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ORIGINAL ARTICLE

Phase II study of high-sensitivity genotyping of *KRAS*, *NRAS*, *BRAF* and *PIK3CA* to ultra-select metastatic colorectal cancer patients for panitumumab plus FOLFIRI: the ULTRA trial

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Background: Several studies show the importance of accurately quantifying not only *KRAS* and other low-abundant mutations because benefits of anti-EGFR therapies may depend on certain sensitivity thresholds. We assessed whether ultra-selection of patients using a high-sensitive digital PCR (dPCR) to determine *KRAS*, *NRAS*, *BRAF* and *PIK3CA* status can improve clinical outcomes of panitumumab plus FOLFIRI.

Patients and methods: This was a single-arm phase II trial that analysed 38 *KRAS*, *NRAS*, *BRAF* and *PIK3CA* hotspots in tumour tissues of irinotecan-resistant metastatic colorectal cancer patients who received panitumumab plus FOLFIRI until disease progression or early withdrawal. Mutation profiles were identified by nanofluidic dPCR and correlated with clinical outcomes (ORR, overall response rate; PFS, progression-free survival; OS, overall survival) using cut-offs from 0% to 5%. A quantitative PCR (qPCR) analysis was also performed.

Results: Seventy-two evaluable patients were enrolled. *RAS* (*KRAS*/*NRAS*) mutations were detected in 23 (32%) patients and *RAS*/*BRAF* mutations in 25 (35%) by dPCR, while they were detected in 7 (10%) and 11 (15%) patients, respectively, by qPCR. *PIK3CA* mutations were not considered in the analyses as they were only detected in 2 (3%) patients by dPCR and in 1 (1%) patient by qPCR. The use of different dPCR cut-offs for *RAS* (*KRAS*/*NRAS*) and *RAS*/*BRAF* analyses translated into differential clinical outcomes. The highest ORR, PFS and OS in wild-type patients with their lowest values in patients with mutations were achieved with a 5% cut-off. We observed similar outcomes in *RAS/BRAF* wild-type and mutant patients defined by qPCR.

Conclusions: High-sensitive dPCR accurately identified patients with *KRAS*, *NRAS*, *BRAF* and *PIK3CA* mutations. The optimal *RAS/BRAF* mutational cut-off for outcome prediction is 5%, which explains that the predictive performance of qPCR was not improved by dPCR. The biological and clinical implications of low-frequent mutated alleles warrant further investigations.

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Key words: digital PCR, FOLFIRI, metastatic colorectal cancer, panitumumab, patient selection, PCR

Introduction

Panitumumab is a fully human monoclonal antibody that binds to epidermal growth factor receptor (EGFR), inhibiting EGFR pathway and tumour growth. 5-fluorouracil-based chemotherapy and panitumumab improved survival outcomes in metastatic colorectal cancer (mCRC) [1, 2], although the absolute benefits are limited and disease course depends on *RAS* mutations [3].

Mutated *KRAS* exon 2 was initially claimed responsible for panitumumab resistance [4], but subsequent analyses revealed the detrimental effect of other *KRAS* (exons 3/4) and *NRAS* (exons 2/3/4) mutations [5]. Treatment indication was then restricted to patients without mutant *RAS* (*KRAS*/*NRAS*) exons 2/3/4 in 2013. However, not all wild-type *RAS* tumours respond to panitumumab and additional predictive markers should be identified. Despite the need for further evidence, patients with mutated *BRAF* exon 15 appear less likely to benefit from EGFR antibodies [6, 7] and those with *PIK3CA* exon 20 mutations may exhibit worse outcomes [8]. Indeed, tumours with *RAS*, *BRAF* or *PIK3CA* mutations display a shared gene expression pattern that can explain resistance to anti-EGFR drugs [9]. Extended mutational analyses may therefore contribute to optimizing the identification of patients that are more likely to respond to anti-EGFR therapies.

Mutational testing remains challenging as no clearly standardized procedures have been established and an increasing number of techniques have been recently developed, including hypersensitive techniques that may ultra-select patients more accurately [10]. Digital PCR (dPCR) is an increasingly applied technique based on a sample split into hundreds of smaller reactions followed by target PCR amplifications. Nanofluidic dPCR improved the sensitivity of detecting *KRAS*-mutant alleles in clinical samples, reaching 0.05%–0.1%, and allows a better tumour classification with a commercially available platform [11]. Subsequent extended *RAS* and *BRAF* hotspot analyses suggested a threshold of 1% of mutated alleles to predict anti-EGFR therapy response, though the optimal cut-off for the clinical setting remains to be defined [12, 13].

In light of the above, we prospectively assessed whether ultra-selection of mCRC patients using high-sensitive nanofluidic dPCR genotyping of *KRAS*, *NRAS*, *BRAF* and *PIK3CA* can improve the selection of patients and so optimize clinical outcomes of panitumumab plus FOLFIRI treatment.

