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ORIGINAL ARTICLE

Ultra-selection of metastatic colorectal cancer patients using next-generation sequencing to improve clinical efficacy of anti-EGFR therapy

J. Vidal[1], B. Bellosillo[2], C. Santos Vivas[3], P. García-Alfonso[4], A. Carrato[5], M. T. Cano[6], R. García-Carbonero[7], E. Élez[8], F. Losa[9], B. Massutí[10], M. Valladares-Ayerbes[11†], J. M. Viéitez[12‡], J. L. Manzano[13], D. Azuara[3], J. Gallego[14], S. Pairet[2], G. Capellá[15], R. Salazar[3], J. Tabernero[8], E. Aranda[6] & C. Montagut[1*]

[1]Medical Oncology Department, Hospital del Mar-IMIM, CIBERONC Instituto de Salud Carlos III, Barcelona; [2]Pathology Department, Hospital del Mar, Barcelona; [3]Translational Research Laboratory, Medical Oncology Department, Catalan Institute of Oncology (ICO), ICO-Bellvitge Biomedical Research Institute (IDIBELL)-CIBERONC Instituto de Salud Carlos III, L'Hospitalet de Llobregat, Barcelona; [4]Medical Oncology Department, Hospital Gregorio Marañón, Madrid; [5]Medical Oncology Department, Hospital Ramón y Cajal, IRYCIS, CIBERONC Instituto de Salud Carlos III, Alcala University, Madrid; [6]Medical Oncology Department, IMIBIC, Reina Sofía Hospital, University of Cordoba, CIBERONC Instituto de Salud Carlos III; [7]Oncology Department, Hospital Universitario 12 de Octubre, Instituto de Investigación Sanitaria Hospital 12 de Octubre, (imas12), UCM, CNIO, CIBERONC Instituto de Salud Carlos III, Madrid; [8]Medical Oncology Department, Vall d'Hebron University Hospital and Institute of Oncology (VHIO), CIBERONC Instituto de Salud Carlos III, Universitat Autònoma de Barcelona, Barcelona; [9]Medical Oncology Department, Hospital Sant Joan Despí - Moisés Broggi, Barcelona; [10]Medical Oncology Department, Hospital General Universitario, Alicante; [11]Medical Oncology Department, Complejo Hospitalario Universitario de A Coruña, A Coruña; [12]Medical Oncology Department, Hospital Universitario Central de Asturias, Oviedo; [13]Medical Oncology Department, ICO, Badalona, Barcelona; [14]Medical Oncology Department, Hospital General Universitario de Elche, Alicante; [15]Faculty of Medicine, Department of Clinical Sciences, Translational Research Laboratory, ICO-IDIBELL, L'Hospitalet de Llobregat, University of Barcelona, CIBERONC Instituto de Salud Carlos III, Barcelona, Spain

Correspondence to: Dr Clara Montagut, Medical Oncology Department, Hospital del Mar, Passeig Marítim 25-29, 08003 Barcelona, Spain. Tel: +34-932483137; E-mail: cmontagut@parcdesalutmar.cat

[†]Present address: Medical Oncology Department, IMIBIC, Reina Sofía Hospital University of Cordoba, CIBERONC Instituto de Salud Carlos III Spain.
[‡]Present address: MD Anderson Cancer, Madrid, Spain.

Background: Extended *RAS* analysis is mandatory in metastatic colorectal cancer (mCRC) patients. The optimal threshold of *RAS* mutated subclones to identify patients most likely to benefit from antiepidermal growth factor receptor (EGFR) therapy is controversial. Our aim was to assess the clinical impact of detecting mutations in *RAS*, *BRAF*, *PIK3CA* and $EGFR^{S492R}$ in basal tissue tumour samples by using a highly sensitive next-generation sequencing (NGS) technology in mCRC patients treated with chemotherapy plus anti-EGFR or anti-vascular endothelial growth factor.

