *EGFR* gene in 53 archival cytologic preparations with specimens obtained by brush abrasion of the respiratory mucosa. The results of cytologic evaluation by a pathologist, which included determination of the histologic NSCLC subtype, percentage of tumor cells, and total cell numbers, are shown in table II. Almost one-half (24 of 53; 45%) of investigated cytologic preparations contained a minimum abundance of tumor cells, ranging from 5% to 20%. The cytologic preparations with the percentage of tumor cells ranging between 25% and 95% represented 54% of samples (29 of 53).

In the HRMA, eight of the investigated samples showed melting curves identical to those of positive controls containing mutation in exon 19 and visible drift of normalized and differential curves compared with samples containing the wild-type *EGFR* gene (figure 2a). Five samples showed melting curves identical to positive controls containing *EGFR* mutation in exon 21 (figure 2b). These samples were specified as positive.

The positive results of HRMA were confirmed by direct sequencing (figure 2). Direct sequencing detected the deletion of 15 nucleotides in exon 19 in three of eight positive HRMA

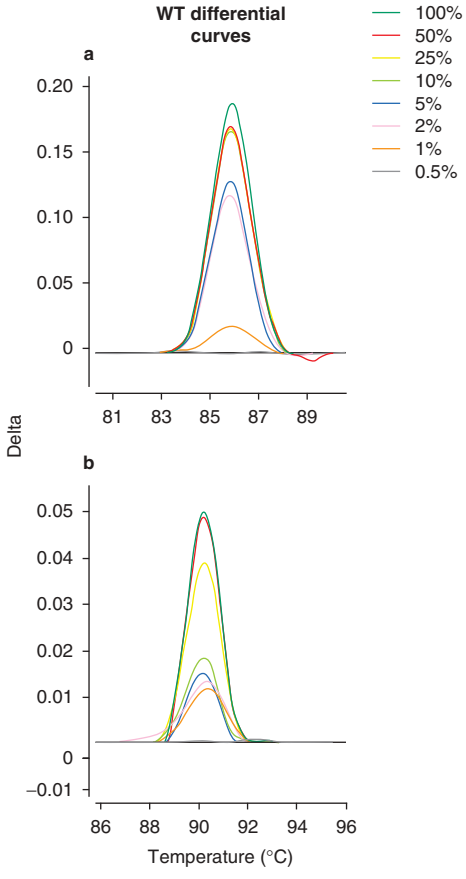

Fig. 1. Determination of sensitivity of the high-resolution melting analysis (HRMA) method for the detection of activating mutations of the epidermal growth factor receptor (*EGFR*) gene. The differential curves represent different DNA dilutions for (**a**) exon 19 (delE746_A750) and (**b**) exon 21 (L858R). The color of each curve corresponds to the dilution of the DNA as shown in the graph key. **WT** = wild-type.

Table II. Results of cytologic evaluation and epidermal growth factor receptor (*EGFR*) mutation status

No.	Histology	Proportion of tumor cells (%)	HRMA		Mutant-enriched PCR	Sequence analysis
			exon 19	exon 21		
1	Epidermoid	5	Positive	WT	Positive	Del746-750
2	Adenocarcinoma	20	WT	Positive	Positive	L858R
3	Adenocarcinoma	5	Positive	WT	Positive	Del746-750
4	Adenocarcinoma	10	Positive	WT	Positive	Del746-750
5	Adenocarcinoma	10	WT	Positive	Positive	L858R
6	Adenocarcinoma	10	WT	Positive	Positive	L858R
7	Adenocarcinoma	30	Positive	Positive		Del746-750 L858R
8	Adenocarcinoma	5	Positive	WT	Positive	Del746-750
9	Adenocarcinoma	25	Positive	WT		Del746-750
10	Adenocarcinoma	10	Positive	WT	Positive	Del746-750
11	Adenocarcinoma	50	Positive	WT		Del746-750
12	Adenocarcinoma	80	WT	Positive		L858R

HRMA = high-resolution melting analysis; **WT** = wild type.

results (37.5%). This method also confirmed the presence of missense mutation in exon 21 in two of five positive HRMA samples (40%). The sensitivity of detection of activating *EGFR* mutations by sequencing analyses from cytologic specimens was 38.5%.