Patients and methods

Study design and participants

This was an open-label, single-arm, phase II trial conducted in the Departments of Medical Oncology at 12 Spanish hospitals according to the Declaration of Helsinki and national regulations. It was approved by the ethics committee and all patients gave their written informed consent before enrolment.

The study included patients aged ≥18 years with histologically confirmed colorectal adenocarcinoma, wild-type *KRAS* exon 2 as per the local or central laboratory conventional technique (wild-type *KRAS* and *NRAS* exons 2/3/4 after the approval of a protocol amendment on 25 July 2013), with ≥1 initially measurable and unresectable metastatic lesion, Karnofsky performance status ≥70% and adequate bone marrow, renal, hepatic and metabolic functions. Patients must have received irinotecan-based chemotherapy for mCRC for ≥6 weeks and have exhibited disease progression during this treatment or within the 6 months after its end. DNA extracted from tumour blocks must also be suitable for high-sensitive analysis. Previous treatment with anti-EGFR antibodies or small-molecule EGFR tyrosine kinase inhibitors was not allowed.

Patients received panitumumab 6 mg/kg over a 60-min intravenous infusion on day 1 in 2-week cycles. FOLFIRI was intravenously administered on day 1 in 2-week cycles according to the following schema: irinotecan 180 mg/m^2 over 30–90-min infusion, leucovorin 400 mg/m^2 over 120-min infusion, 5-fluorouracil 400 mg/m^2 bolus, 5-fluorouracil 2400 mg/m^2 over 46-h infusion. Doses of panitumumab, irinotecan and 5-fluorouracil could be reduced/delayed in case of adverse events (AEs) as per protocol. Treatment continued until disease progression or unacceptable toxicity. Patients were subsequently followed every 3 months to document progression and survival. The study ended 1 year after the last patient enrolment.

Tumour assessments were conducted every 10 ± 2 weeks until disease progression according to the Response Evaluation Criteria in Solid Tumors version 1.1. Overall response rate (ORR) was defined as the proportion of patients with a partial or complete response. Progression-free survival (PFS) was measured from study inclusion to progression or death, and overall survival (OS) from enrolment to death. Toxicity was assessed at every study visit according to the Common Toxicity Criteria for Adverse Events version 4.0.

Mutational analysis

Mutational analysis was conducted at the Institut Català d'Oncología (L'Hospitalet de Llobregat, Spain). DNA was extracted from formalin-fixed paraffin-embedded tumour tissues (primary tumour or metastasis) and a 38-hotspot panel of *KRAS* (exons 2/3/4), *NRAS* (exons 2/3/4), *BRAF* (exon 15) and *PIK3CA* (exon 20) mutations was assessed using a conventional quantitative PCR (qPCR) machine (LightCycler® 480; Roche Applied Science) and a nanofluidic dPCR platform (Digital Array™ and BioMark™ Real-Time PCR System; Fluidigm Europe) as described previously [11, 12]. Mutations assessed in this study are described in supplementary Table S1, available at *Annals of Oncology* online. Investigators remained blind to mutational status until the end of study treatment.

Statistical analysis

The primary end point was ORR, which was calculated according to *RAS* (*KRAS*/*NRAS*), *RAS*/*BRAF* and *RAS*/*BRAF*/*PIK3CA* status (as per protocol amendment) assessed by dPCR. ORRs were correlated with dPCR cut-offs from 0% to 5%, estimating odds ratios, 95% confidence intervals (CIs) and *P*-values. The sample size was calculated assuming 55% of *RAS/BRAF/PIK3CA* ('ultra') wild-type patients, with an expected ORR of

30% versus 5% in those with mutations. Considering a type I error of 15%, type II error of 20%, loss rate of 10%, the estimated sample size was 82 patients. It was required 45 *KRAS/NRAS* and *BRAF* wild-type patients.

Secondary end points included PFS and OS, which were correlated with the previously described mutational status based on dPCR cut-offs from 0% to 5%. They were calculated using the Kaplan–Meier method and log-rank test, estimating hazard ratios, 95% CIs and *P*-values.

Missing data were not considered in the analyses. All statistical analyses were performed using the Statistical Package for the Social Sciences version 18.0, with a significance level of 0.05.

Results

Patient characteristics and treatment exposure

Ninety-six patients were consecutively recruited from November 2012 to July 2015, 24 of whom were screening failures (supplementary Figure S1, available at *Annals of Oncology* online). Thus, 72 patients were finally included in the study; their baseline characteristics are described in Table 1. The median 5-flourouracil-free interval was 1.7 months (range 0.9–6.4), the median irinotecan-free interval was 1.6 months (range 0.9–5.6) and the median oxaliplatin-free interval was 3 months (range 0.9–37.4).

