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KRAS mutation detection by high-resolution melting analysis significantly predicts clinical benefit of cetuximab in metastatic colorectal cancer

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BACKGROUND: Anti-epidermal growth factor receptor (EGFR) monoclonal antibodies are restricted to *KRAS* wild-type (WT) metastatic colorectal cancers (mCRCs), usually identified by direct sequencing, that may yield false negative results because of genetic heterogeneity within the tumour. We evaluated the efficiency of high-resolution melting analysis (HRMA) in identifying *KRAS*-mutant (MUT) tumours.

METHODS: We considered 50 mCRC patients scored as *KRAS*-WT by direct sequencing and treated with cetuximab-containing chemotherapy, and tested the correlations between HRMA findings and response rate (RR), progression-free (PFS) and overall survival (OS).

RESULTS: Aberrant melting curves were detected in four (8%) cases; gene cloning confirmed these mutations. Response rate (RR) of HRMA *KRAS*-WT patients was 28.3%. There was no response in HRMA *KRAS*-MUT patients. Disease control rate (responsive plus stable disease) was 58.7% in HRMA *KRAS*-WT patients and 25% in HRMA *KRAS*-MUT patients. There was no correlation between HRMA *KRAS* status and RR ($P = 0.287$) or disease control ($P = 0.219$). Median PFS (4.8 *vs* 2.3 months; hazard ratio (HR) = 0.29, $P = 0.02$) and OS (11.0 *vs* 2.7 months; HR = 0.11, $P = 0.03$) were significantly longer for the HRMA *KRAS*-WT than for HRMA *KRAS*-MUT patients.

CONCLUSIONS: High-resolution melting analysis identified 8% more *KRAS*-MUT patients not responding to cetuximab-containing regimens, suggesting that HRMA may be more effective than direct sequencing in selecting patients for anti-EGFR antibodies.

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The epidermal growth factor receptor (EGFR)-targeting monoclonal antibodies cetuximab and panitumumab lack efficacy in metastatic colorectal cancer (mCRC) harbouring a *KRAS* mutation (Allegra *et al*, 2009). Thus, regulatory authorities in the North America and Europe recommended *KRAS* mutation testing on tumour tissue before therapy (van Krieken *et al*, 2008; Allegra *et al*, 2009; NCCN Guidelines, V2.0, 2009). This strategy has led to substantial health system savings (Vijayaraghavan *et al*, 2011). *KRAS* gene mutations can be detected by several methods (van Krieken *et al*, 2008); each has limitations and a gold standard methodology is lacking (Bellon *et al*, 2011). There are no Federal Drug Administration-approved *KRAS* mutation detection assays, and the European Medicines Agency does not recommend any particular methodology (Bellon *et al*, 2011). Dideoxy sequencing (direct sequencing) is the *KRAS* mutation detection method used in most of the laboratories participating in the European *KRAS* external quality assessment scheme (Bellon *et al*, 2011). Its sensitivity is low; thus, *KRAS* mutations may be missed when the number of neoplastic cells after selective tissue microdissection is small. Conversely, direct sequencing is usually reliable in samples containing $\geqslant 30\%$ of tumour cells (Tol *et al*, 2010). However, even when a sufficient amount of DNA is extracted from a tissue area rich in neoplastic cells, direct sequencing may yield false-negative results, because of the uneven tissue distribution of mutant (MUT) cells. Indeed, *KRAS* intratumoural heterogeneity led to discordant results when several specimens from the same tumour were analysed by direct sequencing (Baldus *et al*, 2010; Richman *et al*, 2011). Richman *et al* (2011) described discordances among different tumour blocks, whereas Baldus *et al* (2010) reported discordant results between the tumour centre and the invasion front. These problems related to *KRAS* intratumoural heterogeneity may be overcome by increasing the sensitivity of methods used to detect *KRAS* mutations. Reliable identification of *KRAS* mutations has become impelling given the recent finding that also tumours harbouring only a few *KRAS*-mutated cells fail to respond to cetuximab (Bando *et al*, 2011; Molinari *et al*, 2011).

High-resolution melting analysis (HRMA) is a highly sensitive and cost-effective screening method that allows rapid in-tube detection of DNA sequence variations based on specific sequence-related melting profiles of PCR products (Reed *et al*, 2007).