In the remaining five samples with positive HRMA findings in exon 19 of the *EGFR* gene and three cases with a positive HRMA result in exon 21, confirmation by sequencing analysis was not possible, because of the low percentage of tumor cells, ranging from 5% to 20%. When the wild-type genes were selectively eliminated using the mutant-enriched PCR restriction cleavage, we were able to confirm the HRMA results by the sequencing analysis (figure 2). Results of the mutant-enriched PCR were simultaneously analyzed by indirect determination in the 12% PAGE, which showed 100% correspondence with the HRMA results.

The highest frequency of the mutation in exon 19 occurrence was detected in samples with the histologic subtype of adenocarcinoma, and it was found in 87.5% (seven of eight). In one case we detected the sequence changes in exon 19 in the sample with the histologic subtype of squamous cell carcinoma. The mutation in exon 21 of the *EGFR* gene was detected only in samples with the histologic subtype of adenocarcinoma. In one case of adenocarcinoma, we detected both activating mutations of the *EGFR* gene.

Discussion

In this study we established and validated the method of the *EGFR* mutation detection from cytologic preparations via HRMA. With the use of dilutions of DNA from tumor cell lines containing mutations in the *EGFR* gene (delE746_A750 and L858R) we demonstrated high sensitivity of this method. We were able to detect activating mutations of the *EGFR* gene in specimens containing at least 1% of mutant DNA.

Occurrence of the *EGFR* mutations was detected via the HRMA method in 24% (13 of 53) of the archival cytologic preparations we studied. The mutation in exon 19 of the *EGFR* gene was detected in 15% (eight of 53) and in exon 21 in 9% (five of 53) of the specimens. The positive findings were confirmed by sequencing analysis. Sensitivity of the detection of activating mutations by sequencing analysis was only 38.5% in comparison with the HRMA method. In our study we were able to detect activating mutations through sequencing analysis only in five of 13 cases. The low sensitivity of the sequencing analysis is probably caused by the low percentage of tumor cells. Thus, if we wanted to prevent false negative findings, determination of the percentage of tumor cells in cytologic preparations was necessary.

In several studies in which the HRMA method was used for detection of *EGFR* mutations, cases of false negative or false positive determinations have been mentioned.[18,19] Therefore, close cooperation of the genetic laboratory and the pathologist is recommended during investigation of cytologic and biologic preparations.

Studies comparing the HRMA method with the sequencing analysis have claimed that the sensitivity of these methods was comparable.[20-22] It was indicated that the sensitivity of the HRMA in comparison with the sequencing analysis was 83% in

