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A fast, sensitive and accurate high resolution melting (HRM) technology-based assay to screen for common K-ras mutations

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Abstract. *Background*: Increasing evidence points to a negative correlation between K-ras mutations and patient's response to, or survival benefit after, treatment with EGFR-inhibitors. Therefore, rapid and reliable assays for mutational analysis of the K-ras gene are strongly needed.

Methods: We designed a high resolution melting (HRM) technology-based approach followed by direct sequencing to determine K-ras exon 1 (codons 12/13) tumour genotype.

Results: Reconstruction experiments demonstrated an analytical sensitivity of the K-ras exon 1 HRM assay following sequencing of 1.5–2.5% of mutated DNA in a background of wild-type DNA. Assay reproducibility and accuracy were 100%. Application of the HRM assay following sequencing onto genomic DNA isolated from formalin-fixed paraffin-embedded tumour specimens of non-small cell lung cancer ($n = 91$) and colorectal cancer ($n = 7$) patients revealed nucleotide substitutions at codons 12 or 13, including a homozygous mutation, in 33 (34%) and 5 (5%) cases, respectively. Comparison to conventional nested-PCR following cycle-sequencing showed an overall high agreement in genotype findings (kappa value of 0.96), with more mutations detected by the HRM assay following sequencing.

Conclusions: HRM allows rapid, reliable and sensitive pre-screening of routine diagnostic specimens for subsequent genotyping of K-ras mutations, even if present at low abundance or homozygosity, and may considerably facilitate personalized therapy planning.

Keywords: HRM, direct cycle sequencing, G12, G13, K-ras, EGFR, genotype, codon, (nested-)PCR, formalin-fixed paraffin-embedded, molecular diagnostics, TKI, receptor tyrosine kinase inhibitors

1. Introduction

The introduction of novel classes of therapeutic agents for treating cancer (i.e., monoclonal antibodies, small molecules) that interfere with tyrosine kinase receptors such as the epidermal growth factor receptor (EGFR), is rapidly changing clinical practice. Since these new therapeutics have proven to be effective only in subgroups of patients [1–8], development of robust assays that identify these patients in a reliable and rapid manner is of utmost importance. These assays may help clinicians to proactively select those cancer patients who will derive the greatest clinical benefit from targeted therapies, while ineffective therapy is spared for those with a contraindication based on the genetic make-up of their tumour. Such molecular assays will be a first step towards personalized cancer treatment.

An important negative predictor of success to EFGR-targeted therapy is the presence of K-ras mutations, particularly in codons 12 and 13 [1]. These mutations may lead to the constitutive activation of the Ras protein, and may render tumour cells independent of EGFR signalling and thereby resistant to EGFR-targeted therapies. Known activating K-ras mutations are mainly found in codons 12 and 13, and to a lesser extent also in, e.g., codons 59, 61 and 63. As the latter

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occur at low frequency and patients containing these mutations in their tumour have not been extensively studied in clinical trials on EGFR-targeted therapies so far, the predictive effect of these mutations with respect to EGFR-targeted therapies is still tentative.

Given that mutations in K-ras codons 12 and 13 can serve as a biomarker of response to EGFR-targeted therapies, a sensitive, rapid and reliable method for detection of tumour K-ras genotype to be used in clinical practice is of great demand. A variety of molecular assays providing K-ras mutation information is available, including (nested-)PCR following cycle sequencing [9], restriction fragment length polymorphism (RFLP) analysis [10] and point-EXACCT [11]. Major disadvantages of these assays are that they lack sufficient sensitivity, are rather time-consuming and/or expensive to be used on the large scale for molecular diagnostic purposes.

The speed and sensitivity of detecting genomic mutations can nowadays be improved by high resolution melting (HRM) technology [12–14]. HRM assays are proposed as pre-screening assays to decide which specimens should be subjected to sequence analysis for confirmation of the nucleotide change. HRM is based on a PCR in the presence of a fluorescent intercalating DNA dye with subsequent melting analysis and continuous monitoring of the change in fluorescence caused by the release of the dye from a DNA duplex as it is denatured by increasing temperature. The melting profile of a DNA duplex depends, amongst others, on its GC content, length, sequence and heterozygosity. A change in the shape of the melting curve as compared to a wild-type (WT) sequence allows for mutation scanning. In this context, several studies have developed HRM assays for K-ras genotyping [15–17] demonstrating HRM to be a valid approach to efficiently pre-screen for presence of K-ras mutations.