Patients received a median of 11 (1–42) cycles of panitumumab, 10 (1–42) of irinotecan, 9 (1–35) of 5-fluorouracil bolus and 10 (1–42) of 5-fluorouracil infusion. Their median relative dose intensities were 81% (38%–104%), 78% (25%–103%), 70% (0%–102%) and 74% (13%–102%), respectively. Grade 3/4 toxicities were reported in 48 (67%) patients (supplementary Table S2, available at *Annals of Oncology* online) and treatment discontinuations were mainly due to disease progression ($n = 47$, 65%) or AEs ($n = 8$, 11%).

Mutational profiles

Primary tumour and liver metastasis samples were available for mutational analysis in 63 and 9 patients, respectively. Specific mutations in the population of patients included in the study are shown in supplementary Table S3, available at *Annals of Oncology* online. Mutations in *RAS*, *BRAF* or *PIK3CA* were detected in 12 (17%) patients by conventional qPCR and in 26 (36%) by nanofluidic dPCR (detection limited by the technique sensitivity). qPCR found *RAS* mutations in 7 (10%) patients (*KRAS*: $n = 5$; *NRAS*: $n = 2$), while dPCR detected them in 23 (32%) (*KRAS*: $n = 20$; *NRAS*: $n = 5$) (Table 2). *RAS* or *BRAF* mutations were detected in 11 (15%) patients using qPCR and in 25 (35%) using dPCR. Two of the four patients with *BRAF* mutant tumours by qPCR showed additional *KRAS* mutations at low allele fraction. As only one *PIK3CA* mutation (H1047R) was detected in 1 (1%) patient by qPCR and in 2 (3%) by dPCR, it was not considered in the efficacy outcome analyses.

Efficacy outcomes according to mutational profiles

In the *RAS* wild-type population by qPCR ($N = 65$), radiologic tumour response was evaluable in 64 of 65 patients. An inverse correlation between the proportion of mutant allele and tumour response is shown in supplementary Figure S2, available at *Annals of Oncology* online, although it did not reach statistical significance (*P*-value = 0.058). The median percentage of mutated DNA was 0.8% for responders (0.1%–6.1%) and 9.6% for non-responders (0.4%–48%). *RAS* mutations were detected in 7 of 31 responders (23%) by qualitative dPCR genotyping. In 4 of these 7 responders (57%), the major mutant allele fraction was below 1% and in 6 of these 7 responders (86%), the major mutant allele fraction was below 5% (Figure 1). When analysed by qPCR, ORRs were 48% in patients with wild-type *RAS* and 51% in those with wild-type *RAS*/*BRAF* and no patient with mutations showed a response. The bottom line is that when analytical sensitivity is increased beyond 5%, response rate significantly increases in new 'ultra' mutant but not in the new 'ultra' wild-type groups, respectively, which remarkably worsens the prediction ability of the mutational status (Table 3).

As mentioned previously, four of the *RAS* wild-type tumours harboured *BRAF* mutations, detected either by qPCR or dPCR. In an attempt to exclude the potential negative role of *BRAF* mutations, we evaluated the predictive value of low-frequent *RAS*-mutant alleles in the *RAS*/*BRAF* wild-type population assessed by qPCR. Results were similar to those observed in the *RAS* wild-type population, since the higher sensitive cut-off did not improve the predictability to treatment response (supplementary Table S4, available at *Annals of Oncology* online).

At database lock (July 2016), the median follow-up was 12.3 (0.7–34.2) months. Patients showed a median PFS of 7.1 months (95% CI, 5.8–8.5) and OS of 13.7 months (95% CI, 9.4–18). Survival outcome analyses according to *RAS* and *RAS*/*BRAF* status by qPCR and dPCR cut-offs in the *RAS* wild-type population are described in Table 4. When using dPCR, the 5% cut-off showed the highest PFS in wild-type patients with the lowest PFS in those with mutated *RAS* (7.6 versus 4.0 months, *P*-value = 0.048) and *RAS*/*BRAF* (8.8 versus 4.0 months, *P*-value < 0.001). These figures were similar to those observed when using qPCR, with the exception of a slightly higher PFS in wild-type *RAS*/*BRAF* patients by dPCR compared to qPCR (8.8 versus 7.6 months). We also evaluated survival in the *RAS*/*BRAF* wild-type population by qPCR according to different *RAS* mutant allele fractions. We observed that the presence of *RAS* mutations at frequency below 5% did not improve survival outcomes, since PFS was similar in *RAS* wild-type and mutant patients (supplementary Table S5, available at *Annals of Oncology* online).

