

Optimization of *RAS/BRAF* Mutational Analysis Confirms Improvement in Patient Selection for Clinical Benefit to Anti-EGFR Treatment in Metastatic Colorectal Cancer



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Abstract

In metastatic colorectal cancer (mCRC), recent studies have shown the importance to accurately quantify low-abundance mutations of the *RAS* pathway because anti-EGFR therapy may depend on certain mutation thresholds. We aimed to evaluate the added predictive value of an extended *RAS* panel testing using two commercial assays and a highly sensitive and quantitative digital PCR (dPCR). Tumor samples from 583 mCRC patients treated with anti-EGFR- ($n = 255$) or bevacizumab- ($n = 328$) based therapies from several clinical trials and retrospective series from the TTD/RTICC Spanish network were analyzed by cobas, *therascreen*, and dPCR. We evaluated concordance between techniques using the Cohen kappa index. Response rate, progression-free survival (PFS), and overall survival (OS) were correlated to the mutational status and the mutant allele fraction (MAF). Concor-

dance between techniques was high when analyzing *RAS* and *BRAF* (Cohen kappa index around 0.75). We observed an inverse correlation between MAF and response in the anti-EGFR cohort ($P < 0.001$). Likelihood ratio analysis showed that a fraction of 1% or higher of any mutated alleles offered the best predictive value. PFS and OS were significantly longer in *RAS/BRAF* wild-type patients, independently of the technique. However, the predictability of both PFS and OS were higher when we considered a threshold of 1% in the *RAS* scenario (HR = 1.53; CI 95%, 1.12–2.09 for PFS, and HR = 1.9; CI 95%, 1.33–2.72 for OS). Although the rate of mutations observed among techniques is different, *RAS* and *BRAF* mutational analysis improved prediction of response to anti-EGFR therapy. Additionally, dPCR with a threshold of 1% outperformed the other platforms. *Mol Cancer Ther*; 16(9); 1999–2007. ©2017 AACR.

Introduction

In metastatic colorectal cancer (mCRC), antibodies against the epidermal growth factor receptor (EGFR), cetuximab and panitumumab, vascular endothelial growth factor (VEGF) binding

antibodies or other VEGF trap therapies, including bevacizumab and aflibercept, as well as regorafenib, a multiple tyrosine kinase inhibitor (1–5), have demonstrated to increase survival. However, the efficacy of these targeted agents in the clinic has been variable

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and limited, adding toxicity and high costs. In this scenario, it is necessary to identify subgroups of patients more likely to benefit from these therapies.

Exon 2 *KRAS* mutations were firstly identified as mechanisms of resistance to cetuximab and panitumumab (6, 7). However, factors other than exon 2 *KRAS* mutations (codons 12 and 13) have been further recognized as mechanisms of intrinsic resistance (8). *KRAS* mutations in exons 3 and 4 (codons 59–61, 117, and 146) as well as mutations in the same regions of the related oncogene *NRAS* have been recognized (9) and validated as predictive biomarkers to such anti-EGFR therapies (1, 2).

Although Sanger sequencing has been considered the gold standard in clinical genetic testing, no clearly standardized procedures for *RAS* mutational testing have been established. An increasing number of hotspots targeted qualitative techniques have been developed with different levels of sensitivity and specificity (10–12), although only some of them are commercially available (13). Digital PCR is a highly sensitive and quantitative method that has been recently applied with the purpose of analyzing the role of minor mutated *KRAS/NRAS* subclones in patients with mCRC treated with anti-EGFR drugs (14, 15), suggesting that a threshold of 1% of mutated subclones is the optimal cutoff to distinguish patients more likely to benefit from cetuximab or panitumumab. However, data reported on *RAS* mutations analyzed by BEAMing in the *KRAS* exon 2 wild-type cohort of patients enrolled in the CRYSTAL trial suggested that mCRC patients with tumors bearing mutations between 0.1% and <5% benefited from the addition of cetuximab to FOLFIRI in the first-line setting (1). There is therefore a need for identifying a mutation detection threshold that is clinically relevant to select patients for anti-EGFR therapy.

We previously reported the mutational profile of the *RAS* pathway genes in a series of 102 patients with mCRC treated with anti-EGFR based therapy in the refractory setting using two platforms with different analytical sensitivity, a quantitative and highly sensitive digital PCR (Fluidigm platform) and a conventional real-time PCR (Lightcycler 480, Roche; ref. 14). Digital PCR identified a higher number of mutations respect to the real-time PCR, but even more interesting, digital PCR helped us to optimize the selection of patients who had better outcomes after anti-EGFR treatment. Patients with tumors harboring *RAS* and *BRAF* mutations in a fraction of <1% seemed to benefit from cetuximab or panitumumab. In the present study, we aimed to confirm our previous results in a larger series of mCRC. Our objectives were to evaluate and compare the sensitivity to detect point mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* by means of three different mutational tests and additionally, to evaluate whether a cutoff point could be defined for optimal patient selection.

Materials and Methods

Patients and samples

We retrospectively analyzed formalin-fixed paraffin-embedded (FFPE) primary tumors obtained from surgical resections (partial or complete colectomy) or diagnostic biopsies from 583 patients with mCRC treated with anti-EGFR ($n = 255$) or bevacizumab ($n = 328$) regimens from retrospective series and several clinical trials from the TTD/RTICC Spanish network (clinical trial identifiers: NCT01071655, NCT00958386, NCT00885885, NCT01126112, and NCT01704703). Patients received treatment between February 2003 and September 2014. Main clinicopathologic characteristics

of the patients are described in Table 1. Three patients were excluded because of invalid results in the mutational analyses. Data on *KRAS* exon 2 mutational status were available before biological treatment in 135 patients. The *therascreen* *KRAS* PCR Kit (Qiagen), cobas *KRAS* Mutation Test kit, and digital PCR (in two cases included in the clinical trial NCT01704703) were used for the analysis. Pathological diagnosis was verified on the basis of H&E-stained sections, and carcinomas were classified according to UICC TNM classification system. Before DNA extraction, stained slides were reviewed by a pathologist to estimate overall neoplastic cell content and a minimum 15% of tumor cell content was considered to be a valid sample for analysis. Tumor response was extracted from medical records evaluated by attending physician, according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Patients with stable disease (SD) or disease progression (PD) were defined as nonresponders. Independent ethics committees at each participating center reviewed and approved the protocol and the study was carried out according to the principles of the Declaration of Helsinki. Committees allowed a waiver in individual patient consent for retrospective biomedical research studies which included the permission to use patients' samples without informed consent in case of death.