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High-resolution melting analysis identifies *KRAS* mutations even in a small fraction of alleles in a background of wild-type (WT) DNA (Krypuy *et al*, 2006; Simi *et al*, 2008; Ma *et al*, 2009; Borras *et al*, 2011). Discrepancies between direct sequencing and the more sensitive HRMA have been reported but the impact of these discrepancies on treatment has not been evaluated (Deschoolmeester *et al*, 2010).

We have evaluated the therapeutic effects of cetuximab in patients in whom a *KRAS* mutation was missed by direct sequencing and detected by HRMA. The aim of our study was to determine whether HRMA was more effective than gene sequencing in identifying patients who would not benefit from anti-EGFR treatment.

PATIENTS AND METHODS

Patients

We retrospectively selected patients by the following criteria: (1) histological evidence of colorectal adenocarcinoma; (2) codon 12 and 13 *KRAS* gene WT status assessed by our laboratory-validated direct sequencing assay – our laboratory is registered in the European Society of Pathology *KRAS* external quality control scheme (http://kras.eqascheme.org); (3) at least one prior chemotherapy regimen (without cetuximab) for metastatic disease; (4) availability of a sufficient (>200 ng) amount of stored genomic DNA; and (5) confirmation of the *KRAS*-WT status by a second independent direct sequencing analysis. A total of 50 patients met these inclusion criteria, 35 males and 15 females, with a median age of 61 years (range 29–77 years). Disease status was evaluated in all patients by total body CT scan before treatment onset and every 2 months thereafter.

A total of 25 patients received cetuximab associated with irinotecan alone, and the other 25 received cetuximab associated with FOLFIRI or FOLFOX. Details of the patients' characteristics are listed in Table 1. Cetuximab was administered at the initial dose of 400 mg m^{-2} followed by weekly infusions of 250 mg m^{-2} together with chemotherapy, until unacceptable toxicity or disease progression. After internal Ethic Committee approval, the DNA of the 50 patients underwent HRMA to detect *KRAS* mutations.

Table 1 Patients' characteristics

	HRMA WT	*HRMA* MUT	Total
Gender			
Male	31	4	35
Female	15	—	15
Age (median)	61	66	
Primary tumour			
Colon	30	1	31
Rectum	16	3	19
No. of prior lines of treatment			
1	26	2	28
2	20	2	22
Sites of metastases			
Liver only	18	2	20
Lung only	8	1	9
Multiple site	20	1	21
Cetuximab-containing regimen			
Irinotecan	23	2	25
FOLFIRI	20	2	22
FOLFOX	3	—	3

Abbreviations: HRMA = high-resolution melting analysis; MUT = mutant; WT = wild type.

Sample macrodissection and DNA extraction

For each case included in this study, a representative hematoxylin- and eosin-stained (H&E) slide has been reviewed and the area with the highest content of neoplastic cells and the lesser degree of necrosis has been marked and isolated from two 20 μm formalin-fixed paraffin-embedded corresponding unstained slides. In all cases, care was taken to ensure that the neoplastic cell content in the tissue area isolated for DNA extraction represented at least 30% of the total cell population.

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions. DNA was resuspended in 50 μl of molecular biology water. The DNA quantity was assessed by using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Milan, Italy); the average amount of extracted DNA was 250 ng μl^{-1} (range 80–550 ng μl^{-1}). The 260/280 absorbance ratio was used to evaluate the DNA purity (mean value 1.93; range 1.7–2.0).

All samples were collected before the patient underwent chemotherapy or radiation therapy.

High-resolution melting analysis design and PCR conditions

The primer pairs leading to a short amplicon of 114 bp (FW 5′-GC CTGCTGAAAATGACTGA-3′; RV 5′-TTGGATCATATTCGTCCA CCAA-3′) have been validated in a previous HRMA study (Deschoolmeester *et al*, 2010). The reaction mixture was prepared in a 20 μl final volume containing 1X HRMA Melt Doctor Master Mix (Applied Biosystems, Foster City, CA, USA) including a modified SYTO-9 as fluorescent DNA intercalating dye, 400 nM of each primer, 10 ng of genomic DNA and PCR grade water. All PCR reactions were performed in duplicate. PCR for HRMA was performed in 0.2-ml tubes on the 7500 fast Real-Time PCR System (Applied Biosystems). The PCR reaction was run as follows: 1 cycle of 95 °C for 10 min; 40 cycles in the following sequence: 95 °C for 15 s, 60 °C for 1 min; 1 cycle of 95 °C for 15 s; and a melt from 60 to 95 °C increasing 0.1 °C per second.