Patients and methods: Five hundred and eighty-one tumour samples from untreated mCRC patients from 7 clinical studies were collected. Mutational analysis was carried out by standard-of-care (therascreen pyro) with a sensitivity detection of 5% mutant allele fraction (MAF), and compared with NGS technology using 454GS Junior platform (Roche Applied Science, Germany) with a sensitivity of 1%. Molecular results were correlated with clinical outcomes.

Results: After quality assessment, 380 samples were evaluable for molecular analysis. Standard-of-care mutational analysis detected *RAS*, $BRAF^{V600E}$ or *PIK3CA* mutations in 56.05% of samples compared with 69.21% by NGS ($P = 0.00018$). NGS identified coexistence of multiple low-frequency mutant alleles in 96 of the 263 mutated cases (36.5%; range 2–7). Response rate (RR), progression-free survival (PFS) and overall survival (OS) were increasingly improved in patients with *RAS* wild-type, *RAS/BRAF* wild-type or quadruple (*KRAS/NRAS/BRAF/PIK3CA*) wild-type tumours treated with anti-EGFR, assessed by standard-of-care. No additional benefit in RR, PFS or OS was observed by increasing the detection threshold to 1% by NGS. An inverse correlation between the MAF of the most prevalent mutation detected by NGS and anti-EGFR response was observed ($P = 0.039$). $EGFR^{S492R}$ mutation was not detected in untreated samples.

Conclusions: No improvement in the selection of patients for anti-EGFR therapy was obtained by adjusting the mutation detection threshold in tissue samples from 5% to 1% MAF. Response to anti-EGFR was significantly better in patients with quadruple wild-type tumours.

Key words: colorectal cancer, anti-EGFR, NGS, RAS mutations, mutant allele fraction, quadruple wild-type

Introduction

The addition of targeted biological therapies to conventional chemotherapy for metastatic colorectal cancer (mCRC) resulted in a significant improvement in efficacy results. Thus, monoclonal antibodies (mAbs) that inhibit the activation of the epidermal growth factor receptor (EGFR)—cetuximab and panitumumab—and the vascular endothelial growth factor—bevacizumab—are currently part of the standard treatment of mCRC. Extended *RAS* analysis is mandatory in mCRC patients to refine the most appropriate population to be treated with anti-EGFR therapy [1].

Despite biomarker selection of patients for these therapies, the efficacy of cetuximab or panitumumab in routine clinical practice has demonstrated to be variable and limited. Therefore, other potential strategies to increase the benefit from anti-EGFR therapies are needed. One of these strategies is based on the validation of other predictive biomarkers of response beyond *RAS* such as *BRAF, PIK3CA* and *EGFR* extracellular domain (ECD). Currently, the mutational status of *BRAF* is recommended as prognostic biomarker [2] but conflicting data exist regarding whether genomic alterations in *BRAF* are true biomarkers for anti-EGFR resistance. Previous studies indicate that *PIK3CA* mutations can be related with primary resistance to anti-EGFR mAbs [3]. Taken together, a higher response rate (RR) to anti-EGFR treatment has been shown in *RAS*, *BRAF* and *PIK3CA* wild-type tumours [4, 5]. However, results are contradictory and *PIK3CA* mutations have not been introduced in routine clinical practice. Finally, mutations in the *EGFR* ECD have been described as a mechanism of acquired resistance to anti-EGFR therapy [6, 7] but its role in primary resistance has not been ascertained yet [8].

Another strategy to improve patients' selection to anti-EGFR treatment is the use of highly sensitive methods able to detect the presence of DNA mutations even when they are uncommon. Pyrosequencing is nowadays widely used for the determination of *RAS* mutations in the clinics with a cut-off sensitivity between 2.3% and 5% [9] but alternative techniques with a higher sensitivity such as next-generation sequencing (NGS) are available. There is a need to establish the optimal threshold of *RAS* mutant allele fraction (MAF) to identify mCRC patients most likely to benefit from anti-EGFR treatment.