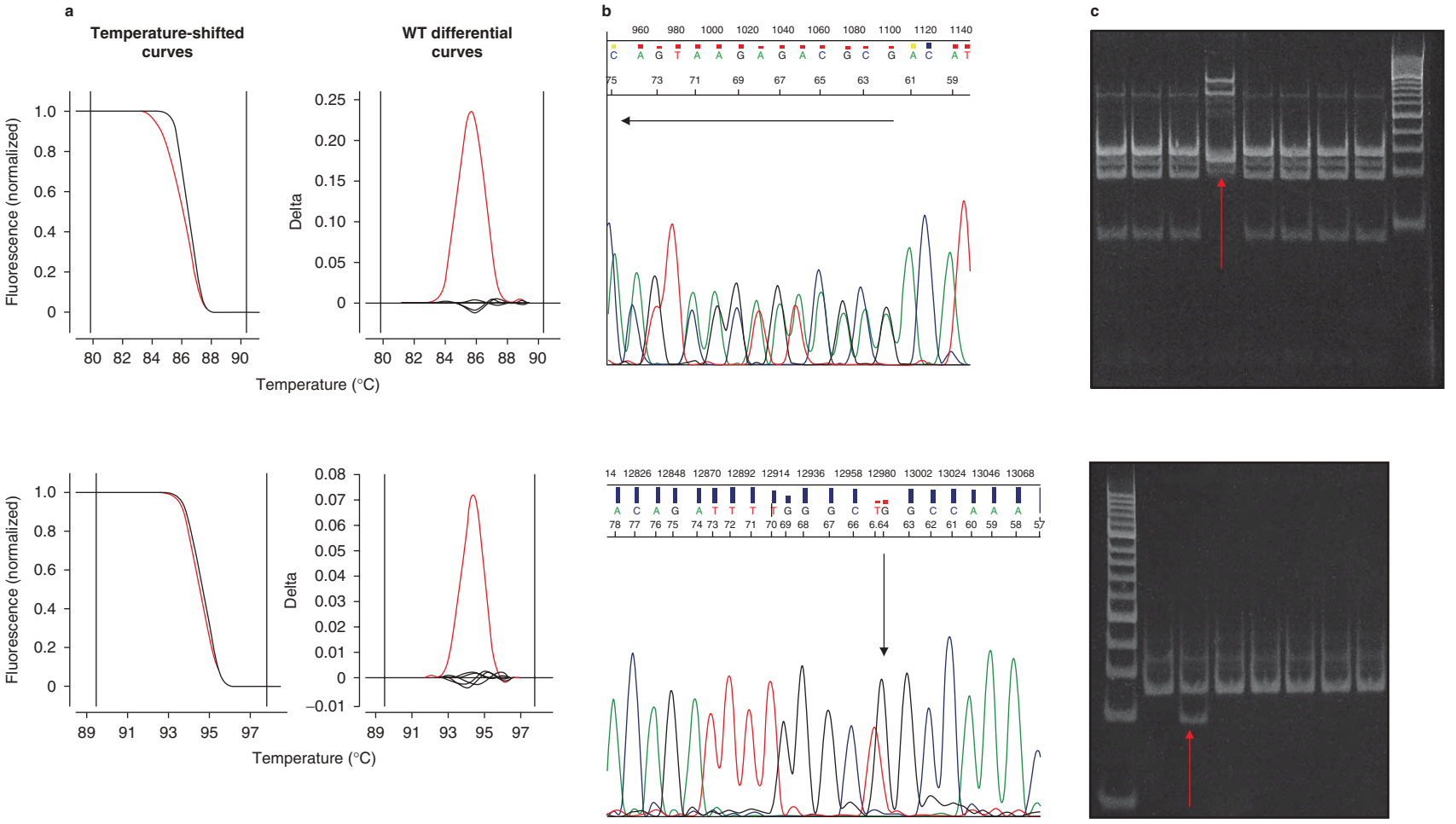

Fig. 2. Confirmation of the positive high-resolution melting analysis (HRMA) results for epidermal growth factor receptor (*EGFR*) mutations in exon 19 (delE746_A750) and exon 21 (Leu858Arg). (**a**) Results of HRMA for each exon, showing the presence of the respective mutations. (**b**) Results of sequencing analysis of tumor-enriched samples via mutant-enriched PCR, confirming the presence of the mutations. (**c**) Results were also confirmed indirectly by different mobility of mutant-enriched PCR products via polyacrylamide gel electrophoresis. **WT** = wild-type.

the case of mutation in exon 19 and 100% in the case of mutation in exon 21.[22] However, in these studies the sequencing analysis was performed on the tumor material. Taking into account that during diagnostics, most patients with NSCLC are in a progressed or metastatic stage of the disease, the most frequently available material in routine practice is the cytologic preparation. Our results imply that sequencing analysis is inappropriate for detection of *EGFR* mutations in cytologic preparations. The choice of the sequencing analysis method is subject to the presence of more than 25% tumor cells in the investigated material[23,24] and it requires the use of laser microdissection in cases where a lower proportion of tumor cells is present.[25]

Since the activating mutations in the samples with a minimum percentage of tumor cells could not be confirmed by sequencing analysis, we decided to utilize the mutant-enriched PCR method. The method is derived from the nested PCR and uses the restriction cleavage for selective elimination of the wild-type genes and amplification of only mutated genes. Asano et al.,[17] who established the mutant-enriched PCR method for the detection of activating mutations of the *EGFR* gene, confirmed the high sensitivity of this method via dilutions. They were able to detect mutations in exons 19 and 21 of the *EGFR* gene in the sample with a 10^3–fold to 10^4–fold excess of wild-type genes.[17]

In our study, we used the mutant-enriched PCR method for reamplification of mutated genes in samples with a minimum percentage of tumor cells. Consequently, we were able to confirm the HRMA results by sequencing analysis. The mutant-enriched PCR method is more time consuming than HRMA. However, it also allows indirect determination of the mutation occurrence by detection of the PCR products in 12% PAGE. Recently, various types of reactive buffers (e.g. FastDigest buffer [Fermentas International Inc.]) have become available, which allow visualization of restriction cleavage products directly in agarose gel, and thus the process is significantly less time consuming. In this study we compared the results of indirect determination of the *EGFR* mutations via the mutant-enriched PCR method with sequencing analysis after enrichment of the mutated genes. The sensitivity of this method was 100%.