Analytical sensitivity of HRMA and interpretation of the results

Cancer cell lines with known *KRAS* mutations were first used to validate the HRMA methodology and then applied to the patients' DNA. H441 and HCT116 were used as reference for mutations in *KRAS* codon 12 (p.Gly12Val, heterozygous) and 13 (p.Gly13Asp, heterozygous), respectively. DNA obtained from the PC-9 cell line was used as reference for *KRAS*-WT. To assess the analytical sensitivity of HRMA, DNA extracted from H441 was variably mixed with *KRAS*-WT DNA obtained from the PC-9 cell line at proportions of 50, 12.5 and 3%. Any given dilution was tested by both HRMA and direct sequencing. The latter was carried out as described previously (Troncone *et al*, 2010). High-resolution melting analysis data were analysed with the 7500 fast Real-Time HRMA Software v 2.0.1 (Applied Biosystems) and evaluated by a molecular geneticist (UM) and molecular pathologist (GT). The normalised and the difference plots were used to analyse the data both in cell lines and in patients. The normalised plot was generated by monitoring the dissociation of the fluorescent dye from double-stranded DNA as the temperature increased. The dye used (modified SYTO-9) can only fluoresce when it is intercalated into double-strand DNA. The normalised plot shows the degree of reduction in fluorescence over a temperature range (60–95 °C). All samples, including the WT, were plotted according to their melting profiles. In the difference plot, the melting profiles of each sample were compared with that of the WT that was converted to a horizontal line. Significant deviations from the horizontal line (relative to the spread of the WT controls) were indicative of sequence changes within the amplicons analysed. Samples with

aberrant melting curves were recorded as HRMA *KRAS* mutation (HRMA *KRAS*-MUT)-positive.

PCR products featuring an aberrant melting curve were further processed to confirm the patient mutational status and to identify the mutation type. To this end, PCR products were subcloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In total 30 plasmids were purified and sequenced using the BigDye Terminator kit (Applied Biosystems), and run on the ABI 3730 analyser (Applied Biosystems) with M13 forward and reverse primers. Sequence data were analysed using a Mutation Surveyor (SoftGenetics, State College, PA, USA). The sample was scored as a true positive HRMA *KRAS* mutation when the mutation was found in at least one clone. In all cases scored as an HRMA *KRAS* mutation, the corresponding H&E-stained slide was retrieved from the files, and the area from which DNA had been extracted was microscopically reviewed to assess tumour cell abundance.

Measured outcomes

The RR was evaluated according to RECIST criteria (version 2.0) (Eisenhauer *et al*, 2009). Progression-free survival (PFS) was defined as the time from the first administration of cetuximab to the first evidence of disease progression or death from any cause. Overall survival (OS) was considered the time from the first administration of cetuximab to death from any cause.

Statistical analysis

Fisher's exact test was used to correlate the treatment response to *KRAS* status. Progression-free survival and OS data were plotted as Kaplan–Meier curves and the differences between the groups categorised by HRMA-identified *KRAS* status were compared by the log-rank test. A P level $\leqslant 0.05$ was considered statistically significant. All analyses were performed with IBM SPSS Statistics 18 software package (SPSS Inc., Chicago, IL, USA).

RESULTS

High-resolution melting analysis results

High-resolution melting analysis was able to discriminate the p.Gly12Val (H441) and the p.Gly13Asp (HCT116) DNA from *KRAS*-WT DNA (PC-9). Figure 1 shows a difference plot of the HRMA data using WT DNA as a baseline, and the corresponding electropherograms. Regarding HRMA sensitivity testing, Figure 2 shows the difference plots obtained with H441 cell dilutions of 50, 12.5 and 3%. We were able to detect as little as 3% of MUT p.Gly12Val DNA in a WT background.