The aim of the present study was to assess the clinical relevance of highly sensitive NGS to detect point mutations in *KRAS* (exons 2, 3 and 4), *NRAS* (exons 2, 3 and 4), $BRAF^{V600E}$, $EGFR^{S492R}$ and *PIK3CA* in tissue tumour samples from mCRC patients.

Materials and methods

Study design

A retrospective analysis was carried out with formalin-fixed paraffin-embedded untreated tumour samples at diagnosis from mCRC patients from five Spanish clinical trials and two Spanish retrospective series treated with anti-EGFR therapy or bevacizumab recruited between February 2003 and September 2014 (see clinical trials identifiers and regulatory aspects in supplementary material, available at *Annals of Oncology* online).

Tumour tissue analysis and mutational assessment

DNA from tumour tissue was extracted from two sections of 15 µm using the QIAamp DNA Mini kit (Qiagen). The cases in which no fragments larger than 200 bp were obtained were considered of insufficient quality to perform NGS analysis.

Mutations in exons 2, 3 and 4 from *KRAS* and *NRAS*, *PIK3CA* and exon 12 of *EGFR* were analysed by NGS in a 454 GS Junior platform (Roche Applied Science) (details in supplementary material, available at *Annals of Oncology* online). Therascreen pyro sequencing was carried out according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Statistical analysis

Chi-square or Fisher's exact tests were used to compare the overall RR (ORR) between wild-type and mutated patients and other comparisons done for categorical variables. MAF levels according to overall response were compared using Kruskal–Wallis test for non-parametric data. Cox proportional regression models were used to compare progression-free survival (PFS) and overall survival (OS) according to mutational status evaluated through different techniques adjusting for age, gender and chemotherapy line. Data analysis was carried out using IBM SPSS Statistics v.21.

Results

Patient clinical and treatment characteristics

As a whole, treatment naïve tumour samples of 581 patients were collected from 7 studies. Tumour specimens were analysed for *RAS/BRAF/PIK3CA* mutations by therascreen pyro as standard-of-care. Among 581 samples, 443 DNA samples had adequate quality to be assessed by NGS. Sufficient coverage to evaluate the mutational *RAS/BRAF/PIK3CA* status was obtained in 380 patients (supplementary Figure S1, available at *Annals of Oncology* online): 161 treated with anti-EGFR and 215 patients treated with bevacizumab. Mutations in $EGFR^{S492R}$ were also analysed by NGS. Baseline clinical and treatment characteristics of the assessable population ($N = 380$) was broadly similar to those of the subset of patients treated with an anti-EGFR based therapy (Table 1).

Mutational profiling of tumour samples

In the assessable population, there were significant differences in the proportion of patients with an *RAS*, $BRAF^{V600E}$ or *PIK3CA* mutated tumour detected through both sequencing platforms ($P = 0.00018$). Using standard-of-care therascreen pyro sequencing technique, mutations in *RAS*, $BRAF^{V600E}$ or *PIK3CA* were detected in 213 of the 380 samples (56.05%). Analysis by NGS increased the number of detected mutations up to 263 (69.21%) (supplementary Figure S2, available at *Annals of Oncology*