Discussion

The identification of predictive biomarkers to EGFR inhibitors to select patients more likely to benefit from these therapies is still a matter of concern. The expanded mutational analysis to *KRAS* and *NRAS* exon 2, 3 and 4 has been widely explored in retrospective and prospective studies associated to clinical trials and its assessment is mandatory before anti-EGFR therapies, whereas there is still insufficient data for *BRAF* [6, 7], although it is highly recommended by international guidelines [14]. The role of additional low-abundant mutations in the EGFR pathway genes rather than *RAS* exon 2, 3 and 4 mutations has been addressed previously in retrospective studies suggesting that the optimal cut-off for patient selection is >1% [12, 13, 15, 16]. However,

Table 1. Baseline patient characteristics (overall population and patients with *RAS* wild-type population by qPCR)

Characteristics	Value Overall population *N* = 72	Value *RAS* wild-type by qPCR *N* = 65
Median age, years (range)	62 (38–83)	62 (38–83)
Gender, *n* (%)		
Male	51 (71)	46 (71)
Female	21 (29)	19 (29)
Tumour stage at initial diagnosis, *n* (%)		
II	4 (5.6)	4 (6.2)
III	16 (22.2)	14 (21.5)
IV	52 (72.2)	47 (72.3)
Karnofsky performance status, *n* (%)		
70–80	18 (25)	14 (21.5)
90–100	54 (75)	51 (78.5)
Primary tumour site, *n* (%)		
Right colon	10 (14)	9 (13.8)
Left colon	31 (43)	31 (47.7)
Rectum	31 (43)	25 (38.5)
Primary tumour surgery, *n* (%)		
Yes	53 (74)	49 (75.4)
No	19 (26)	16 (24.6)
Number of metastatic sites, *n* (%)		
<3	49 (68)	45 (69.2)
≥3	23 (32)	20 (30.8)
Adjuvant chemotherapy (non-metastatic disease)		
Flouropyrimidine monotherapy	4 (5.6)	4 (6.1)
Flouropyrimidine + oxaliplatin	12 (16.7)	11 (17)
Observation	4 (5.6)	3 (4.6)
Previous therapies for metastatic disease		
Neoadjuvant/adjuvant		
Oxaliplatin-based chemotherapy	6 (8.3)	5 (7.7)
Irinotecan-based chemotherapy	1 (1.4)	0 (0)
Folfoxiri-based chemotherapy	1 (1.4)	1 (1.5)
First line		
Oxaliplatin-based chemotherapy	30 (41.7)	25 (38.5)
Irinotecan-based chemotherapy	38 (52.7)	36 (55.4)
Folfoxiri-based chemotherapy	4 (5.6)	4 (6.2)
Second line		
Oxaliplatin-based chemotherapy	7 (9.7)	6 (9.2)
Irinotecan-based chemotherapy	27 (37.5)	23 (35.4)
Flouropyrimidine monotherapy	2 (2.8)	2 (3.1)
Third line		
Irinotecan-based chemotherapy	4 (5.6)	4 (6.2)
Oxaliplatin-based chemotherapy	1 (1.4)	1 (1.5)
Fourth line and beyond		
Irinotecan-based chemotherapy	3 (4.2)	2 (3.1)
Others	1 (1.4)	1 (1.5)

there is a need for further validation. In this sense, we report the results of the first published clinical trial addressing this question.

Nanofluidic dPCR allowed the identification of higher rates of gene mutations, most of them located in *KRAS* exons 2/3. It is remarkable that additional low-abundant mutations in *KRAS* exon 4, *NRAS* and/or *PIK3CA* were detected in more than 20% of patients, similar to data published previously [12, 13, 15, 16].

Table 2. Mutation distribution (*N* = 72)

	Conventional qPCR, *n* (%)		Nanofluidic dPCR, *n* (%)	
	Wild-type	**Mutation**	**Wild-type**	**Mutation**
KRAS	**67 (93)**	**5 (7)**	**52 (72)**	**20 (28)**
Exon 2-codon 12–13	71 (99)	1 (1)	62 (86)	10 (14)[a]
Exon 3-codon 58–61	70 (97)	2 (3)	65 (90)	7 (10)[a]
Exon 4-codon 117	72 (100)	0 (0)	71 (99)	1 (1)
Exon 4-codon 146	70 (97)	2 (3)	69 (96)	3 (4)
NRAS	**70 (97)**	**2 (3)**	**67 (93)**	**5 (7)**
Exon 2-codon 12–13	71 (99)	1 (1)	69 (96)	3 (4)[a,b]
Exon 3-codon 59–61	71 (99)	1 (1)	69 (96)	3 (4)[a,b]
Exon 4-codon 117	72 (100)	0 (0)	72 (100)	0 (0)
Exon 4-codon 146	72 (100)	0 (0)	71 (99)	1 (1)
BRAF	**68 (94)**	**4 (6)**	**68 (94)**	**4 (6)**
Exon 15-codon 600	68 (94)	4 (6)	68 (94)	4 (6)
PIK3CA	**71 (99)**	**1 (1)**	**70 (97)**	**2 (3)**
Exon 20-codon 1043–1047	71 (99)	1 (1)	70 (97)	2 (3)

dPCR, digital PCR; qPCR, quantitative PCR.
[a]One patient exhibited two mutations: one in codon 12 and another in codon 59.
[b]One patient exhibited two mutations: one in codon 12 and another in codon 61.