We then used this sensitive method to assess the HRMA exon 2 *KRAS* mutational status of the selected 50 patients. Four (8%) samples with aberrant melting curves were detected. Figure 3 shows the discordance between the results of HRMA and direct sequencing. The microscopic review of the H&E-stained sections confirmed that, in all cases, the DNA had been extracted from a tissue area corresponding to the tumour bulk containing an invasive neoplastic component of 80% ($n=2$), 60% ($n=1$) and 30% ($n=1$) without significant necrosis. As HRMA is a screening method that requires further analysis to identify the type of mutation, we validated the results by PCR product cloning; 30 clones were purified and sequenced. Two to three mutated

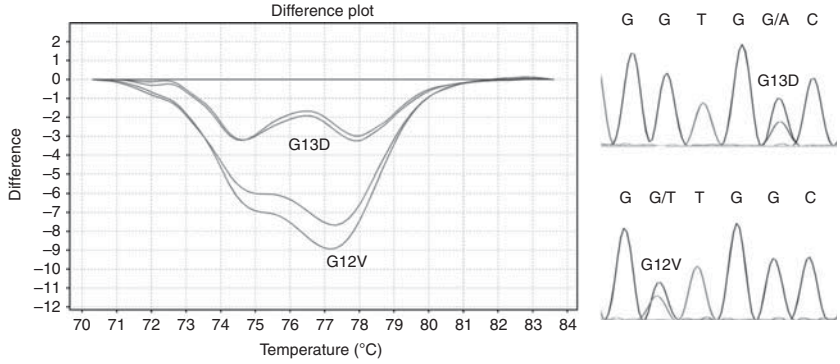

Figure 1 High-resolution melting analysis of *KRAS* exon 2 codons 12 and 13. The horizontal line indicates WT control, and the p.Gly13Asp p.Gly12Val labelled lines indicate in duplicate the mutated DNA. The corresponding sequencing analysis electropherograms are reported on the right.

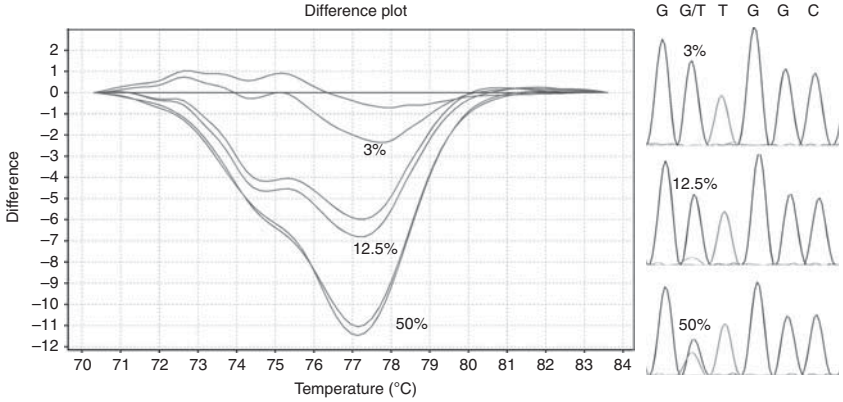

Figure 2 High-resolution melting analysis difference plots generated by serial dilutions of DNA from H441 cells that harbour the *KRAS* p.Gly12Val mutation, mixed with the *KRAS* WT PC-9 cell line DNA at proportions of 50, 12.5 and 3% are shown on the left. The corresponding sequencing analysis electropherograms are shown on the right.