DNA extraction and primary PCR reactions

DNA from FFPE tissues was extracted using two comparable methods: the cobas DNA Sample Preparation Kit (Roche Molecular Diagnostics) and the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA quality and concentration were measured with a NanoPhotometer (Implen GmbH).

Mutations in *KRAS* (exons 2/3/4), *NRAS* (exons 2/3/4), *BRAF*, and *PIK3CA* were detected by two commercial assays (Roche cobas test and Qiagen *therascreen* pyrosequencing kit) and by quantitative nanofluidic dPCR (sensitivity of about 0.05%–0.1%) of genomic DNA as previously described (18). For the cobas analysis, DNA extracted by the cobas kit was used, and for *therascreen* pyro and dPCR analysis DNA extracted by Qiagen kit was used.

The cobas *KRAS* Mutation Test kit, the cobas 4800 *BRAF* V600 Mutation Test, and the cobas *PIK3CA* Mutation Test were used for detection of somatic mutations in *KRAS*, *BRAF*, and *PIK3CA* genes. The cobas *KRAS* Mutation Test kit is a PCR assay designed to detect 21 somatic mutations in codons 12, 13 (exon 2), and 61 (exon 3). The cobas 4800 *BRAF* V600 Mutation Test detects the *BRAF* V600E mutation and the cobas *PIK3CA* Mutation Test identifies 17 mutations in exons 1, 4, 7, 9, and 20. In brief, 50 ng of DNA (for *KRAS* and *PIK3CA*) or 125 ng of DNA (*BRAF*) was amplified as well as a negative and positive control, according to the manufacturer's instructions. Mutations were detected using the cobas 4800 System (Roche Molecular Systems Inc.) with automated result interpretation software.

For somatic mutations in *NRAS* codons 12–13, 59–61, 117, and 146 as well as *KRAS* codons 117 and 146, we used a probe-based assay, LightMix Kit *KRAS-NRAS* (TIB MOLBIOL). Six different reactions were performed for each sample. Fifteen microliters of every reaction mix was added to 5 μ L of sample or control DNA for a final reaction volume of 25 μ L according to the manufacturer's instructions. All reactions were performed using the Roche Diagnostics LightCycler FastStart DNA Master Hybridization Probe reaction mix and run on a LightCycler 480 instrument (Roche Molecular Systems Inc.). The thermocycling

Table 1. Clinicopathologic characteristics of the patients included in the efficacy analysis

Variable	No. of patients (anti-EGFR + bev) N = 580 (100%)	No. of patients (anti-EGFR) N = 255 (44%)	No. of patients (bev) N = 325 (56%)
Age (years)			
Median (range)	65 (21–88)	65 (32–88)	65 (21–86)
Gender			
Male/female	382 (65.86)/192 (33.1)	185 (72.55)/70 (27.45)	197 (60.61)/122 (37.54)
Unknown	6 (1.03)	0 (0)	6 (1.85)
Tumor location			
Right colon	169 (29.14)	53 (20.78)	116 (35.69)
Left colon	55 (9.48)	25 (9.8)	30 (9.23)
Rectum	217 (37.41)	93 (36.47)	124 (38.15)
Colon unspecified	132 (22.76)	81 (31.76)	51 (15.69)
Unknown	7 (1.2)	3 (1.17)	4 (1.23)
TNM stage			
I–II	5 (0.86)/45 (7.76)	3 (1.18)/18 (7.06)	2 (0.62)/27 (8.3)
III–IV	136 (23.45)/381 (65.69)	66 (25.88)/161 (63.13)	70 (21.53)/220 (67.7)
Unknown	13 (2.24)	7 (2.74)	6 (1.85)
Primary tumor resection			
Yes/No	459 (79.14)/54 (9.31)	172 (67.45)/18 (7.05)	287 (88.3)/36 (11.07)
Unknown	67 (11.55)	65 (25.5)	2 (0.62)
Metastasis resection			
Yes/no	168 (28.97)/342 (58.97)	66 (25.88)/123 (48.24)	102 (31.38)/219 (67.38)
Unknown	70 (12.06)	66 (25.88)	4 (1.23)
Treatment intention			
First-line	355 (61.21)	84 (32.94)	271 (83.38)
Second-line	95 (16.38)	68 (26.67)	27 (8.31)
Neoadjuvant	34 (5.86)	28 (10.98)	6 (1.85)
Other	96 (16.55)	75 (29.41)	21 (6.46)
Biological treatment			
Cetuximab	120 (20.69)	120 (47.06)	—
Panitumumab	135 (23.28)	135 (52.94)	—
Bevacizumab	325 (56.03)	—	325 (100)
Chemotherapy regimens—clinical trials			
NCT01071655 (SETICC)	70 (12.07)	—	70 (21.54)
NCT00958386 (SPECTRA)	33 (5.69)	33 (12.94)	—
NCT00885885 (PLANET)	42 (7.24)	42 (16.47)	—
NCT01126112 (FRAIL)	15 (2.59)	15 (5.88)	—
NCT01704703 (ULTRA)	32 (5.52)	32 (12.55)	—
Chemotherapy regimens—no clinical trials			
FU based chemotherapy + bevacizumab			
1st line/2nd line	201 (34.66)/27 (4.66)	—	201 (61.85)/27 (8.31)
3rd line and beyond	12 (2.07)	—	12 (3.69)
Other	15 (2.59)	—	15 (4.61)
FU based chemotherapy + cetuximab			
1st line/2nd line	36 (6.21)/7 (1.21)	36 (14.12)/7 (2.74)	—
3rd line and beyond	2 (0.34)	2 (0.78)	—
Other	8 (1.38)	8 (3.14)	—
FU based chemotherapy + panitumumab			
1st line/2nd line	7 (1.21)/1 (0.17)	7 (2.74)/1 (0.4)	—
Irinotecan + anti-EGFR			
1st line/2nd line	7 (1.21)/23 (3.96)	7 (2.74)/23 (9.02)	—
3rd line and beyond	22 (3.79)	22 (8.63)	—
Other	2 (0.34)	2 (0.78)	—
Anti-EGFR monotherapy	18 (3.1)	18 (7.06)	—
Tumor response			
Complete response/partial response	27 (4.66)/180 (31.03)	10 (3.92)/80 (31.37)	17 (5.23)/100 (30.77)
Stable disease/progressive disease	216 (37.24)/88 (15.17)	87 (34.12)/46 (18.04)	129 (39.7)/42 (12.92)
Not evaluable	69 (11.9)	32 (12.55)	37 (11.38)