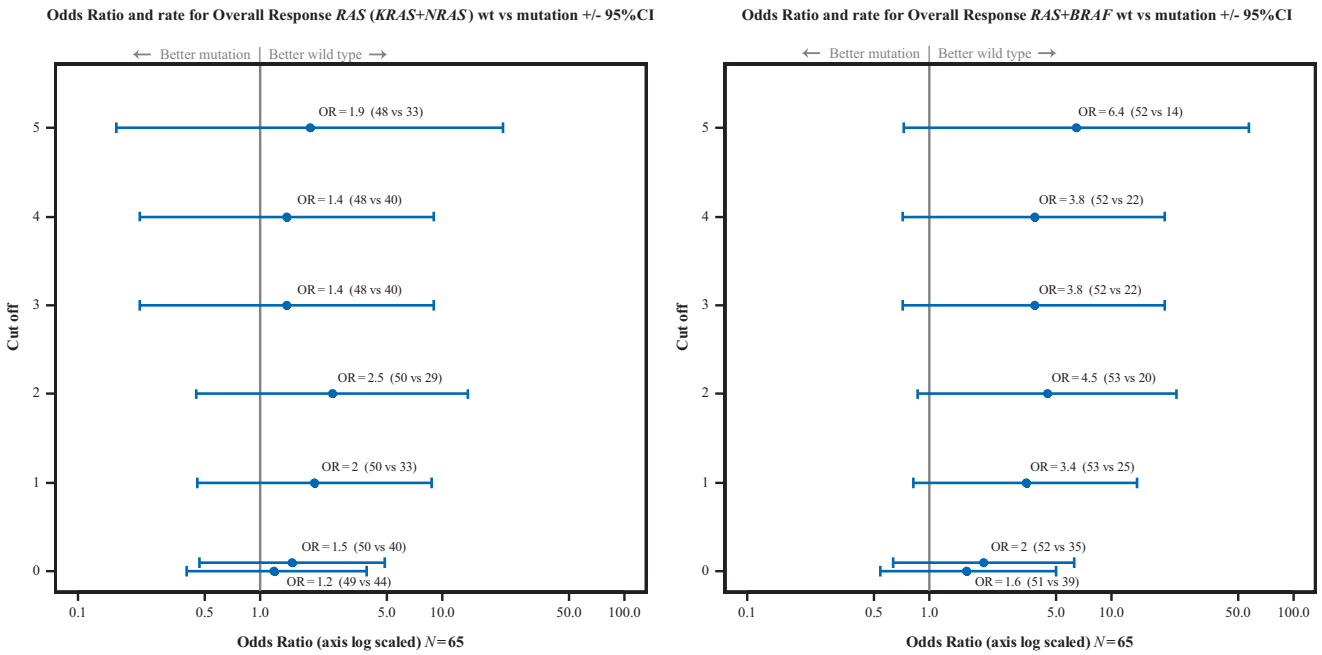

Figure 1. Overall response rates in wild-type versus *RAS* and *RAS/BRAF* mutations detected by nanofluidic digital PCR (*N* = 65). Odds ratios and their 95% confidence intervals are represented, along with the description of overall response rates for each cut-off value. CI, confidence interval; OR, odds ratio; vs, versus; wt, wild-type.

In terms of efficacy, we have shown that *BRAF* mutations are clearly associated with poor outcome, independently of the technique used, as reported previously from randomized clinical trials [5, 17]. Additionally, extended mutational *RAS* (*KRAS/NRAS*) and *RAS/BRAF* status translated into differential clinical outcomes defined by dPCR sensitivity cut-offs.

This is in line with the more evident benefit of anti-EGFR therapies in patients with wild-type *RAS/BRAF* tumours observed in retrospective extended analyses of tumour tissues from case series and clinical trials using dPCR [12, 13]. However, identifying optimal fractions of mutated alleles that predict anti-EGFR-therapy outcomes remain challenging. In this phase II clinical

Table 3. Tumour response according to mutational status in the *RAS* wild-type population by qPCR (*N* = 65)