Based on the HRM assay principle of detecting heteroduplex sequences, a disadvantage of the assay may be seen in the inability to detect homozygous K-ras mutations. Although occurring at low frequency, misdetection of patients with homozygous K-ras mutations in their tumour as WT may lead to an unfavourable therapy selection and consequently may have marked clinical consequences. Based on a recent improvement of the HRM protocol towards the inclusion of a WT-probe in order to distinguish homozygous mutations as well [18], we developed an HRM technology-based PCR with the addition of a WT-probe and direct sequencing confirmation possibility, to detect and genotype K-ras exon 1 (codons 12 and 13) mutations in routinely used formalin-fixed paraffin-embedded (FFPE) tissue. The assay comprises real-time amplification of genomic DNA isolated from patient FFPE tumour material with target specific primers in the presence of a WT-specific probe and a fluorescent intercalating DNA dye using an asymmetric PCR amplifying a 117 bp product following fluorescent melting curve analysis of both PCR amplicons and amplicon-probe duplexes to discriminate between WT and mutant DNA. HRM PCR products can be directly sequenced by cycle sequencing to identify the specific genotype. In this study, we compared the HRM assay following sequencing to conventional nested-PCR following cycle-sequencing [9] for genotyping of K-ras mutations in FFPE tissue collected for routine diagnostics.

2. Materials and methods

2.1. Study population

FFPE tissue sections containing more than 20% tumour cells from 91 non-small cell lung cancer (NSCLC) patients (78 adenocarcinoma, 2 squamous cell carcinoma, 11 large cell carcinomas), and 7 colorectal adenocarcinoma (CRC) patients diagnosed in the period 2006–2008 were selected from the files of the Pathology Department (VU University medical center, Amsterdam, The Netherlands). This study followed the ethical guidelines of the Institutional Review Board of the VU University Medical Centre.

2.2. Isolation of genomic DNA

The tumour region, as indicated by a pathologist, was macro-dissected from 10 μm FFPE sections, digested for 20 h at 55°C in 250 μl 0.5 mg/ml proteinase K in 0.25% SDS, 10 mM Tris HCl (pH 7.4), following DNA isolation by easyMAG (Biomérieux, France) according to the recommendations of the manufacturer. DNA concentration was measured on a ND1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). All DNA isolates were set to 10 ng/μl in Nuclisens easyMAG extraction buffer 3 prior to use. DNA isolated from human lung cancer cell lines Calu-1 (homozygous G12C mutation) and HCT116 (heterozygous G13D mutation) as well as dilution series thereof (i.e., 20, 15, 10, 5, 2.5 and 1.25% of mutant DNA in a background of wild-type (WT) DNA extracted from human foreskin keratinocytes) were used as positive controls throughout the study.

2.3. Nested polymerase chain reaction (PCR) and sequencing of K-ras exon 1

Nested polymerase chain reactions (PCR) following cycle-sequencing were carried out essentially as previously described [9], except that the following K-ras exon 1 internal forward primer was used: 5′-TGTAAAACGACGGCCAGTCACATTTTCATTATT TTTATTAT AAGGC-3′. All sequences were read in forward and reverse direction. All mutations found were confirmed in both readings.

2.4. High resolution melting (HRM) PCR for K-ras exon 1 with direct sequencing confirmation

The K-ras exon 1 primers and probe sequences are shown in Table 1. PCR for HRM analysis was performed in the presence of the dye LCGreen-Plus on a LightCycler480 (Roche, Mannheim, Germany). The reaction mixtures with a final volume of 10 μl contained: 500 nM forward primer, 100 nM reverse primer, 500 nM unlabelled probe with a 3′-conjugated C3 spacer, and 10 ng genomic DNA in 1× LightScanner Master mix (Idaho Technologies, Salt Lake City, UT, USA). The cycling and melting conditions were as follows: one cycle of 2 min 95°C; 50 cycles of 30 s 95°C; 30 s 64°C; 30 s 72°; and one cycle of 60 s 72°C, 20 s 95°C, 20 s 55°C; with a final melting in two steps: Melt 1 (55–78°C at 0.06°C/s) followed by Melt 2 (78–95°C, 0.06°C/s) and continuously recording of the fluorescent level. The change of fluorescence is converted to a melting peak by plotting the negative derivative of the fluorescent signal corresponding to the temperature ($-\mathrm{d}F/\mathrm{d}T$) on the LightCycler software. K-ras mutations are identified by the comparison of each patient's peak plot with that of WT (i.e. DNA isolated from human foreskin keratinocytes) and mutant (i.e. DNA isolated from cell lines HCT116 or Calu-1) reference DNA. Normalized and temperature-shifted difference plots of the LightCycler 480 software release 1.5 and LightCycler 480 Gene Scanning Software were additionally used to aid mutation identification. The HRM assay was performed in duplicate on each specimen showing complete agreement in all cases analysed.