conditions were as follows: Uracil-DNA glycosylase treatment at 40°C for 20 minutes, denaturation at 95°C for 10 minutes, amplification of target DNA; 60 cycles at 95°C for 5 seconds, 58°C for 5-second acquisition, 58°C for 10 seconds and 72°C for 15 seconds; melting at 95°C for 20 seconds, 58°C for 20 seconds, 40°C for 20 seconds and then a gradual increase in temperature to 85°C with continuous acquisition before cooling at 40°C. Genotype calling based on melting curves was performed using the

LightCycler 480 software release 1.5.1 (Roche), and mutations are identified by a melting peak only in case of the presence of a mutation.

The PyroMark Q24 system (Qiagen) was used for pyrosequencing analysis of KRAS, NRAS, and BRAF mutations. In brief, PCR templates for pyrosequencing were amplified from 10 ng of DNA using *therascreen* KRAS, NRAS, RAS, extension and BRAF Pyro Kit according to the manufacturer's instructions. The biotinylated

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PCR products were placed in 24-well plates and bound to streptavidin-coated sepharose beads (GE Healthcare). The PCR products were denatured, and the nonbiotinylated fragments were washed from the beads using the Pyromark Q24 Vacuum Workstation (Qiagen). The beads were then resuspended in annealing buffer containing 0.4 pmol/L of the sequencing primer. Raw data files were imported into Pyromark Q24 software (version 2.0; Qiagen) for further analysis following pyrosequencing. For mutations in *PIK3CA* we used the PI3K Mutation Test Kit (Qiagen), a real-time PCR assay for the detection of 4 somatic mutations in exons 9 and 20. The kit uses a real-time PCR assay based on ARMS PCR technology combined with Scorpions detection technology. The PI3K Mutation Test Kit includes a control assay and 3 mutation assays to assess the total DNA in a sample and the presence or absence of mutated DNA. Twenty nanograms of DNA were amplified as well as negative and positive controls, using the LightCycler 480 software release 1.5.1 (Roche) and mutations were identified calculating ΔCt (Delta Ct) according to the manufacturer's instructions.

The dPCR analysis methods are described in detail in our previous study (14), and specific primers and probes are specified in Supplementary Table S1.

Statistical analysis

We performed a survival analysis using the Cox proportional regression model and calculated the hazard ratio (HR) for progression-free survival (PFS) and overall survival (OS), described in detail in our previous study (14).

Independently from the survival analysis, in order to evaluate the best sensitivity threshold for mutations to predict treatment response in the anti-EGFR cohort, we calculated the likelihood ratio of positive test analysis. In this analysis, we only included patients with evaluable radiological response ($N = 223$). In order to compare the predictive value of different mutational profiles, we defined three different scenarios for analysis: (i) *KRAS* (exon 2); (ii) *RAS* (*KRAS* exons 2/3/4 and *NRAS* exons 2/3/4); and (iii) *RAS* and *BRAF*.

The concordance between techniques was calculated with the Cohen's kappa coefficient. Data were analyzed with statistical software R version 3.1.2.

Results

Mutational profiling detected by cobas, *therascreen* pyro, and highly sensitivity dPCR

We performed mutational analyses of *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* through three different platforms. The average time for testing all genes per 24 samples was 14 hours for cobas, 35 hours for *therascreen* pyro, and 65 hours for digital PCR. Mutations in any of the genes analyzed were detected in 331 tumors (56.77%) by cobas, in 325 tumors (55.74%) by *therascreen*, and in 361 tumors (61.92%) by dPCR (Table 2). *BRAF* mutations were most frequently in *KRAS* exon 2 wild-type tumors, although both mutations were detected in 7.4% tumors by cobas, 8.3% tumors by *therascreen*, and 13.3% by dPCR. Specific mutations of individual exons in the population of patients are shown in Supplementary Table S2.