		Conventional qPCR		Nanofluidic dPCR																	
				Cut-off 0%			*Cut-off 0.1%*			*Cut-off 1%*			*Cut-off 2%*			*Cut-off 3%*			*Cut-off 4%*			*Cut-off 5%*		
		wt	*P*-value	mut	wt	*P*-value	mut	wt	*P*-value	mut	wt	*P*-value	mut	wt	*P*-value	mut	wt	*P*-value	mut	wt	*P*-value	mut	wt	*P*-value	mut
RAS (*KRAS* + *NRAS*)	CR (%)	0.0	–	0.0	0.0	0.843	0.0	0.0	0.745	0.0	0.0	0.624	0.0	0.0	0.362	0.0	0.0	0.850	0.0	0.0	0.850	0.0	0.0	0.549	0.0
	PR (%)	47.7		0.0	49.0		43.8	50.0		40.0	50.0		33.3	50.0		28.6	48.3		40.0	48.3		40.0	48.4		33.3
	SD (%)	36.9		0.0	36.7		37.5	36.0		40.0	35.7		44.4	36.2		42.9	36.7		40.0	36.7		40.0	37.1		33.3
	PD (%)	13.8		0.0	12.2		18.8	12.0		20.0	12.5		22.2	12.1		28.6	13.3		20.0	13.3		20.0	12.9		33.3
RAS + *BRAF*	CR (%)	0.0	0.067	0.0	0.0	0.773	0.0	0.0	0.604	0.0	0.0	0.241	0.0	0.0	0.130	0.0	0.0	0.164	0.0	0.0	0.164	0.0	0.0	0.076	0.0
	PR (%)	50.8		0.0	51.1		38.9	52.1		35.3	52.8		25.0	52.7		20.0	51.8		22.2	51.8		22.2	51.7		14.3
	SD (%)	36.1		50.0	34.0		44.4	33.3		47.1	34.0		50.0	34.5		50.0	35.7		44.4	35.7		44.4	36.2		42.9
	PD (%)	11.5		50.0	12.8		16.7	12.5		17.6	11.3		25.0	10.9		30.0	10.7		33.3	10.7		33.3	10.3		42.9

CR, complete response; dPCR, digital PCR; mut, mutation; PD, progressive disease; PR, partial response; qPCR, quantitative PCR; SD, stable disease; wt, wild-type.

trial, sensitivity of *RAS/BRAF* mutational analysis of dPCR cut-offs for clinical outcome prediction was 5%, as the presence of mutations <5% was not associated with inferior response rate or survival. We have to point out that it was not possible to evaluate the predictive/prognostic value of *BRAF* minor subclones since *BRAF*-mutant allele fraction was >10% in all cases. Increasing analytical sensitivity using dPCR may entail drawbacks in terms of patient selection, as clinical outcomes observed in patients with *RAS* and *BRAF* mutations were higher when using lower cut-offs. This effect was less evident and comparable to conventional qPCR when a 5% dPCR cut-off was used. However, previous retrospective analyses have reported even lower optimal thresholds when using nanofluidic dPCR [12, 13].

Other types of high-sensitive dPCR have also faced the challenging scenario of assessing the most appropriate threshold to identify patients most likely to benefit from anti-EGFR therapies [15, 16]. While an analysis of the clinical relevance of *KRAS*-mutated subclones assessed by picodroplet dPCR reported a 1% threshold in mCRC patients treated with cetuximab or panitumumab [15], another outcome analysis using BEAMING dPCR showed that mCRC patients with *RAS* mutation signals from 0.1% to 5% may benefit from adding cetuximab to FOLFIRI [16]. Despite the current evidence suggests different optimal cut-offs for outcome prediction, it is noteworthy that a minimum of mutant alleles is required to confer primary resistance to anti-EGFR drugs. Several hypotheses beyond those related to the retrospective nature of the studies, the heterogeneity of patient populations and analytical methods could explain the slight discrepancies among publications. Intratumour heterogeneity has been recently described as a mechanism likely to affect the response to anti-EGFR [18, 19], pointing out the potential cooperative effect of EGFR pathway genes mutations on outcome, even though these mutations were present in a low frequency. The clonal selection under treatment pressure has also been involved in the treatment efficacy to target agents such as anti-EGFR [20–22]. Moreover, additional factors such as tumour site (right versus left colon) and other molecular alterations could be responsible for intrinsic resistance [9, 23–26].

The authors acknowledge certain study limitations that should be considered when interpreting its findings, including the impossibility to assess the role of *PIK3CA* status due to the low number of mutations. In addition, initially enrolled patients may have had mutations in *KRAS* 3/4 exons and *NRAS* 2/3/4 exons, while patients must have had no mutation in *KRAS* and *NRAS* exons 2/3/4 after the approval of protocol amendment on 25 July 2013. The analysis of *KRAS*/*RAS* status to assess patient eligibility could have been performed either by the local laboratory conventional technique or by conventional qPCR in the central laboratory. Nonetheless, tumour tissues from enrolled patients were analysed by conventional qPCR in the central laboratory to ensure homogeneity in study results. As expected, 10% of tumour samples harboured mutations in *KRAS* exon 3 or 4 or in *NRAS* exon 2, 3 or 4 by standard of care, which translated into a *RAS* wild-type population of 65 patients.