Table 1. Demographic and clinical data

	Total population *N* = 581	Assessable patients *N* = 380	Patients treated with anti-EGFR *N* = 161
Age, median [range]	65 [21–88]	65 [32–88]	64 [32–88]
Gender, male, *n* (%)	383 (65.9)	243 (63.9)	114 (70.8)
Tumour location, *n* (%)			
Right	123 (21.1)	81 (21.4)	29 (18.0)
Left	55 (9.4)	36 (9.4)	19 (11.8)
Transversal	46 (7.9)	27 (7.1)	6 (3.7)
Rectum	218 (37.5)	140 (36.8)	56 (34.8)
Not specified	139 (23.9)	95 (25)	51 (31.7)
Stage, *n* (%)			
I + II	50 (8.6)	31 (8.2)	17 (10.5)
III	137 (23.6)	89 (23.4)	37 (23.0)
IV	381 (65.6)	253 (66.6)	103 (64.0)
Unknown	13 (2.3)	7 (1.8)	4 (2.5)
Location M1, *n* (%)			
Liver	390 (67.1)	256 (67.4)	107 (66.5)
Lymph node	97 (16.7)	67 (17.6)	31 (19.3)
Peritoneum	97 (16.9)	63 (16.6)	26 (16.1)
Lung	193 (33.2)	128 (33.7)	44 (27.3)
Others	87 (15)	57 (15)	23 (14.3)
Treatment line, *n* (%)			
First line	390 (67.2)	257 (67.6)	71 (44.1)
Second line	98 (16.9)	60 (15.8)	44 (27.3)
≥Third line	50 (8.6)	37 (9.7)	21 (13.0)
Unknown	43 (7.4)	26 (6.8)	25 (13.0)
Biological treatment, *n* (%)			
Anti-EGFR	253 (43.5)	161 (42.4)	161 (100–0)
Bevacizumab	324 (55.8)	215 (56.6)	–
Other	4 (0.7)	4 (1)	–

online). NGS identified multiple additional low-frequency mutant alleles in the same sample in 96 of the 263 mutated cases (36.5%; range 2–7) (see Clinical characteristics and treatment outcome in supplementary Table S3, available at *Annals of Oncology* online). In four $BRAF^{V600E}$ mutant cases, coexistence of low-frequency *RAS* mutations (MAF <5%) were detected; two of them harboured three *RAS* concomitant mutations ($KRAS^{G13D}$, $NRAS^{Q61R}$ and $NRAS^{Q61K}$ in one case, $KRAS^{G12D}$, $NRAS^{G12D}$ and $NRAS^{G13S}$ in the other case).

The percentage of mutant alleles of the most prevalent mutation in each patient by NGS ranged from 1.06% to 78.25%, and varied according to each specific mutation. *KRAS* exon 2 mutations showed the highest median MAF (23.88%, range 1.06%–73.40%), followed by mutations in *KRAS* exons 3–4 (19.2%, range 1.48%–78.25%), $BRAF^{V600E}$ (18.88%, range 2.48%–44.69%), *NRAS* [14.3% (range 1.37%–41.88%) and 15.5% (range 1.23%–54.50%) for exon 2 and exons 3 and 4, respectively], and *PIK3CA* with the lowest MAF (3.7%, range 1.28%–26.12%) (supplementary Figure S3, available at *Annals of Oncology* online). No mutations were detected by NGS in $EGFR^{S492R}$.

RR according to the mutational profile

Radiological tumour response was evaluable in 137 out of 161 patients treated with anti-EGFR therapy (85.09%). Complete response (CR) or partial response (PR) occurred in 51 (31.68%) patients (4 CRs, 47 PRs). Stable disease (SD) and progressive disease (PD) were observed in 55 (40.15%) and 31 (22.63%) patients, respectively.

RR to anti-EGFR treatment was evaluated according to mutational status profile as identified by standard-of-care therascreen pyro test (Table 2). *KRAS* exon 2 wild-type population presented an RR of 34.8% versus 5.9% for *KRAS* exon 2 mutant population ($P = 0.014$). Determination of mutations in all *RAS* (*KRAS* and *NRAS* exons 2–4) and all $RAS/BRAF^{V600E}$ increased the RR up to 37.5% and 40.6% in wild-type [versus 18.4% ($P = 0.016$) and 16.7% ($P = 0.002$) for population with mutated tumours], respectively. When considering all mutations (all$RAS/BRAF^{V600E}$/*PIK3CA*), RR was 41.5% in quadruple wild-type tumour patients versus 17.9% in the population with mutated samples ($P = 0.004$).