Uni-directional sequencing of HRM PCR products was performed using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), M13 forward (−20) primer 5′-GTAAAACGACGGCCAG-3′, Big Dye XTerminator purification kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

3. Statistics

The level of agreement between genotype findings of HRM assay following sequencing and nested-PCR following sequencing was determined using kappa statistics. Genotyping results were considered concordant in case of complete genotype agreement between both assays, and discordant in case of genotype disagreement. Cases in which one or both assays could not reveal a sequence (i.e., not to determine (NTD)) were excluded from kappa statistics. Level of statistical significance was set at 0.05. All analyses were performed using SPSS 15 software.

4. Results and discussion

In response to the great demand for fast, reliable and sensitive molecular detection assays to help clinicians to predict response to, or survival benefit after, EGFR-targeted therapy in patients with cancers like NSCLC and CRC, we developed a HRM assay for rapid and reliable pre-screening of K-ras exon 1 mutational status of patient's tumour tissue. The HRM technique is based on an asymmetrical PCR in the presence of an unlabelled wild-type oligoprobe and a fluorescent intercalating DNA dye with subsequent melting analysis of both PCR amplicons and amplicon-probe duplexes, wherein the presence of a mutation is reflected by an altered decrease of the fluorescence as compared to WT-sequences [12–14]. In our de-

Table 1
HRM primers and probes

Name	Sequence (5′–3′)
HRM-forward primer	GTAAAACGACGGCCAGTCACATTTTCATTATTTTTATTATAAGGC
HRM-reverse primer	GATTCTGAATTAGCTGTATCGTCAAG
HRM-WT probe	CTTGCCTACGCCACCAGCTCCAACT

Note: The M13 moiety is underlined.