In the subset of patients treated with bevacizumab, we did not detect any mutation in 55 of 328 (16.77%) tumors. No valid results were obtained in 3 tumors. As expected, the majority of tumors harbored mutations in *KRAS* exon 2, independently of the

Table 2. Summary of the mutational status of tumors included in the study population detected by the different techniques

	Cobas N (%)	<i>Therascreen</i> Pyro N (%)	Digital PCR N (%)
<i>KRAS</i> (exon 2)			
WT	370 (63.46%)	372 (63.81%)	317 (54.37%)
MUT	209 (35.85%)	208 (35.68%)	261 (44.77%)
NV	4 (0.69%)	3 (0.51%)	5 (0.86%)
<i>RAS</i> (<i>KRAS</i> + <i>NRAS</i> , exons 2/3/4)			
WT	306 (52.49%)	304 (52.14%)	245 (41.95%)
MUT	273 (46.83%)	276 (47.34%)	332 (57.02%)
NV	4 (0.68%)	3 (0.51%)	6 (1.03%)
<i>RAS</i> + <i>BRAF</i>			
WT	283 (48.63%)	275 (47.17%)	223 (38.25%)
MUT	296 (50.68%)	305 (52.32%)	355 (60.89%)
NV	4 (0.69%)	3 (0.51%)	5 (0.86%)
<i>RAS</i> + <i>BRAF</i> + <i>PIK3CA</i>			
WT	246 (42.2%)	254 (43.57%)	217 (37.22%)
MUT	331 (56.77%)	325 (55.74%)	361 (61.92%)
NV	6 (1.03%)	4 (0.69%)	5 (0.86%)

Abbreviations: WT, wild-type tumor; MUT, mutated tumor; NV, no valid results.

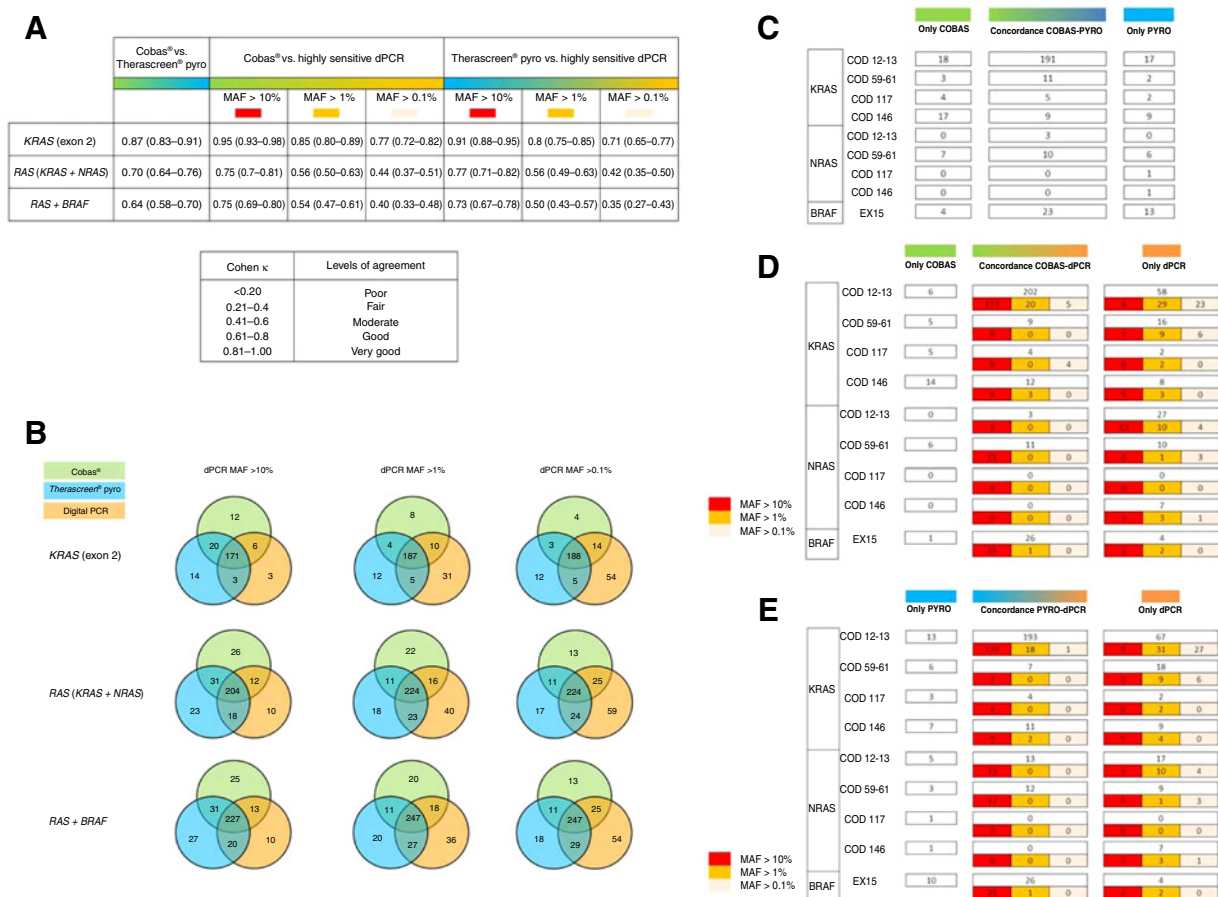
technique. Extended *RAS*, *BRAF*, and *PIK3CA* analysis increased the number of mutated tumors as referred in Supplementary Table S3.

In the cohort of patients treated with cetuximab or panitumumab, no mutations were detected in 102 of 255 (40%) tumors with none of the three techniques used in the study. There was an invalid result in the cobas test and two invalid results in the dPCR test. One hundred and one of 255 (39.6%) tumors harbored one mutation detected by cobas and 18 of them showed multiple mutations. One hundred of 255 (39.2%) tumors harbored one mutation detected by *therascreen* pyro and 15 of them showed multiple mutations. Mutations analyzed by both techniques were frequently located in *KRAS* followed by *PIK3CA*, *NRAS*, and *BRAF* as described in Supplementary Table S3. Analysis by dPCR increased the number of patients bearing mutations up to 119 of 255 (46.7%) and identified multiple additional low-frequency mutant alleles in 34 cases. In this setting, *KRAS* mutations were further overrepresented (81/255; 31.8%) followed by *NRAS* (33/255; 12.9%), *BRAF* (17/255; 6.7%), and *PIK3CA* (8/255, 3.1%; Supplementary Table S3). Sixty-four tumors scored as positive with the three techniques, and the percentage of mutant alleles by quantitative nanofluidic dPCR ranged from 0.2% to 64.5% (median, 46.3%). The percentage of mutant alleles in the 57 tumors detected only by dPCR ranged from 0.04% to 41.5% (median, 2.1%).

