Cellular Oncology 31 (2009) 329–333
DOI 10.3233/CLO-2009-0489
IOS Press

A panel of high resolution melting (HRM) technology-based assays with direct sequencing possibility for effective mutation screening of EGFR and K-ras genes

D.A.M. Heideman [a,*], F.B. Thunnissen [a], M. Doeleman [a], D. Kramer [a,**], H.M. Verheul [b], E.F. Smit [c], P.E. Postmus [c], C.J.L.M. Meijer [a], G.A. Meijer [a] and P.J.F. Snijders [a]

[a] *Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands*
[b] *Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands*
[c] *Department of Pulmonary Diseases, VU University Medical Center, Amsterdam, The Netherlands*

Abstract. *Background*: Increasing data from clinical trials support EGFR and K-ras mutation status as predictive markers of tumour response to EGFR-targeted therapies. Consequently, rapid and reliable mutation screening assays are demanded to guide rational use of EGFR-targeted therapies.

Methods: In this study, we describe the development of high resolution melting (HRM) technology-based assays with direct sequencing confirmation possibility for mutation screening of the EGFR gene (exons 19, 20 and 21) in routine diagnostic specimens, and compared assay findings to those of conventional nested-PCR following cycle-sequencing.

Results: In reconstruction experiments, each HRM assay following sequencing demonstrated a sensitivity of $\leqslant$5% of mutated DNA in a background of wild-type DNA. The panel of EGFR HRM assays following sequencing applied to a series of genomic DNA samples isolated from 68 FFPE NSCLC specimens correctly identified all EGFR mutations that were previously found by nested-PCR following cycle-sequencing. The HRM approach additionally scored two mutations not detected by the conventional assay.

Complementary HRM following sequencing for K-ras revealed three mutations. EGFR and K-ras mutations were mutually exclusive.

Conclusions: The panel of designed HRM assays with direct reflex sequencing possibility provides an effective method for mutation screening of EGFR and K-ras genes in routine diagnostic specimens, thereby allowing the selection of the treatment of choice in clinical practice.

Keywords: HRM, direct cycle sequencing, K-ras, epidermal growth factor receptor, genotype, codon, (nested-) PCR, formalin-fixed paraffin-embedded, molecular diagnostics, TKI, receptor tyrosine kinase inhibitors, anti-EGFR monoclonal antibodies, EGFR-targeted therapies, personalized therapy

Increasing evidence points to the mutational status of EGFR and K-ras genes being effective molecular predictors of patient's response to and survival benefit after treatment with inhibitors of the EGFR axis (i.e., anti-EGFR monoclonal antibodies and EGFR tyrosine kinase inhibitors (TKI)) [1,2]. As a consequence, mutation screening tests for K-ras and EGFR genes are important as their results may provide a valuable guidance for clinicians to make decisions on EGFR-targeted therapies. Previously, we reported a high resolution melting (HRM) technology-based assay with direct sequencing confirmation possibility, for detection and genotyping of K-ras exon 1 (codons 12 and 13) mutations in routine pathological specimens (i.e., formalin-fixed paraffin-embedded (FFPE) tissue and cytology specimens) [3]. The HRM assay princi-

*Corresponding author: D.A.M. Heideman, PhD, Department of Pathology, VU University Medical Center, de Boelelaan 1117, 1081 HV Amsterdam, The Netherlands. Tel.: +31 20 444 4023; Fax: +31 20 444 2964; E-mail: dam.heideman@vumc.nl.

**Current address: Department of Dermatology, University Medical Centre Groningen, Groningen, The Netherlands.

ple is a real-time PCR amplification of genomic DNA with target specific primers in the presence of a fluorescent intercalating DNA dye (i.e., LCGreen-Plus) following fluorescent melting curve analysis of PCR amplicons to discriminate between wild-type (WT) and mutant DNA. The HRM assay is particularly suited as pre-screening test to decide whether or not a specimen should be subjected to subsequent cycle sequencing in order to identify the specific nucleotide alteration(s). By use of PCR primers that contain M13-tags genotyping of mutations can be directly performed by sequencing of the PCR product generated during the HRM procedure, obviating the need for performing additional PCR reactions.

Herein, we describe the same approach to detect and genotype genomic mutations in EGFR (i.e., exons 19, 20 and 21) and demonstrate superior characteristics of the HRM-based assays as compared to a conventional method using nested-PCR followed by cycle-sequencing [4]. These EGFR HRM-based assays combined with the previously developed K-ras assay [3] provide a fast, comprehensive mutation pre-scanning method easily applicable to routine diagnostic specimens that afford direct sequencing as reflex test for mutation verification.