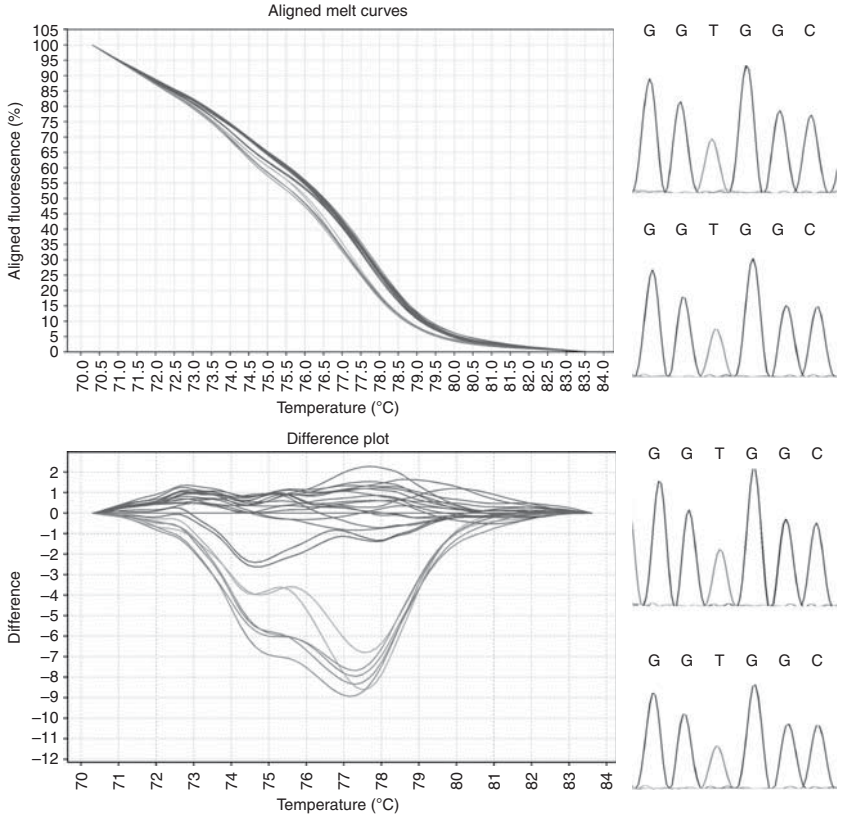

Figure 3 Aligned (normalised) melting curves and difference plots of the four HRMA-mutated samples; each sample is in duplicate. Upper lines (in the − 1 to 2 difference range) correspond to WT controls (PC-9-derived DNA); lower lines correspond to HRMA mutated samples (from top to bottom: p.Gly13Asp, p.Gly12Val and the p.Gly12Asp). Corresponding WT sequencing electropherograms are shown on the right. In particular, from top to bottom, the first electropherogram shows the p.Gly13Asp HRMA-mutated sample, the second the p.Gly12Asp HRMA-mutated samples and the third and fourth show two p.Gly12Val HRMA-mutated samples.

clones were detected in all four samples showing aberrant melting curves (p.Gly12Val $n=2$; p.Gly12Asp $n=1$ and p.Gly13Asp $n=1$).

When we compared the number of mutated clones with the percentage of neoplastic cells in the four HRMA-MUT cases, the percentage of mutated tumour cells was: 8% (3/30 mutated clones; 80% of neoplastic cells), 5% (2/30 mutated clones; 80% of neoplastic cells), 4% (2/30 mutated clones; 60% of neoplastic cells) and 3% (3/30 mutated clones; 30% of neoplastic cells), respectively. The number of mutated clones did not correlate with the percentage of neoplastic cells.

Response to treatment

In all, 13 of the 46 (28.3%) HRMA *KRAS*-WT patients responded to cetuximab treatment: 1 (2.2%) complete response and 12 (26.1%) partial responses. Conversely, none of the HRMA *KRAS*-MUT patients responded to treatment (all had received an irinotecan-containing regimen). Three of the four had disease progression as best response. Stable disease was obtained in 14/46 (30.4%) and in 1/4 patients (25%) in HRMA *KRAS*-WT and MUT patients, respectively. The mutated patient who achieved a stable disease had a p.Gly13Asp mutation. The disease control rate (objective responses plus stable disease) was 58.7% (27/46 patients) in HRMA *KRAS*-WT patients and 25% (1/4 patients) in *KRAS*-MUT patients. No statistically significant correlations were observed between HRMA *KRAS* status and RR ($P=0.287$) or disease control rate ($P=0.219$).

Survival

The median PFS was significantly longer in HRMA *KRAS*-WT patients (4.8 months) than in HRMA *KRAS*-MUT patients (2.3 months; Figure 4); hazard ratio (HR) = 0.29, 95% confidence interval 0.10–0.88, $P=0.02$. Similarly, the median OS was 11.0 months in HRMA *KRAS*-WT *vs* 2.7 in HRMA *KRAS*-MUT (Figure 5); HR = 0.11, 95% confidence interval 0.03–0.38, $P=0.03$.