In conclusion, although the predictive role of *PIK3CA* status within this extended mutational analysis remains unclear, the

Table 4. Survival according to mutational status in the *RAS* wild-type population (*N* = 65)

	Conventional qPCR	Nanofluidic dPCR						
		Cut-off 0%	Cut-off 0.1%	Cut-off 1%	Cut-off 2%	Cut-off 3%	Cut-off 4%	Cut-off 5%
***RAS* (*KRAS*/*NRAS*)**								
PFS, months								
wt/mut (*n*/*n*)	65/–	49/16	50/15	56/9	58/7	60/5	60/5	62/3
Median (wt/mut)	7.4/–	7.2/7.4	7.6/7.4	7.6/7.4	7.6/6.7	7.6/7.4	7.6/7.4	7.6/4.0
HR (95% CI)	–	0.9 (0.5–1.6)	0.9 (0.5–1.7)	0.8 (0.4–1.8)	1.3 (0.6–2.9)	1.0 (0.4–2.5)	1.0 (0.4–2.5)	3.3 (1.0–11.0)
P-value	–	0.741	0.818	0.657	0.513	0.996	0.996	0.048
OS, months								
wt/mut (*n*/*n*)	65/–	49/16	50/15	56/9	58/7	60/5	60/5	62/3
Median (wt/mut)	13.9/–	11.7/17.4	11.8/16.1	12.5/16.1	13.9/16.1	13.9/16.1	13.9/16.1	13.9/16.1
HR (95% CI)	–	0.6 (0.3–1.2)	0.7 (0.3–1.4)	0.6 (0.3–1.7)	0.8 (0.3–2.1)	0.8 (0.3–2.2)	0.8 (0.3–2.2)	1.5 (0.4–4.7)
P-value	–	0.142	0.294	0.367	0.689	0.620	0.620	0.534
RAS*/*BRAF								
PFS, months								
wt/mut (*n*/*n*)	61/4	47/18	48/17	53/12	55/10	56/9	56/9	58/7
Median (wt/mut)	7.6/1.8	7.6/6.7	7.6/6.7	7.6/5.5	8.1/4.6	8.1/4.6	8.1/4.6	8.8/4.0
HR (95% CI)	6.0 (2.0–17.7)	1.0 (0.6–1.8)	1.0 (0.6–1.9)	1.1 (0.6–2.2)	1.7 (0.9–3.4)	1.7 (0.8–3.4)	1.7 (0.8–3.4)	5 (2.1–11.7)
P-value	0.001	0.965	0.879	0.732	0.123	0.160	0.160	<0.001
OS, months								
wt/mut (*n*/*n*)	61/4	47/18	48/17	53/12	55/10	56/9	56/9	58/7
Median (wt/mut)	16.1/6.2	11.8/16.1	12.5/16.1	13.9/13.7	15.6/8.4	16.2/8.4	16.2/8.4	16.2/7.3
HR (95% CI)	8.1 (2.6–25.5)	0.7 (0.4–1.4)	0.8 (0.4–1.7)	1.0 (0.5–2.2)	1.3 (0.6–2.8)	1.5 (0.7–3.3)	1.5 (0.7–3.3)	2.8 (1.3–6.4)
P-value	<0.001	0.349	0.619	0.951	0.528	0.290	0.290	0.012

CI, confidence interval; dPCR, digital PCR; HR, hazard ratio; mut, mutation; OS, overall survival; PFS, progression-free survival; qPCR, quantitative PCR; wt, wild-type.

optimal *RAS*/*BRAF* mutational cut-off for outcome prediction is 5% within the range of analytical sensitivity of conventional methods, which explains that the predictive performance of qPCR was not improved by dPCR in our study. Further research on the biological role and clinical relevance of low-frequent mutations (1%–5%) is warranted. Larger datasets may be important to re-assess the effect of ultra-selection, since minimal but significant effect would need greater numbers to be proven.