The levels of concordance between techniques for mutation detection are shown in Fig. 1. The concordance was good or very good between cobas and *therascreen* pyro and also with dPCR when mutant allele fraction (MAF) considered is $>10\%$. These levels decreased slightly considering $MAF > 1\%$. The concordance is even lower when we compared cobas or *therascreen* with dPCR with a threshold of $MAF > 0.1\%$.

Response rate

Radiological tumor response was evaluable in 514 patients (88%). Complete or partial response occurred in 209 patients (35.8%). Tumor stabilization was observed in 216 patients (37%) and disease progression in 89 patients (15.3%). We then assessed the response rate according to biological therapy. In the group of

**Figure 1.**

Concordance of mutation detection by cobas, *therascreen* pyro, and highly sensitive dPCR. **A**, Cohen kappa index measures the agreement between techniques in mutation detection. **B**, Venn diagram for the results of three tests together. **C**, Number of mutations detected by cobas and *therascreen* pyro. **D**, Number of mutations detected by cobas and digital PCR. **E**, Number of mutations detected by *therascreen* pyro and digital PCR. MAF, mutant allele fraction.

patients treated with bevacizumab, the response rate was not associated with *RAS*/*BRAF* mutational status.

In the group of patients treated with anti-EGFR therapy, radiological tumor response was evaluable in 223 of the 255 patients. Regarding the treatment administered in this specific cohort, 30 patients (13.45%) received cetuximab or panitumumab monotherapy (23 of them were chemo-naïve for metastatic disease), 75 patients (33.63%) received an irinotecan + anti-EGFR regimen and 118 patients (52.91%) were treated with a fluoropyrimidine-based chemotherapy regimen (78 of them in the first-line or neoadjuvant/post-metastectomy setting). Overall response rate (ORR) in this subgroup of patients was 40.3%. We further evaluated response rate according to mutational profile (Table 3). *KRAS* exon 2 wild-type population identified by cobas test presented an ORR of 42.7% [76 responders (89.4%) received anti-EGFR in combination with chemotherapy—19 and 24 patients in the neoadjuvant/post-metastectomy and first-line setting, respectively—and 9 responders were treated with anti-EGFR monotherapy]. Four of the *KRAS* exon 2 mutated patients responded to anti-EGFR regimens [chemotherapy and anti-EGFR in the neoadjuvant/post-metastectomy setting (3/4) or in first line]. Extended *RAS* identified 16 additional *RAS* mutant mCRC

patients with tumor response, 10 of them treated with anti-EGFR in combination with chemotherapy in the neoadjuvant or first-line setting and 2 of them treated only with panitumumab in first line. *BRAF* genotyping identified 8 additional mutated cases, 2 of them responders (both treated in combination with chemotherapy in first line). *RAS*/*BRAF* analysis by cobas increased the response rate up to 45.8% and 26.9% in wild-type and mutated populations, respectively. Analysis of *KRAS* exon 2 by *therascreen* pyro kit identified 24 mutated cases, 5 of them responders (one of them treated with anti-EGFR monotherapy in first line, two of them treated in combination with chemotherapy in the neoadjuvant and post-metastectomy setting, and the other 2 patients treated in second and third line with chemotherapy). This reclassification translated into an ORR of 42.7% in the *KRAS* exon 2 wild-type subgroup [73 responders (91.2%) received a combination of chemotherapy and anti-EGFR—18 and 24 patients in the neoadjuvant/post-metastectomy and first-line setting, respectively—and 11 responders received cetuximab or panitumumab alone]. Extended *RAS* identified 15 additional mutated tumors with radiological response (5 and 6 patients treated in first-line and neoadjuvant/post-metastectomy setting, respectively). *BRAF* genotyping identified 11 additional mutated cases (2 of

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Table 3. ORR in the anti-EGFR group (N = 223)

		Highly sensitive digital PCR															
		Cobas				Therascreen Pyro				Cutoff 0.1%				Cutoff 1%			
		WT	RR	WT	MUT	RR	WT	MUT	RR	WT	MUT	RR	WT	MUT	RR	WT	MUT
<i>KRAS</i> (exon 2)	CR	9	42.7	1	17.4	8	42.7	2	20.8	9	42	1	34	9	43.2	1	24.2
	PR	76		3		77		3		65		15		73		7	
	SD	78		9		78		9		72		15		75		12	
	PD	36		10		36		10		30		16		33		13	
<i>RAS</i> (<i>KRAS</i> ex 2/3/4 + <i>NRAS</i> ex 2/3/4)	CR	9	44.8	1	27.1	8	46.3	2	24.6	9	47.1	1	29.9	9	48.4	1	22.9
	PR	64		15		67		13		55		25		65		15	
	SD	63		24		61		26		49		38		54		33	
	PD	27		19		26		20		23		23		25		21	
<i>RAS</i> + <i>BRAF</i>	CR	9	45.8	1	26.9	8	48.3	2	25	8	48.8	2	29.6	8	50.4	2	23.2
	PR	62		17		63		17		53		27		63		17	
	SD	61		26		57		30		46		41		51		36	
	PD	23		23		19		27		18		28		19		27	

NOTE: The improvement in response prediction gained by assessing the mutation status of each gene.