The HRM primer sequences for the various targets are shown in Table 1. HRM assays were designed to yield relatively small amplimers of at maximum 168 bp, thereby favouring reproducible target amplification from FFPE tissue. To limit interference of mutation detection by common single nucleotide polymorphisms (SNPs), exon 20, known to contain a very common SNP (c.2361G>A) (www.ncbi.nlm.nih.gov/SNP; ID: ss24778961), was divided into two HRM assays (region 20p and region 20d) with the SNP falling within the primer binding region. The used primers, either or not with the acceptance of a nucleotide mismatch, allowed targeting both nucleotides representing the SNP. To allow direct sequencing of HRM products, M13-tagged primers were designed. This approach has advantage over previously reported HRM assays for EGFR [5–10] that need separate PCR reactions for sequencing. The HRM conditions and subsequent unidirectional sequencing procedures were essentially as described before [3].

Reconstruction experiments using a dilution series of DNA isolated from either cell lines or patient material containing known EGFR mutations (i.e., cell line H1650 containing a deletion in exon 19 (delE746-L750; K745K), cell line H1975 containing L858R/T790M and tissue EK1472 containing an insertion in exon 20 (D770-N771insSVQ) in a background of WT DNA (i.e., DNA isolated from SiHa cell line) demonstrated an analytical sensitivity for detection of mutations for the HRM assays of $\leqslant 5\%$, independent of the exon targeted (Fig. 1A–D). Subsequent direct sequencing of the HRM PCR products confirmed the presence of the respective nucleotide change(s) with similar sensitivity (Fig. 1E). Triplicate analyses demonstrated each assay in the panel to perform at a reproducible manner.

On the basis of pilot experiments on a series of 10 FFPE tissue-derived WT DNAs, as verified by nested-PCR following cycle sequencing [4], thresholds for each HRM assay were defined for scoring a specimen as having an aberrant melting profile and directing reflex sequencing for mutation confirmation. During the course of this study, we also included in subsequent sequencing analysis samples with a read out value just below the chosen thresholds (i.e., 6, 6, 6

Table 1
HRM primers per exon

Exon		Name	Amplicon size	Sequence (5′–3′)
19		19-FP	136 bp	GTAAAACGACGGCCAGCGTCTTCCTTCTCTCTCTGTCAT
		19-RP		ACACAGCAAAGCAGAAAC
20	region 20p	20p-FP	110 bp	GTAAAACGACGGCCAGCCACACTGACGTGCCTCT
		20p-RP		AGCTGCGTGATGAGT*TGCA
	region 20d	20d-FP	82 bp	CACCTCCACCGTGCAG*CTC
		20d-RP		GTAAAACGACGGCCAGCAGGTACTGGGAGCCAATA
21		21-FP	168 bp	GTAAAACGACGGCCAGTCCCATGATGATCTGTCCCTCACAG
		21-RP		TGCCTCCTTCTGCATGGTATTCTT

Notes: The M13 moiety is underlined; FP – forward primer; RP – reverse primer;
*indicates the position of the common SNP in exon 20 (c.2361G>A).