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References

1. Douillard JY, Siena S, Cassidy J et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. JCO 2010; 28(31): 4697–4705.
2. Peeters M, Price TJ, Cervantes A et al. Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. JCO 2010; 28(31): 4706–4713.
3. Liang RF, Zheng LL. The efficacy and safety of panitumumab in the treatment of patients with metastatic colorectal cancer: a meta-analysis from five randomized controlled trials. Drug Des Dev Ther 2015; 9: 4471–4478.
4. Amado RG, Wolf M, Peeters M et al. Wild-type *KRAS* is required for panitumumab efficacy in patients with metastatic colorectal cancer. JCO 2008; 26(10): 1626–1634.
5. Douillard JY, Oliner KS, Siena S et al. Panitumumab-FOLFOX4 treatment and *RAS* mutations in colorectal cancer. N Engl J Med 2013; 369(11): 1023–1034.
6. Pietrantonio F, Petrelli F, Coinu A et al. Predictive role of *BRAF* mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: a meta-analysis. Eur J Cancer 2015; 51(5): 587–594.
7. Rowland A, Dias MM, Wiese MD et al. Meta-analysis of *BRAF* mutation as a predictive biomarker of benefit from anti-EGFR monoclonal antibody therapy for *RAS* wild-type metastatic colorectal cancer. Br J Cancer 2015; 112(12): 1888–1894.
8. Huang L, Liu Z, Deng D et al. Anti-epidermal growth factor receptor monoclonal antibody-based therapy for metastatic colorectal cancer: a meta-analysis of the effect of *PIK3CA* mutations in *KRAS* wild-type patients. Arch Med Sci 2014; 10: 1–9.
9. Tian S, Simon I, Moreno V et al. A combined oncogenic pathway signature of *BRAF*, *KRAS* and *PI3KCA* mutation improves colorectal cancer classification and cetuximab treatment prediction. Gut 2013; 62(4): 540–549.
10. Van Krieken JH, Rouleau E, Ligtenberg MJ et al. *RAS* testing in metastatic colorectal cancer: advances in Europe. Virchows Arch 2016; 468(4): 383–396.
11. Azuara D, Ginesta MM, Gausachs M et al. Nanofluidic digital PCR for *KRAS* mutation detection and quantification in gastrointestinal cancer. Clin Chem 2012; 58(9): 1332–1341.
12. Azuara D, Santos C, Lopez-Doriga A et al. Nanofluidic digital PCR and extended genotyping of *RAS* and *BRAF* for improved selection of metastatic colorectal cancer patients for anti-EGFR therapies. Mol Cancer Ther 2016; 15(5): 1106–1112.
13. Santos C, Azuara D, Garcia-Carbonero R et al. Optimization of *RAS*/*BRAF* mutational analysis confirms improvement in patient selection for clinical benefit to anti-EGFR treatment in metastatic colorectal cancer. Mol Cancer Ther 2017; 16(9): 1999–2007.
14. Glynne-Jones R, Wyrwicz L, Tiret E et al. Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2017; 28(Suppl 4): iv22–iv40.
15. Laurent-Puig P, Pekin D, Normand C et al. Clinical relevance of *KRAS*-mutated subclones detected with picodroplet digital PCR in advanced colorectal cancer treated with anti-EGFR therapy. Clin Cancer Res 2015; 21: 1087–1097.
16. Van Cutsem E, Lenz HJ, Kohne CH et al. Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and *RAS* mutations in colorectal cancer. JCO 2015; 33(7): 692–700.
17. Van Cutsem E, Kohne CH, Lang I et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor *KRAS* and *BRAF* mutation status. JCO 2011; 29(15): 2011–2019.
18. Normanno N, Rachiglio AM, Lambiase M et al. Heterogeneity of *KRAS*, *NRAS*, *BRAF* and *PIK3CA* mutations in metastatic colorectal cancer and potential effects on therapy in the CAPRI GOIM trial. Ann Oncol 2015; 26(8): 1710–1714.
19. Diaz LA Jr, Williams RT, Wu J et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 2012; 486: 537–540.
20. Dienstmann R, Elez E, Argiles G et al. Analysis of mutant allele fractions in driver genes in colorectal cancer - biological and clinical insights. Mol Oncol 2017; 11(9): 1263–1272.
21. Morelli MP, Overman MJ, Dasari A et al. Characterizing the patterns of clonal selection in circulating tumor DNA from patients with colorectal cancer refractory to anti-EGFR treatment. Ann Oncol 2015; 26(4): 731–736.
22. Vidal J, Muinelo L, Dalmases A et al. Plasma ctDNA *RAS* mutation analysis for the diagnosis and treatment monitoring of metastatic colorectal cancer patients. Ann Oncol 2017; 28(6): 1325–1332.
23. Tejpar S, Stintzing S, Ciardiello F et al. Prognostic and predictive relevance of primary tumor location in patients with *RAS* wild-type metastatic colorectal cancer: retrospective analyses of the CRYSTAL and FIRE-3 trials. JAMA Oncol 2017; 3(2): 194–201.
24. Misale S, Di Nicolantonio F, Sartore-Bianchi A et al. Resistance to anti-EGFR therapy in colorectal cancer: from heterogeneity to convergent evolution. Cancer Discov 2014; 4(11): 1269–1280.
25. Sadanandam A, Lyssiotis CA, Homicsko K et al. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. Nat Med 2013; 19(5): 619–625.
26. Cremolini C, Morano F, Moretto R et al. Negative hyper-selection of metastatic colorectal cancer patients for anti-EGFR monoclonal antibodies: the PRESSING case-control study. Ann Oncol 2017; 28(12): 3009–3014.