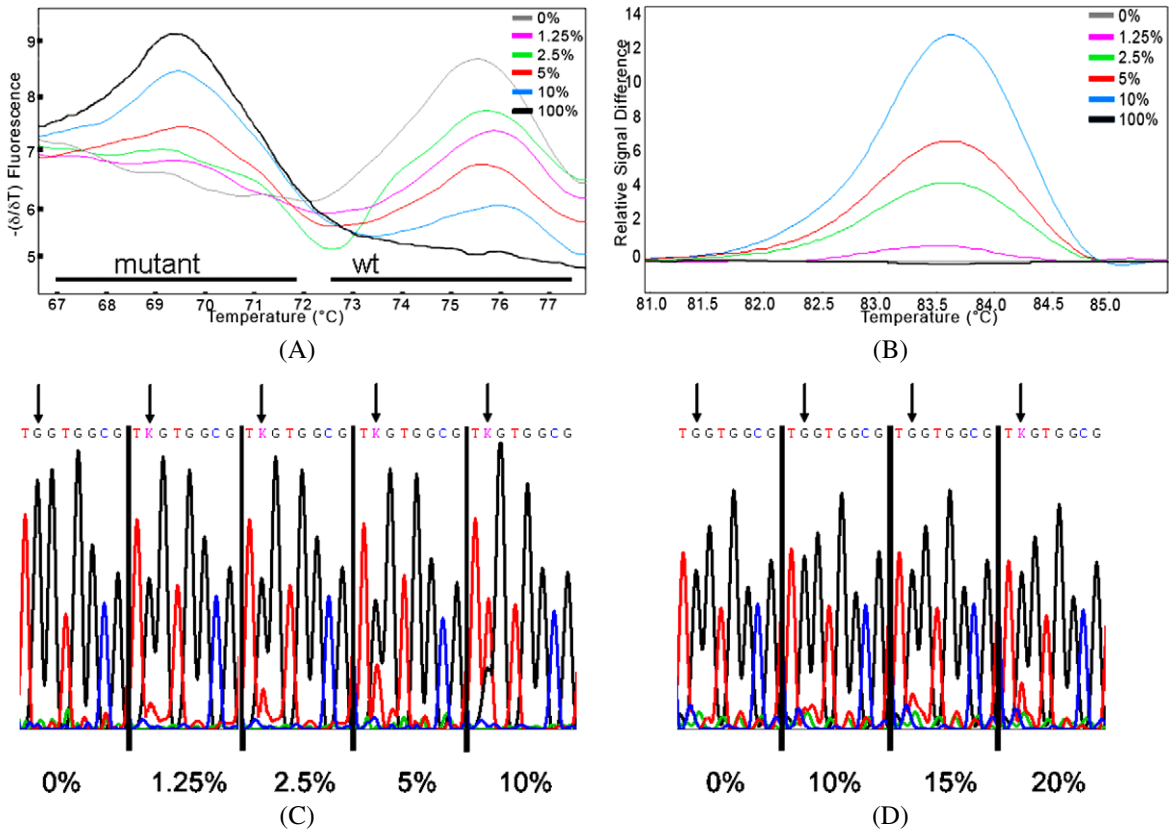

Fig. 1. HRM assay for K-ras exon 1. The designed HRM assay encompasses a pre-screening approach. The HRM assay is based on an asymmetrical PCR with an unlabelled wild-type probe in the presence of a fluorescent intercalating DNA dye with subsequent melting analysis of both PCR amplicons and amplicon-probe duplexes. The pre-screen assay may be followed by direct sequencing of HRM PCR products showing an aberrant melt out to reveal K-ras exon 1 genotype. Shown are representative HRM assay findings on a dilution series of Calu-1, containing a homozygous G12C mutation (i.e., 0, 1.25, 2.5, 5, 10 and 100% indicated by grey, purple, green, red, blue and black lines, respectively) in a background of WT-DNA. (A) The separation of the probe is examined in the first melting range (Melt 1: 55–78°C). The probe melts off at a lower temperature (i.e., 68–72°C) when a mutation is present as compared to WT (i.e., 74–78°C). Due to the WT-sequence of the probe, besides heterozygous mutations also homozygous mutations are detected (see black line of 100% Calu-1 DNA). (B) The normalised and temperature shifted melting plot is used to best visualise the difference in melt out of amplicons (Melt 2; melting range: 78–95°C). The relative signal difference as compared to a reference WT-DNA (indicated in grey) indicates the level of altered melting of amplicons. (C) The pre-screen assay may be followed by direct sequencing of HRM PCR products showing an aberrant melt out to find out the nature of the K-ras mutation. Representative examples of 0, 1.25, 2.5, 5, 10% Calu-1 DNA in WT-DNA background are shown. (D) Direct sequencing of conventional nested-PCR products. Representative examples of 0, 10, 15, 20% Calu-1 DNA in WT-DNA background are shown. *Note*: Colours available in the web version only.

sign, both the separation of the probe (melting range of 55–78°C) (Fig. 1(A)) and the amplicon (melting range of 78–95°C) (Fig. 1(B)) are evaluated. Application of the method to a dilution series of DNA from the cell line HCT116 exhibiting heterozygous G13D or Calu-1 exhibiting homozygous G12C, in WT-DNA background revealed that HRM identified the known mutations with a 100% accuracy and an analytical sensitivity down to 2.5% and 1.25%, respectively (shown for Calu-1 in Fig. 1(A) and (B)). Similar sensitivities were found for the sequencing analysis that follows the HRM pre-screening assay (Fig. 1(C)). In comparison, conventional nested-PCR following sequencing [9] required at least 15–20% of mutant DNA in WT-DNA background (Fig. 1(D)). A most likely explanation for the higher sensitivity of HRM following sequencing is the presence of the WT-probe in the reaction that, to a certain extent, negatively interferes with the PCR efficiency of WT-DNA, favouring the PCR amplification of the mutated strand and consequently resulting in a very high sensitivity for mutant sequences. Furthermore, the inclusion of the WT-probe allows for the detection of homozygous mutations as shown for Calu-1 DNA (Fig. 1(A)). Conversely, by melting curve analysis of amplimer duplexes only, homozygous mutations remain undetected (Fig. 1(B)), as is the case

when reactions are performed without WT-probe included [18]. Repeating experiments demonstrated the reproducibility of the HRM assay following sequencing to be 100%, i.e., similar sensitivity levels were obtained in triplicate analyses.