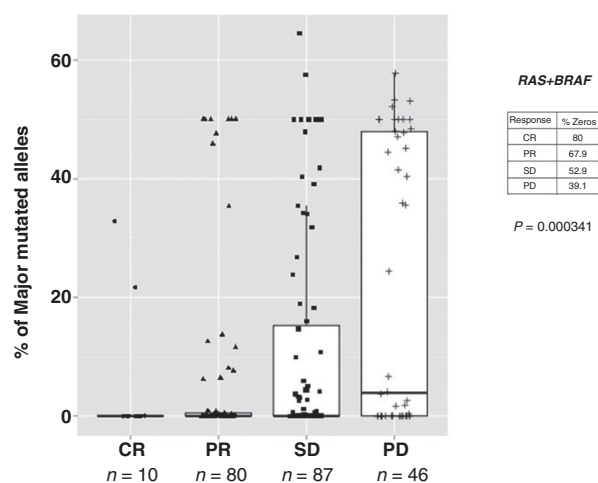
Abbreviations: WT, wild-type tumor; MUT, mutated tumor; RR, response rate; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

them responders and treated in first line). We have to point out that only one of the *RAS/BRAF* mutated tumors that responded was treated with anti-EGFR monotherapy. Extended *RAS* and *BRAF* panel by *therascreen* increased response rate up to 48.3% and 25% in wild-type and mutated populations, respectively. Qualitative dPCR genotyping (limit of detection of the technique, 0.05%–0.1% of mutant alleles) of *KRAS* exon 2 translated into an ORR of 41.7% in the *KRAS* exon 2 wild-type population. Sixty-seven *KRAS* exon 2 wild-type responders (91.8%) received a combination of anti-EGFR and chemotherapy (38 of them as neoadjuvant/postmetastectomy or first-line therapy) and 6 responders received anti-EGFR monotherapy (5 of them in the first-line setting). The extended *RAS* genotyping reclassified 39 additional mutated cases. Nine of these patients responded to treatment (everyone received anti-EGFR monotherapy), that was administered as first-line or neoadjuvant chemotherapy in 5 of them. Finally, the *BRAF* analysis identified 11 additional mutated cases, 3 of them responders (all of them treated with anti-EGFR in the first-line setting). The ORR in the *RAS/BRAF* wild-type subset increased up to 48.8%, whereas ORR in patients reclassified as *RAS/BRAF* mutated by qualitative dPCR was 29.6%. It is noteworthy that most of the *RAS/BRAF*-mutated tumors classified as responders were treated with anti-EGFR and chemotherapy combination (26 out of 29 cases), as described with cobas and *therascreen* pyro. Moreover, approximately 90% of the *KRAS* exon 2 wild-type patients with a partial or complete response received cetuximab or panitumumab in combination with chemotherapy.

An inverse correlation between the proportion of mutant allele and anti-EGFR response is shown in Fig. 2 ($P < 0.0001$). The median percentage of mutated DNA was 12.5% for responders (range, 0.1%–50%) and 34.2% for nonresponders (range, 0.3%–65%) and of note in 10 of 29 responders (34%) that have been scored as positive considering mutated cases by qualitative dPCR genotyping, the major MAF was below 1%.

The likelihood ratio of positive test analysis was performed to estimate the optimal clinically relevant threshold cutoff value of mutant alleles in the prediction of response. For dPCR, the categorization of a fraction of 1% or higher for any mutant allele offered the best likelihood ratio of positive test for all combinations of *RAS* and *BRAF* analysis (Supplementary Table S4) delivering the best balance between sensitivity and specificity. The ORR using the 1% cutoff in the population with *KRAS* exon 2 wild-type tumors was 43.2%, whereas ORR in patients reclassified as *KRAS*

exon 2 mutated tumors was 24.2% (Table 3). The extended *RAS* genotyping reclassified 37 additional mutated cases (8 responders out of 37) which translate into a higher ORR (48.4%) in the *RAS* wild-type cases. The ORR among *RAS* extended mutated reclassified group was 22.9%. Finally, the *BRAF* analysis identified 12 additional mutated cases. The ORR in this *RAS/BRAF* wild-type subset was 50.4%, whereas ORR in patients reclassified as *RAS/BRAF* mutated by qualitative dPCR with 1% cutoff was 23.2%. When compared with qualitative detection of dPCR, the use of 1% cutoff reclassified as wild-type 16 patients: 10 responders and 6 nonresponders. Of the 6 nonresponder cases now reclassified as wild-type, 5 had stable disease while in the remaining 1 disease progressed. We have also calculated ORR with different cutoffs (1% to 5%) but the 1% is the best scenario for the ORR compared with the others (Supplementary Table S5).

**Figure 2.**

Box plot representing the correlation between the proportion of major mutant alleles and the anti-EGFR response. In the y axis: the percentage of major mutated allele (*RAS* + *BRAF*). In the x axis: patients with an evaluable response (N = 223). CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. The mean percentage of mutated alleles in each group is shown by a line (the percentage of mutant alleles are shown in Supplementary Table S3).

Table 4. Survival analyses according to mutational status through different techniques in the anti-EGFR cohort