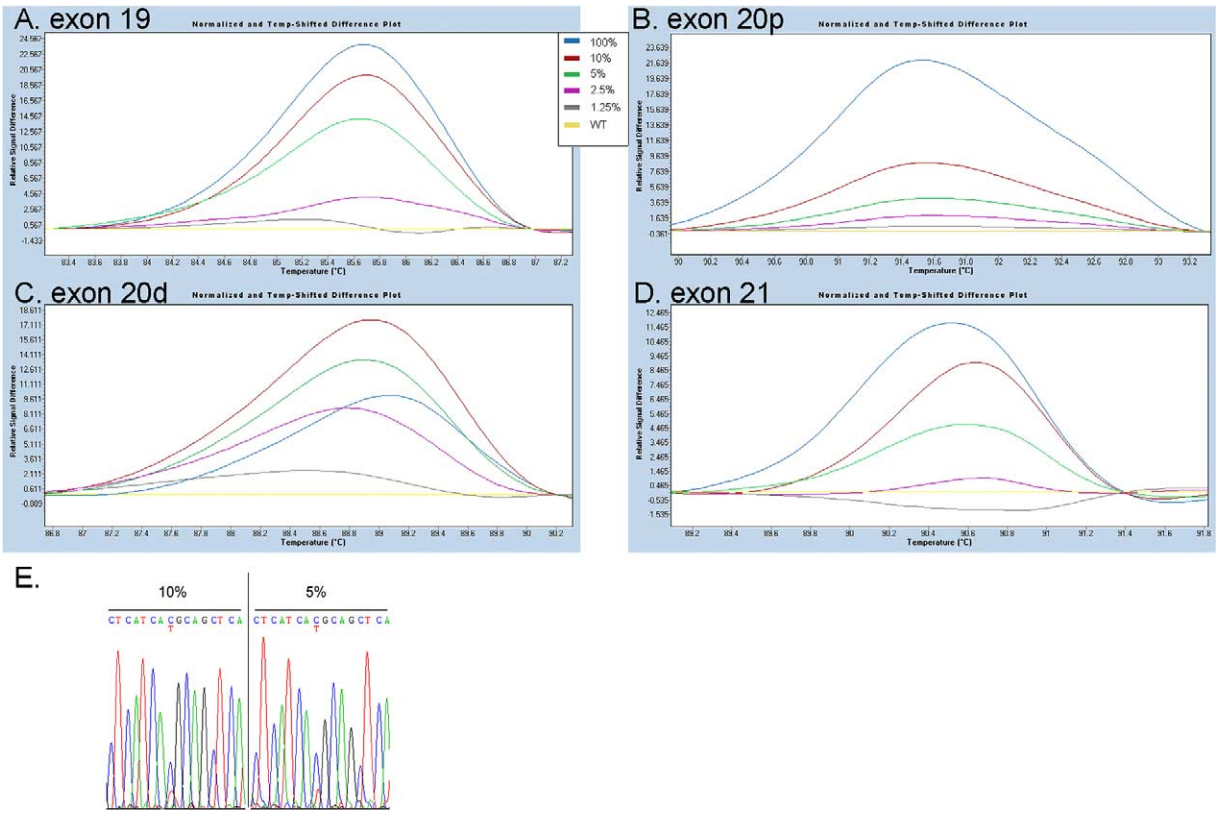

Fig. 1. Panel of HRM assays for EGFR exons 19, 20 and 21. The designed panel of EGFR HRM assays encompasses a pre-screening approach with direct sequencing confirmation possibility. The altered melting of HRM amplicons is best visualized in the normalised and temperature shifted melting plot, in which the relative signal difference of the specimen as compared to a reference WT DNA indicates the likelihood of a mutation. Shown are representative EGFR HRM assay findings for (A) exon 19 on a dilution series of H1650, containing a deletion in EGFR exon 19, (B) exon 20/region 20p (proximal region) on a dilution series of EK1472, containing an insertion in EGFR exon 20, (C) exon 20/region 20d (distal region) on a dilution series of H1975, containing T790M, and (D) exon 21 on a dilution series of H1975, containing L858R. (E) The pre-screen assay may be followed by direct sequencing of HRM PCR products that showed an aberrant melt out to find out the nature of the EGFR mutation. Representative examples of the sequencing results of the HRM products for exon 20/region 20d of 10% and 5% H1975 DNA, respectively, in WT-DNA background is shown, indicating both the T790M mutation.

and 7 specimens in HRM for exon 19, 20p, 20d and 21, respectively). All these cases revealed WT sequences. Consistent with these findings, our HRM assays confidently differentiate between aberrant and WT curves. This 100% negative predictive value of HRM signifies the ability of the assay for mutation pre-screening purposes to decide which specimen should be subjected to subsequent sequence analysis for genotype confirmation.

Next, a series of genomic DNAs isolated from 68 FFPE non-small cell lung cancer (NSCLC) specimens was selected for validation of the developed HRM-based assays for pre-screening purposes and subsequent genotyping in diagnostic setting, in comparison to conventional nested-PCR following cycle sequencing [4]. This series of tumour tissues was selected from the files of the Pathology Department (VU University Medical Center, Amsterdam, The Netherlands) on the basis of prior findings in conventional nested-PCR following cycle sequencing. The series comprised, either or not in combination, 37 EGFR mutations (including 19 deletions in exon 19 of which 3 combined with T790M, 5 insertions in exon 20, 9 L858R and 4 P848L) and 53 SNPs (including 49 SNP c.2361G>A (Q787Q) and 4 SNP c.2508C>T (R836R)). For four specimens of this series, nested-PCR following sequencing could not reveal a sequence and findings were therefore referred to as “not to determine” (NTD). The study followed the ethical guidelines of the Institutional Review Board of the VU University Medical Center (Amsterdam, The Netherlands).