Next, we validated the HRM assay for clinical diagnostic pre-screening purpose by examining a series of 98 FFPE tumour tissues from patients with NSCLC or CRC for K-ras tumour genotype. In this series, the HRM assays revealed 38 patients with an abnormal melting curve, which demonstrated substitutions at codons 12 and 13 in 33 (34%) and 5 (5%) patients, respectively, by subsequent sequencing (Table 2). All specimens with a melting profile like WT-DNA in the HRM assay were confirmed to exhibit a WT-sequence by subsequent sequencing of their HRM PCR products. Noteworthy, in one tumour only the probe melting curve was aberrant (while the amplimer melting curve was alike WT) suggesting the presence of homozygous mutated K-ras allele only, which was confirmed by the sequence analysis (i.e., homozygous G12C) that demonstrated the sole presence of the mutant nucleotide (i.e., presence of T at position 34 instead of G). Though occurring at low frequency, this finding strongly suggests homozygous mutations to be identified by the HRM assay in patient FFPE material as well. Our findings confirm the suitability of the HRM assay as pre-screening assay to decide which specimens should be subjected to sequence analysis for genotype confirmation. This approach may considerably reduce the number of redundant and time-consuming sequencing reactions of specimens containing WT-sequences (i.e., 61% of specimens based on our findings), thereby increasing speed and reducing costs of mutational analysis. No HRM false-negativity (i.e., specimens demonstrating a melting like WT while a mutant sequence was revealed by subsequent cycle sequencing) nor HRM false-positivity (i.e., specimens demonstrating an altered melting while a WT-sequence was revealed by subsequent cycle sequencing) were encountered in our series. The 100% negative predictive value further supports the use of this K-ras HRM assay as pre-screen method.

The findings of HRM following sequencing were compared to the outcome of conventional nested-PCR following cycle sequencing (Table 2). The conventional assay revealed 32 patients with a K-ras exon 1 mutation, with substitutions at codons 12 and 13 in 29 (33%) and 3 (3%) patients, respectively. The HRM assay following sequencing generated remarkably less non-informative data (i.e., NTD) as compared to the conventional assay (5 (5%) vs. 15 (15%)). These findings strongly suggest the HRM assay following sequencing to have less test-failures, which may be explained by FFPE-derived DNA preferentially detected by methods using small amplimers. Among the 83 specimens of which both assays revealed a sequence, an overall high agreement between assay findings was found (kappa value of 0.96). In 81 cases (98%) a complete agreement between assay findings (i.e., exactly the same genotype) was observed, and 2 cases (2%) with discordant findings due to additional mutations found by HRM assay following sequencing while not detected by conventional nested-PCR following sequencing (Table 2). The latter may be explained by the higher sensitivity of HRM technique.

Given its high sensitivity compared to conventional nested-PCR following sequencing, the HRM assay following sequencing may also allow for application to cytology preparations, like bronchoalveo-

Table 2
Comparison of genotype findings of HRM and nested-PCR assays

HRM	Nested-PCR									Total
	WT	G12A	G12C	G12D	G12S	G12V	G13C	G13D	NTD	
WT	49								6	55
G12A	1	5								6
G12C			10						1	11
G12D	1			6					1	8
G12S					1					1
G12V						7				7
G13C							1		1	2
G13D								2	1	3
NTD									5	5
Total	51	5	10	6	1	7	1	2	15	98

Notes: NTD – not to determine. Overall agreement in genotyping: kappa value of 0.96.