On the other hand, highly sensitive NGS analysis detected 30 additional *KRAS* exon2 mutated cases (16 of which responded to anti-EGFR), one extended *RAS* mutated tumour and one *PIK3CA* mutated sample in tumours treated with anti-EGFR therapy. However, RR of patients treated with anti-EGFR was not significantly different in wild-type versus *KRAS* exon 2, all *RAS* or all $RAS/BRAF^{V600E}$ mutated tumour as detected by NGS analysis. Differences in RR reached significance in quadruple wild-type tumours by NGS sequencing (42.4% quadruple wild-type versus 24.2% mutated; $P = 0.015$) (Table 2).

Table 2. Overall response rate in patients treated with anti-EGFR therapy ($n = 161$)

		Therascreen pyro				NGS 454 GS junior			
		WT		MUT		WT		MUT	
		n	ORR %	*n*	ORR %	*n*	ORR %	*n*	ORR %
KRAS 2	CR	4	**34.8**	0	**5.9**	4	**31.3**	0	**32.7**
	PR	46		1		31		16	
	SD	49		6		38		17	
	PD	26		5		22		9	
	Unk	19		5		17		7	
	P	0.014				0.860			
All *RAS*	CR	4	**37.5**	0	**18.4**	3	**35.4**	1	**28.1**
	PR	38		9		25		22	
	SD	35		20		25		30	
	PD	18		13		12		19	
	Unk	17		7		14		10	
	P	0.016				0.313			
All *RAS* or *BRAF*	CR	4	**40.6**	0	**16.7**	3	**37.8**	1	**26.4**
	PR	37		10		25		22	
	SD	33		22		24		31	
	PD	14		17		9		22	
	Unk	13		11		13		11	
	P	0.002				0.121			
All *RAS* or *BRAF* or *PiK3CA*	CR	4	**41.5**	0	**17.9**	3	**42.4**	1	**24.2**
	PR	35		12		25		22	
	SD	30		25		20		35	
	PD	13		18		6		25	
	Unk	12		12		12		12	
	P	0.004				0.015			

WT, wild-type; MUT, mutated; n, number of patients for each subgroup; ORR, overall response rate; CR, complete response; PR, partial response; SD, stable disease; PD, progression disease; Unk, unknown.

An inverse correlation between the MAF of the most prevalent mutation as detected by NGS and anti-EGFR response was observed ($P = 0.039$). The median MAF for responders (CR or PR) was 0.55% and 0.77%, respectively. In patients with SD, the median MAF was slightly higher (median MAF 2.20%), and it significantly increased in patients with PD (median MAF 11.29%) (Figure 1).

RR was also evaluable in 193 of the 215 patients treated with bevacizumab (89.77%). CR or PR occurred in 84 (43.52%) patients (10 CR, 74 PR). SD and PD were observed in 82 (42.49%) and 27 (13.99%) patients, respectively. In patients treated with bevacizumab, the RR was not associated with *RAS*, *BRAF*, and/or *PIK3CA* mutational status and neither with the MAF of the most prevalent mutation detected by NGS ($P = 0.474$) (Figure 1 and supplementary Table S1, available at *Annals of Oncology* online).

PFS and OS according to the mutational profile

A significant benefit in both PFS and OS was observed in patients treated with anti-EGFR therapy and wild-type tumours as assessed by standard-of-care therascreen pyro, except when considering *KRAS* exon 2 mutations alone in the analysis (Table 3 and Figure 2).