Recently, a number of articles validating methods for *EGFR* mutation detection from small tumor biopsies have been published. Mostly, these were non-sequencing methods for mutation detection, such as single-strand confirmation polymorphism (SSCP),[26] restriction fragment length polymorphism (RFLP),[27] peptide nucleic acid (PNA)-locked PCR clamping,[28] TaqMan PCR (Applied Biosystems),[29] amplification refractory mutation system (ARMS),[30] and other methods. Most of them are costly and require specific probes and instruments. The HRMA represents a rapid, specific and cost-effective method for *EGFR* mutation detection from cytologic preparations. It involves no post-PCR processing, which eliminates the possibility of contamination. The great advantage of the HRMA method is time efficiency. This method allows the selection of positive and negative samples in a short time.

The HRMA method is also suitable for detection of *EGFR* mutations from formalin-fixed, paraffin-embedded (FFPE) blocks as well as from samples with very low DNA quantity. The advantage of the HRMA method is the requirement for only a small amount of the template. By dilution assays we found that the HRMA method allows detection of the mutation in the sample with only 1 ng of template.[31] The recovery rate of DNA isolated from the cytologic preparations was variable and it depended on the quality and the density of the coating.

However, the HRMA method also has certain limitations. It is only suitable for analysis of samples isolated from the same type of material in one run. It is also necessary to emphasize that the control sample has to be used for every DNA analysis. DNA isolated from various types of investigated material (peripheral blood, paraffin block, frozen tissue) produced different denaturation HRMA profiles, consequently leading to false positive determinations.[32] For their elimination, it is necessary to analyze patient DNA samples and control samples (containing wild-type *EGFR*) that have been isolated from the same type of the material.

In the course of our experiments, we have observed that for clearer determination of the results by the HRMA method, it is optimal to analyze at least 10 samples by LightScanner in one run. The high sensitivity for the DNA quality represents another disadvantage of the HRMA method. By investigation of DNA isolated from frozen tissues and formalin-fixed tissues, the interaction between formalin and DNA was confirmed. Formalin initiates an irreversible DNA degradation, thus it significantly affects its quality. It was found that the denaturation profile of HRMA is significantly affected by DNA quality. The DNA quality is one of the factors which causes aberrant variations of the HRMA denaturation profile and it can lead to determination of false negative or false positive results.[33]

Conclusion

Until now, no criteria had been developed for detection of activating *EGFR* mutations in cytologic specimens, which would define the percentage of tumor cells sufficient for analysis.

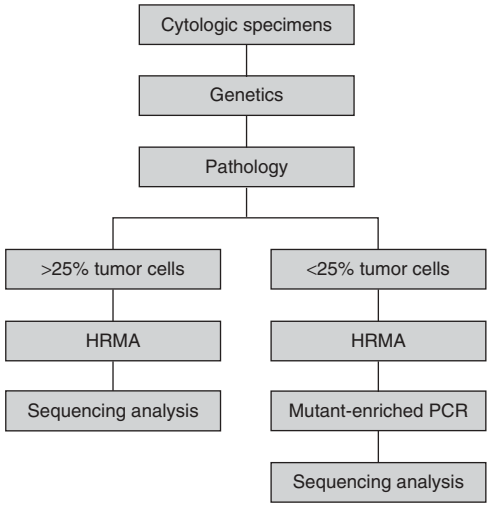

Fig. 3. Proposed algorithm of screening methods for efficient detection of activating epidermal growth factor receptor (*EGFR*) mutations from cytologic specimens. **HRMA** = high-resolution melting analysis.

Cytologic samples should not be specified as inadequate for *EGFR* mutational testing only on the basis of an insufficient amount of tumor cells for sequencing analysis.

In our laboratory, we used the HRMA method for detection of *EGFR* mutations as a pre-screening method in the diagnostic algorithm shown in figure 3. The HRMA represents a highly suitable method for detection of *EGFR* mutations from cytologic preparations. It shows a high sensitivity rate and low material expenses. To confirm positive results of the HRMA, sequencing analysis should be used. Because of the detection limits, sequencing analysis should be used for samples with a percentage of tumor cells higher than 25%. In the samples with a percentage of tumor cells lower than 25%, mutations can be confirmed via sequencing analysis only after enrichment of mutated genes via a mutant-enriched PCR method. The algorithm for *EGFR* mutation detection that we propose is suitable for routine oncologic practice.

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