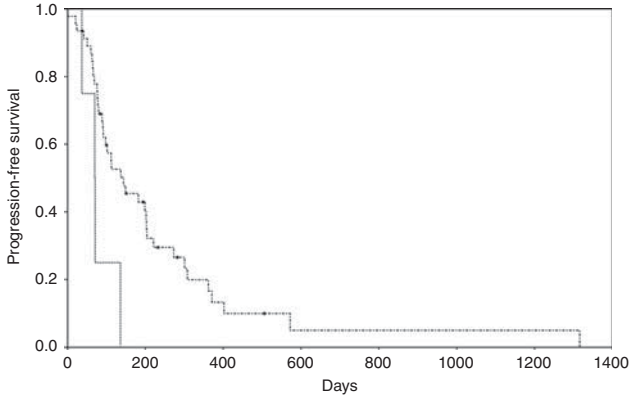

Figure 4 Kaplan–Meier plots of PFS according to *KRAS* status determined by HRMA (WT, dotted line; MUT, pointed line).

DISCUSSION

In this retrospective study, we used HRMA to look for *KRAS* mutations in 50 mCRC patients previously found to be *KRAS*-WT by direct sequencing and treated in a second- or third-line setting with cetuximab-based therapy. High-resolution melting

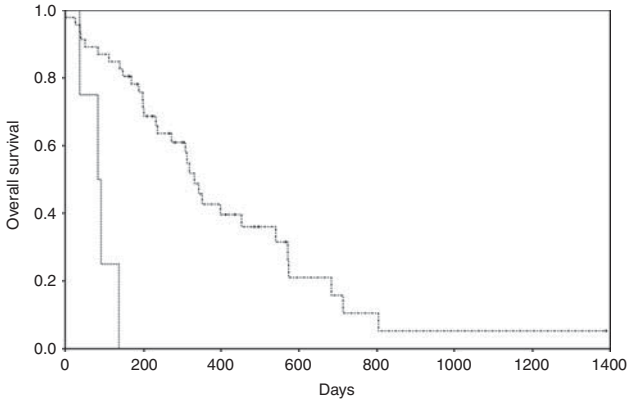

Figure 5 Kaplan–Meier plots of survival according to KRAS status determined by HRMA (WT, dotted line; MUT, pointed line).

analysis-identified mutations in 4/50 patients that had been missed by direct sequencing. None of these four patients responded to cetuximab treatment, and their PFS and OS were very short. Thus, if patient management had been based on HRMA results, a significant percentage (8%) of patients would have been spared useless treatment.

Discrepancy between the results of HRMA and direct sequencing has already been reported (Deschoolmeester *et al*, 2010). *KRAS* mutations can be below the sensitivity level of sequencing detection as a consequence of a low percentage of tumour cells in the sample (Tol *et al*, 2010) or intratumoural genetic heterogeneity (Baldus *et al*, 2010; Richman *et al*, 2011). Our validation experiments conducted with serial dilutions of a mutated cancer cell line and normal DNA showed that direct sequencing had a detection limit of 12.5%, whereas HRMA identified as little as 3% of mutated alleles in a background of WT DNA, which was the smallest dilution tested. Furthermore, direct sequencing may yield false-negative results in cases of heterogeneous tissue distribution of MUT cells (Baldus *et al*, 2010; Tol *et al*, 2010; Richman *et al*, 2011). This heterogeneity is quite common, being found in about 10% of cases and even between adjacent neoplastic areas (Baldus *et al*, 2010; Richman *et al*, 2011). It is noteworthy that, the microscopic review of the H&E sections corresponding to the four discrepant cases confirmed an adequate ($\geqslant$30%) amount of invasive neoplastic component in all instances, thus suggesting that the discordant findings in our patients are due to tumour heterogeneity.