As for NGS analysis, the risk of disease progression and death were significantly lower in the wild-type population only for patients with quadruple-negative tumours (HR =0.67, $P = 0.033$ for PFS; HR =0.59, $P = 0.023$ for OS) (Table 3 and Figure 2). No significant differences were found when *KRAS* exon 2, all *RAS* or *RAS/BRAF*^{V600E} mutations were taken into consideration ($P = 0.540$, 0.315, 0.204 for PFS; $P = 0.902$, 0.085, 0.163 for OS, respectively). An inverse correlation between the MAF of the most prevalent mutation as detected by NGS and anti-EGFR PFS was observed (HR: 1.013, 95% CI 1.001–1.024; $P = 0.031$) (supplementary Figure S4, available at *Annals of Oncology* online).

In patients treated with bevacizumab, mutations in *RAS*, *BRAF*^{V600E} or *PIK3CA* were not associated with PFS, independently of therascreen pyro or NGS analysis. However, patients with *KRAS* exon2 wild-type tumours, all*RAS* wild-type, all*RAS*/*BRAF*^{V600E} wild-type and quadruple wild-type by therascreen pyro had higher OS when treated with bevacizumab compared with mutant tumour patients [HR = 0.67 (95% CI 0.49–0.91), $P = 0.011$; HR = 0.68 (95% CI 0.49–0.95), $P = 0.022$; HR = 0.61 (95% CI 0.43–0.85), $P = 0.004$; HR = 0.58 (95% CI 0.41–0.82),

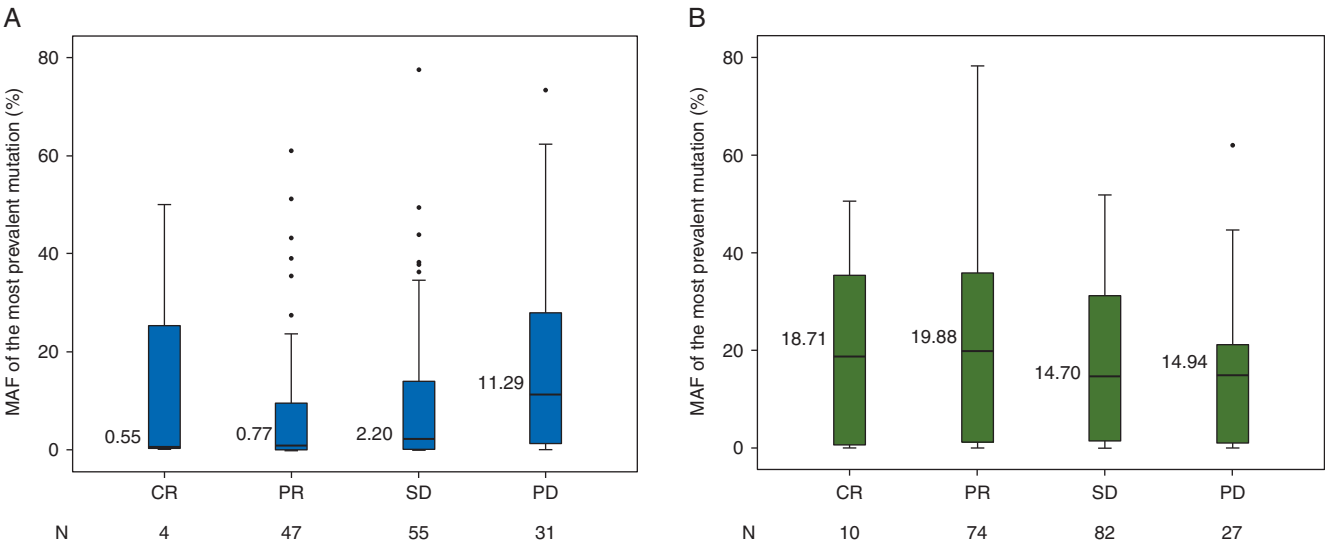

Figure 1. Correlation between MAF detected by NGS and RR to treatment. Box plot representing the correlation between the proportion of major mutant alleles and the best response to treatment. (A) Patients treated with anti-EGFR ($N = 137$) and (B) patients treated with bevacizumab ($N = 193$). MAF, mutant allele fraction; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. The median percentage of mutated alleles in each group is shown by a line. Tumours wild-type are included as MAF=0.