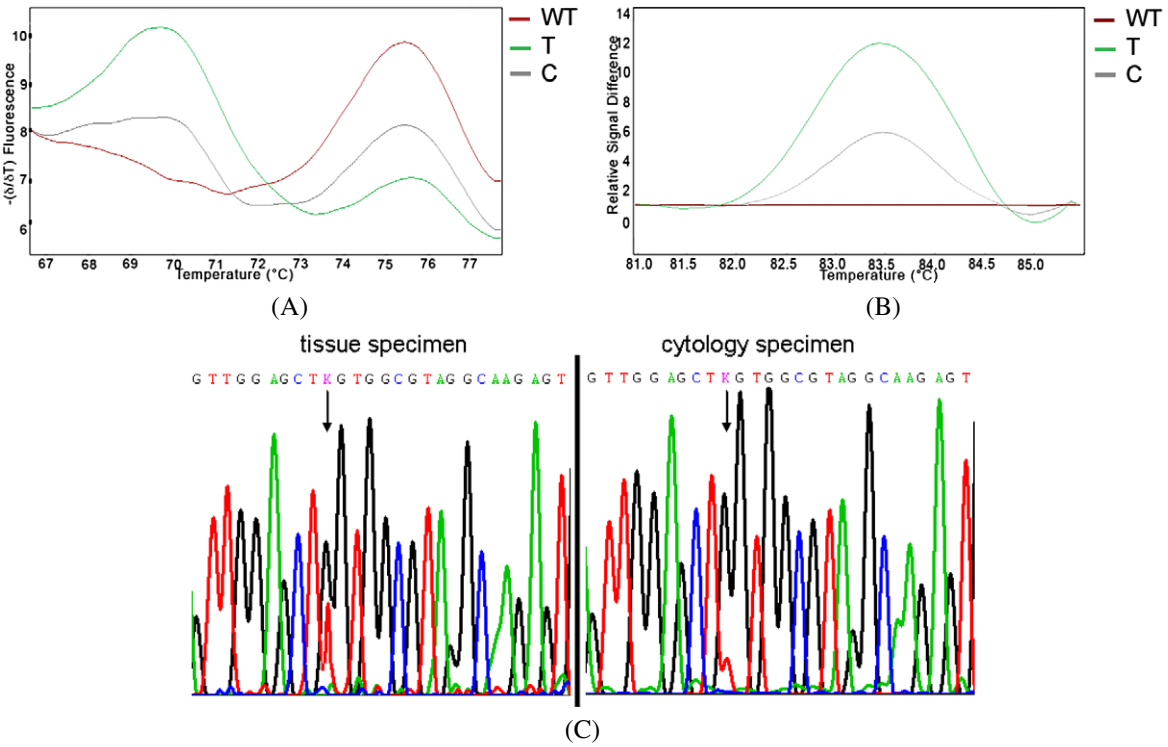

Fig. 2. Suitibility of HRM K-ras exon 1 assay for mutational analysis of cytology specimens containing low tumour cell percentage. The HRM assay was performed using DNA isolated from a BAL specimen (indicated as C or 'cytology specimen') of a patient who presented with G12C (34G > T) K-ras exon 1 mutation in its tumour tissue (indicated as T or 'tissue specimen'). Shown are: (A) the first (probe) melting range of the HRM assay, (B) the normalised and temperature shifted melting plot of the second (amplimer) melting range of the HRM assay, and (C) the sequence confirmation data. WT indicates wild type reference DNA; T indicates tissue specimen; C indicates cytology specimen (i.e. BAL). *Note*: Colours available in the web version only.

lar lavage (BAL), bronchial washing, bronchial brush, transbronchial fine-needle aspirate or sputum specimens, which in general contain a very low target tumour cell fraction. We confirmed this possibility in our study by detection of the similar genotype (i.e., G12C (34G > T) mutation) in the DNA isolate of a BAL specimen of a patient as the genotype revealed in its tumour specimen (Fig. 2).

The K-ras exon 1 genotypes found in our series of tissues are similar to those reported previously (www.sanger.ac.uk). It should however be noted that overall (39%) and genotype specific mutation rates (Table 2) may not directly be compared to previous studies based on the pre-selection bias of this series by treating physicians for features associated with (un)likelihood of response to EGFR-targeted therapies, including clinico-pathological parameters (e.g. 86% of NSCLC are of the adenocarcinoma type; Table 3).

In summary, the HRM assay developed herein is an extremely sensitive, accurate and reliable technique for fast mutational pre-screening and is validated for this purpose. The technique allows high-throughput screening to select for sequencing only those specimens that most likely contain mutant K-ras allele(s).

Table 3

K-ras genotype in relation to tumour type and histology

K-ras genotype	Tumour type				Total
	NSCLC			CRC	
	AdCa	SSC	LCC	AdCa	
WT	43	2	7	3	55
G12A	6				6
G12C	11				11
G12D	6			2	8
G12S	1				1
G12V	5		1	1	7
G13C	1		1		2
G13D	1		1	1	3
NTD	4		1		5
Total	78	2	11	7	98

Notes: NSCLC – non-small cell lung cancer; CRC – colorectal cancer; AdCa – adenocarcinoma; SSC – squamous cell carcinoma; LCC – large cell carcinoma; NTD – not to determine.

The high sensitivity of HRM assay following sequencing allows for the detection of low abundant, therapy-resistant K-ras mutations arising within only a subset of the tumour cell population. This is of great importance since it may predict poorer response or more rapid tumour progression after EGFR-targeted treatment. Effective implementation of the assay into molecular diagnostics will be important to rapidly and accurately assess the likelihood of therapeutic benefit of receptor tyrosine kinase inhibitors in clinical practice. The development of HRM methods to detect other therapy-significant mutations, e.g. in EGFR, is under investigation, and may have significant implications for future personalized therapy.

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