			Cobas		Therascreen		Highly sensitive dPCR qualitative detection		Highly sensitive dPCR cutoff 1%	
			mut	wt	mut	wt	mut	wt	mut	wt
			<i>KRAS</i> (exon 2)	PFS months	Median (mut/wt)	28	226	30	225	56
		HR (95% CI)	3.88	7.03	3.88	7.03	5.22	7.03	4.86	7.03
		<i>P</i>	1.79 (1.12–2.85)		1.44 (0.92–2.25)		1.29 (0.91–1.82)		1.51 (1.02–2.24)	
			0.015		0.113		0.157		0.041	
	OS months	Median (mut/wt)	12.55	18.17	12.55	18.1	13.8	18.1	13.3	18.1
		HR (95% CI)	2.06 (1.27–3.34)		1.38 (0.87–2.18)		1.52 (1.04–2.22)		1.76 (1.14–2.72)	
		<i>P</i>	0.003		0.167		0.031		0.011	
<i>RAS</i> (<i>KRAS</i> ex 2/3/4 + <i>NRAS</i> ex 2/3/4)	PFS months	Median (mut/wt)	70	184	72	183	101	152	82	172
		HR (95% CI)	4.73	7.19	4.73	7.19	5.22	7.2	4.86	7.2
		<i>P</i>	1.21 (0.87–1.67)		1.37 (0.989–1.896)		1.34 (0.99–1.8)		1.53 (1.12–2.09)	
			0.253		0.059		0.051		0.007	
	OS months	Median (mut/wt)	13.64	18.7	12.59	19.06	13.27	18.7	14.89	18.4
		HR (95% CI)	1.72 (1.23–2.40)		1.71 (1.22–2.39)		1.61 (1.16–2.23)		1.9 (1.33–2.72)	
		<i>P</i>	0.002		0.002		0.004		0.0004	
<i>RAS</i> + <i>BRAF</i>	PFS months	Median (mut/wt)	82	172	91	164	115	139	97	157
		HR, (95% CI)	4.43	7.49	4.43	7.62	5.19	7.59	4.38	7.59
		<i>P</i>	1.50 (1.11–2.04)		1.87 (1.37–2.53)		1.54 (1.15–2.05)		1.66 (1.23–2.24)	
			0.009		≤0.001		0.003		0.0008	
	OS months	Median (mut/wt)	13.27	19.19	12.55	20.24	13.8	20.01	13.67	19.72
		HR (95% CI)	1.86 (1.34–2.58)		1.90 (1.38–2.62)		1.72 (1.25–2.39)		2.01 (1.41–2.85)	
		<i>P</i>	≤0.001		≤0.001		0.001		0.0001	

NOTE: Adjusted for age, gender, and chemotherapy lines.

Abbreviations: wt, wild-type tumor; mut, mutated tumor.

Progression-free survival and overall survival

In the overall study population, OS was significantly shorter in the subgroup of patients with *RAS* or *RAS/BRAF* mutations assessed by any of the three platforms (Supplementary Table S6). However, a clear and significant benefit to anti-EGFR treatment in terms of both PFS and OS was observed only in patients with wild-type tumors (Table 4). Patients with *KRAS* exon 2 mutated tumors analyzed by cobas had a significantly increased risk of disease progression and death with an HR of 1.79 (95% CI, 1.12–2.85) and 2.06 (95% CI, 1.27–3.34), respectively. OS was also associated with *KRAS* exon 2 mutation assessed by dPCR. The risk of disease progression was higher when any mutation assessed by any of the three different techniques was considered (HR of 1.50, *P* = 0.009, HR of 1.87, *P* ≤ 0.001 and HR of 1.54, *P* = 0.003 for cobas, *therascreen*, and dPCR, respectively). This association was maintained in the OS analysis.

Interestingly, when we analyzed survival according to different cutoffs, patients with *KRAS* exon 2 mutated tumors with more than 1% of MAF assessed by quantitative nanofluidic dPCR presented an HR of 1.51 (95% CI, 1.02–2.24) for PFS and 1.76 (95% CI, 1.14–2.72) for OS, compared with wild-type patients or with less than 1% of mutant allele fraction. When we extended the analysis to *RAS* and *BRAF* and a threshold of 1%, the 97 mutated cases presented an HR of 1.66 (95% CI, 1.23–2.24) and 2.01 (95% CI, 1.41–2.85) for PFS and OS, respectively. In concordance with likelihood ratio of positive test analysis depicting 1% as the best cutoff value, a 5% cutoff resulted in a poorer discrimination of PFS and OS.

Additionally, we differentiated patients treated with anti-EGFR in two groups: patients treated with anti-EGFR in first-line setting and patients treated with anti-EGFR in second-line or beyond, and evaluated the impact of gene mutations on survival (Supplementary Table S7). Eighty-three patients (32.6%) received cetuximab or panitumumab (60 of them in combination with chemotherapy) as first-line treatment and were defined as "chemosensitives," and 99 patients (38.8%) received anti-EGFR drugs after first-line

treatment failure (89 of them in combination with chemotherapy). *KRAS* exon 2 mutations had no impact on survival in the chemosensitive group. Only *RAS* mutations assessed by dPCR with a threshold of 1% increased the risk of progression compared with wild-type tumors or with less than 1% of MAF (HR = 1.79, 95% CI, 1.05–3.05). We observed similar results when we extended the mutational analysis to *RAS* and *BRAF*. Once again, the cutoff of 1% better discriminated patients likely to benefit from anti-EGFR therapy with an HR of 2.03 (95% CI, 1.20–3.43). In terms of OS, *RAS* and *BRAF* mutations assessed by cobas, *therascreen*, or dPCR were significantly associated with poor outcome. Similar results were observed in the group of patients treated with anti-EGFR drugs after first-line tumor progression, although in this setting, *RAS* and *BRAF* mutations were related to worse PFS, independently of the technique used.

In the subgroup of patients treated with bevacizumab, only *BRAF* mutations detected by *therascreen* and dPCR were associated with shorter overall survival.

Discussion

In this retrospective study, we have analyzed the mutational status of *RAS* pathway genes through different techniques to evaluate and compare the sensitivity of each one. Furthermore, we have demonstrated that anti-EGFR benefit is more evident in patients with *RAS* and *BRAF* wild-type tumors.