Various groups have studied what threshold of *KRAS*-mutated cells within the tumour mass should be considered clinically relevant, and whether cetuximab treatment would be beneficial in patients with tumours harbouring small numbers of mutated cells (Tol *et al*, 2010; Bando *et al*, 2011; Molinari *et al*, 2011; Santini *et al*, 2011). However, to date, only retrospective analyses have been conducted. In a recent study by Bando *et al* (2011) 19% more *KRAS* mutations were detected by a standardised amplification refractory mutation system – Scorpion assay (ARMS/S) method than by direct sequencing. Among the 47 patients with complete clinical information who were *KRAS*-WT by direct sequencing and had been treated with cetuximab alone or combined with irinotecan, the 9 ARMS/S-MUT patients failed to respond and had a significantly shorter PFS and OS than ARMS/S WT patients. Similarly, Molinari *et al* (2011) identified mutations using the highly sensitive MUT-enriched PCR (eME-PCR) method in 55/111 patients (49.5%), while the mutation rate in exon 2 by direct sequencing was 43/111 (38.7%). None of the 12 patients *KRAS*-MUT at eME-PCR responded to anti-EGFR monoclonal antibody-containing therapy. Using pyrosequencing, Santini *et al* (2011) detected *KRAS* mutations in 3/29 patients (10.3%) previously identified as *KRAS*-WT by real-time PCR using allele-specific oligonucleotide primers. However, these three patients showed a stable disease after treatment with cetuximab combined with irinotecan. These contrasting results may be due to the limited number of cases analysed, different populations of patients (one, two or more previous lines of treatment for metastatic disease), different treatment regimens (anti-EGFR monoclonal antibody alone or in combination with chemotherapy) and different mutation detection panels. Moreover, the suitability of RR as end-point may be questionable. In fact, RR in mCRC significantly decreases in second- or third-line treatment, and tumour control or time-to-progression are more reliable indicators of treatment benefit.

In accordance with previous studies (Krypuy *et al*, 2006; Do *et al*, 2008; Ma *et al*, 2009), we confirm that HRMA is a reliable, sensitive and rapid procedure for *KRAS* mutation detection. However, the novelty of our study is the demonstration that HRMA is an effective tool to predict lack of benefit from cetuximab treatment. Furthermore, we have addressed the issue of what method is the most appropriate to confirm HRMA findings. In fact, although HRMA is a highly sensitive, cost-effective screening tool, it should be kept in mind that positive results need confirmation (Reed *et al*, 2007). Most studies of HRMA detection of cancer-specific mutations in tumour biopsies used direct sequencing to confirm positive results (Krypuy *et al*, 2006; Reed *et al*, 2007; Do *et al*, 2008; Simi *et al*, 2008; Ma *et al*, 2009; Deschoolmeester *et al*, 2010), but we argue that direct sequencing is not reliable for validation of positive HRMA results in cases of a low MUT allele concentration. Thus, a more sensitive tool is required to confirm positive HRMA samples. In all our four positive HRMA cases, the *KRAS* mutation was confirmed by subcloning PCR products into TOPO TA vectors. In routine diagnostics, confirmation of positive HRMA results may be obtained with kits approved for *in vitro* diagnostic (IVD) use by the European Community such as the TheraScreen *KRAS* Mutation Kit (DxS-Qiagen, Manchester, UK) and the PyroMark Q24 KRAS Kit (Qiagen, Duesseldorf, Germany). However, these tests are expensive (Kotoula *et al*, 2009), whereas a diagnostic algorithm based on HRMA screening and confirmation by IVD tests is inexpensive, rapid and robust, and can also detect genetic heterogeneity within the tumour, and, hence, correctly identifiy patients who would not respond to cetuximab. Recently, ultra-deep pyrosequencing of *KRAS* amplicons with GS Junior 454 was found to be cost-effect in confirming HRMA *KRAS* genotyping (Borras *et al*, 2011).

In conclusion, HRMA may identify patients who should be excluded from treatment with cetuximab more accurately than direct sequencing. In addition, our results confirm previous studies suggesting that treatment with cetuximab may be ineffective even when a small number of MUT clones are detected by a mutation detection technique more sensitive than direct sequencing. However, prospective studies are needed to investigate the relationship between genetic intratumoural heterogeneity, mutational detection tools and cetuximab treatment outcome.

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Conflict of interest

The authors declare no conflict of interest.

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