In the selected series, HRM followed by sequencing of PCR amplimers with aberrant melting curves was performed blinded for the results of the nested-PCR

following sequencing. We observed aberrant melting profiles in the HRM assays for exons 19, 20p, 20d and 21 in 19, 5, 4 and 18 DNA samples, respectively. WT EGFR exons were scored by HRM in the remaining cases, except for 3 cases in which HRM was unable to generate amplimers for one or more target locations. The latter is likely due to poor DNA quality as it mainly involved exon 21 (i.e., the exon from which the largest fragment is to be amplified).

In all specimens with an aberrant melting profile in one or more of the HRM assays, the respective nucleotide alteration(s) could be identified by cycle-sequencing (Table 2). In total, 38 cases demonstrated EGFR mutations in one or more exons (including 19 deletions in exon 19 of which 4 combined with T790M, 5 insertions in exon 20, 10 L858R, and 4 P848L), and in 4 cases the aberrant melting profile could be attributed to SNP c.2508C > T (R836R). Based on the very low frequency of this SNAP (www.ncbi.nlm.nih.gov/SNP; ID:ss24778993), the designed HRM approach for exon 21 will generate only a small number of unnecessary reflex sequencing reactions.

In comparison to nested-PCR following sequencing, the HRM approach correctly identified all EGFR alterations that were previously found, with the exception of SNP c.2361G > A (Q787Q) consistent with the assay design. Additionally, the HRM-based assays scored two mutations that were not detected by the conventional assay, i.e., a specimen containing T790M besides the previously identified deletion in exon 19 and a specimen harbouring L858R while prior tested WT. The detection of these mutations by HRM whilst not by the conventional assay is likely related to the higher analytical sensitivity of the HRM assay. Both FFPE specimens were estimated to contain a percentage of tumour cells around the threshold for detection of mutations by nested-PCR following sequencing (i.e., 20–30%) [3,4], that may account for missing these mutations by the conventional assay. The latter findings may have marked clinical relevance as both alterations have been described to modify tumour response to EGFR-targeted therapies [11].

Altogether, a high agreement in assay findings restricted to mutations commonly detected by both assays was found (overall kappa of 0.96; and region-specific kappa's of 1.0, 1.0, 0.85 and 0.97 for region 19, 20p, 20d and 21, respectively).

Specimens were also screened for mutations in K-ras exon 1 by HRM according to the method of Kramer et al. [3] revealing 3 cases (4%) with a K-ras exon 1 mutation. Of note, K-ras and EGFR mutations were mutually exclusive in this series.

In conclusion, the designed panel of HRM-based assays allows reliable and sensitive mutation prescreening of EGFR and K-ras with direct mutation verification possibility for use on routine diagnostic specimens. The HRM panel considerably increases the

Table 2
EGFR genotype findings of HRM following sequencing in relation to findings of nested-PCR following sequencing[1]

Nested-PCR	WT	Deletion exon 19*	Deletion exon 19* + T790M	Insertion exon 20**	R836R	P848L	L858R	NTD	Total
HRM									
WT	22[2]							1	23
Deletion exon 19[3]		15							15
Deletion exon 19[3] + T790M		1	3						4
Insertion exon 20[4]				5					5
R836R					4				4
P848L						4			4
L858R	1						9		10
NTD								3[5]	3
Total	23	16	3	5	4	4	9	4	68

Notes: NTD – not to determine;
[1]only EGFR alterations commonly detected by both HRM following sequencing and nested-PCR following sequencing are indicated;
[2]including 3 cases with a K-ras mutation;
[3]comprise delE746-L750; delE746-L750/K745K; delE746-T751/insVA; delL747-E749; delL747-E749/A750P/K754T; delL747-T751 and delL747-S752;
[4]comprise D770-N771insSVD; D770-H771insGG; D770-H771insGF/N771T and V774-C775insHV;
[5]one case is NTD for all exons in both nested-PCR and HRM, and 2 cases are NTD for all exons in nested-PCR, while in HRM only NTD for exon 21 and WT for exon 19 and 20.

speed of mutation analysis by diminishing the number of redundant and time-consuming sequencing reactions of specimens containing WT-sequences. The designed approach is useful in molecular diagnostics to aid the decision on the treatment of choice in clinical practice and saves costs.

Acknowledgements

We thank Sandra Mongera and Douwe Buma of the Department of Pathology, and Mariëlle Gallegos-Ruiz of the Department of Medical Oncology, VU University Medical Center, for excellent technical assistance.

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