Nowadays, expanded *RAS* and *BRAF* analyses should be performed in mCRC patients to establish the best therapeutic strategy. Several commercial platforms are used in clinical practice for *RAS* testing, although none of them is considered the standard of care (13). We have used three different techniques, the commercial cobas and *therascreen* pyro assays and the digital PCR by Fluidigm, for the analysis of mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA*, and we then compared the sensitivity and predictive capability to anti-EGFR- and bevacizumab-based regimens. As

expected, the most common gene mutation was located in *KRAS* exon 2. We have to point out that the panel of hotspots analyzed by each technique was slightly different. In this context, it was expected that the frequency of gene mutations differed between techniques. Nevertheless, dPCR identified mutations in approximately 62% tumors compared with 55% of mutated tumors identified by cobas or *therascreen* due to its higher analytical sensitivity. dPCR is a highly sensitive and quantitative method with a limit of detection or sensitivity < 0.1%. Because of this higher technical sensitivity, dPCR increased the mutation rate up to 62% with a range of MAF between 0.04% and 85.4%. When we analyzed the concordance between tests, we observed a high agreement (Cohen's kappa \approx 0.90) if we considered *KRAS* exon 2 and a limit of detection of 10%. However, the level of agreement decreased when we compared cobas and *therascreen* with dPCR with a limit of detection of 1%–10% or <1% and is even lower when we included in the mutational analysis *RAS* (*KRAS* exons 3, 4 and *NRAS* exon, 2, 3, 4) and *BRAF*. Taking into account that there was no bias in tumor sample selection because we selected one paraffin block per patient with a minimum of tumor cells for DNA extraction and subsequent DNA mutational analysis by the three techniques, our results demonstrated that quantitative dPCR is more sensitive than cobas and *therascreen* for *RAS* testing.

From a practical point of view, the average time for DNA extraction was similar for the two methods used and the amounts of DNA required for the three tests were also very similar. However, we found differences between the three techniques regarding the turnaround time: Cobas significantly outperformed the others and the slowest one was the dPCR which needs an improvement in the automation to increase the competitiveness. Finally, costs of the reagents per case analyzed by the three platforms were very similar.

In terms of treatment efficacy, we have observed that the clinical outcomes of patients treated with bevacizumab are independent of *RAS* mutational status, as previously reported (16). We have also confirmed that *RAS* mutational status is associated with anti-EGFR treatment outcome in the first-line setting and beyond. This is in line with the results published from different clinical trials in metastatic disease. Pivotal studies with anti-EGFR drugs have analyzed the mutational status of *RAS* by different techniques (13). The PRIME study used Sanger sequencing and WAVE-based SURVEYOR Scan Kits from Transgenomic for *RAS* and *BRAF* testing and detected 52% *RAS*-mutated tumors (2). *RAS* mutational analysis in the Crystal study was performed by BEAMing and detected mutations in 43% of tumors (1). Similar *RAS* mutation rates are reported in the literature, but it is remarkable that the analytical sensitivity of the tests is around 5% to 10%. Highly sensitive methods such as digital PCR or BEAMing are able to identify minor mutated sub-clones present in a low proportion (limit of detection < 0.01%; refs. 17, 18). Both methods have demonstrated to improve the selection of patients more likely to benefit from anti-EGFR therapies; however, a discrepancy in the definition of the optimal cutoff for anti-EGFR treatment was observed (1, 14, 15). To identify the fraction of mutated allele that correlates with resistance to anti-EGFR agents is still a challenge. Our results have confirmed the findings previously reported by Laurent-Puig and colleagues and our own group (14, 15). We have observed that *RAS* mutational status assessed by dPCR with a threshold of 1% identifies candidates for cetuximab or panitumumab with better clinical outcomes in terms of ORR, PFS, and OS than any of the platforms and sensitivity thresholds, confirming the results we reported from a smaller series of chemorefrac-

tory mCRC (14). Overall, the response rate in the *RAS/BRAF* wild-type group of patients increased around 5% in both series when we compared the dPCR with a cutoff of 1% with a conventional analytical method (either real-time PCR or cobas). However, we need to be cautious because a relevant percentage of patients with mutated tumors (around 20% across different analyses) responded to anti-EGFR regimens. Recent publications of clinical randomized trials have reported results on response rate in the mutated *RAS* tumors population treated with anti-EGFR and chemotherapy with ORR of 34% to 15% (1, 19). This can be explained by the chemotherapy effect in most of them but not all, and is a fact that must stimulate continuous research for finding better prediction models beyond *RAS* mutational analyses (20).

A number of limitations of our study need to be highlighted. First, there was no validation cohort to validate the mutation rates for each of the three tests, their concordance, and the prediction of disease outcome. Second, this is a retrospective and pooled analysis of a highly heterogeneous series of patients treated with different chemotherapy regimens and biologicals. Finally, we have analyzed only patients treated with bevacizumab or anti-EGFR containing regimens in a nonrandomized fashion. We have therefore only analyzed clinical outcomes of those patients with tumors with and without mutations assessed by different methods. To validate our results, a similar analysis should be performed in prospective and randomized clinical trials (20). Tumors from patients included in the CAPRI GOIM trial were tested for *RAS*, *BRAF*, and *PIK3CA* by next-generation sequencing and suggested that fraction of neoplastic cells carrying a specific molecular alteration (*RAS*, *BRAF*, *PIK3CA*) could correlate with the level of resistance to anti-EGFR agents (21). Additionally, we have recently completed accrual of the prospective TTD ULTRA clinical trial (clinical trial identifier: NCT01704703) which will provide relevant information about the utility of highly sensitive methods in DNA testing for clinical practice.

Disclosure of Potential Conflicts of Interest

B. Massuti has received speakers bureau honoraria from Roche and Merck and is a consultant/advisory board member for Roche. M. Valladares is a consultant/advisory board member for Merck Serono. J.M. Vieitez reports receiving a commercial research grant from Roche and Amgen and is a consultant/advisory board member for Amgen, Roche, Servier, and Shire. J. Tabernero is a consultant/advisory board member for Amgen, Bayer, Pfizer, Roche, Sanofi, Symphogen, Taiho, Takeda, Boehringer-Ingelheim, Celgene, Chugai, Genentech, Lilly, MSD, Merck Serono, and Novartis. G. Capella reports receiving a commercial research grant member for Roche. No potential conflicts of interest were disclosed by the other authors.

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Optimization of *RAS/BRAF* Mutational Analysis Confirms Improvement in Patient Selection for Clinical Benefit to Anti-EGFR Treatment in Metastatic Colorectal Cancer

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