$P = 0.002$, respectively]. No significant differences in OS were found in wild-type versus mutated tumours as assessed by NGS in patients treated with bevacizumab (supplementary Table S2, available at *Annals of Oncology* online).

Discussion

In this work, we aimed to determine the optimal cut-off of mutant detection in untreated tissue samples to predict response to anti-EGFR therapy in mCRC patients.

The molecular profiling of CRC tumours analysed by therascreen pyro reported in the present study is similar to the literature and public databases [10]. As expected, higher rates of mutated tumours were detected by NGS, identifying additional mutations in 13.16% of samples. A major advantage of NGS over traditional mutation detection methods is the ability to simultaneously screen for a panel of mutations. In our study, NGS was able to detect multiple low-frequency mutant alleles in 36.5% of the mutated samples, and identified concomitant low MAF *RAS* and $BRAF^{V600E}$ mutations in four cases. Traditionally, *RAS* and *BRAF* mutations are considered to be mutually exclusive in untreated samples [10], and coexistence of multiple mutations has been described in the setting of acquired resistance to anti-EGFR therapy [7]. Our finding is consistent with the concept of tumour heterogeneity and the coexistence of several sub-clones within the same tumour lesion.

Clinical validation of the predictive value of other EGFR downstream effectors, including *BRAF* and *PIK3CA* mutations, has shown controversial results. In the current study, the best clinical benefit to anti-EGFR therapy was observed in the subgroup of patients with quadruple wild-type tumours. In this molecularly defined population, RR was doubled (41% versus 18%) and the risk of progression and death was significantly decreased to half compared with patients with all$RAS/BRAF^{V600E}/PIK3CA$ mutated tumours (HR 0.52 and 0.39 for PFS and OS, respectively). Notably, clinical outcomes were very similar in all $RAS/BRAF^{V600E}$ wild-type tumours without taking into consideration *PI3KCA* mutations, suggesting a marginal predictive and prognostic role for *PIK3CA* mutations.

Important efforts have been made to define the best MAF cut-off to predict response to anti-EGFR therapy, delivering the best balance between sensitivity and specificity. *RAS* mutational status post hoc analysis of pivotal clinical anti-EGFR trials used sequencing technologies with a sensitivity of 5%–10%. In patients treated with first-line panitumumab plus FOLFOX, an HR for PFS of 0.72 ($P = 0.004$) was detected in *RAS* wild-type tumours by Sanger sequencing and WAVE-based Surveyor Scan Kits (Transgenomic) [11]. In patients treated with first-line cetuximab plus FOLFIRI, analysis of *RAS* mutations by BEAMing with a 5% cut-off showed an HR for PFS of 0.56 ($P < 0.001$) in patients with *RAS* wild-type tumours [12]. Recently, two studies explored the role of dPCR to establish the lower limit of detection of mutations [13, 14]. Laurent-Puig *et al.* analysed *KRAS* and *BRAF* status from 136 wild-type tumours and identified a threshold of 1% of mutant sub-clones as the best predictive value of response. Similar results were obtained by Santos *et al.* in a cohort of 255 patients treated with anti-EGFR therapy analysed by extended *RAS* and $BRAF^{V600E}$. In our study, 50 samples previously classified as *RAS/BRAF/PIK3CA* wild-type were switched to mutant by increasing the threshold of sensitivity detection from 5% to 1% with NGS technology. Contrary to previous results, this did not result in a better prediction of response, and suggests that low abundance of *RAS* mutant alleles does not lead to resistance to anti-EGFR treatment. Interestingly, we observed an inverse correlation between the proportion of mutated DNA alleles and response to anti-EGFR therapy. This supports that cancer does not display a uniform biological mutational pattern and suggests that tumours harbouring low-frequency mutations may respond to anti-EGFR therapy.

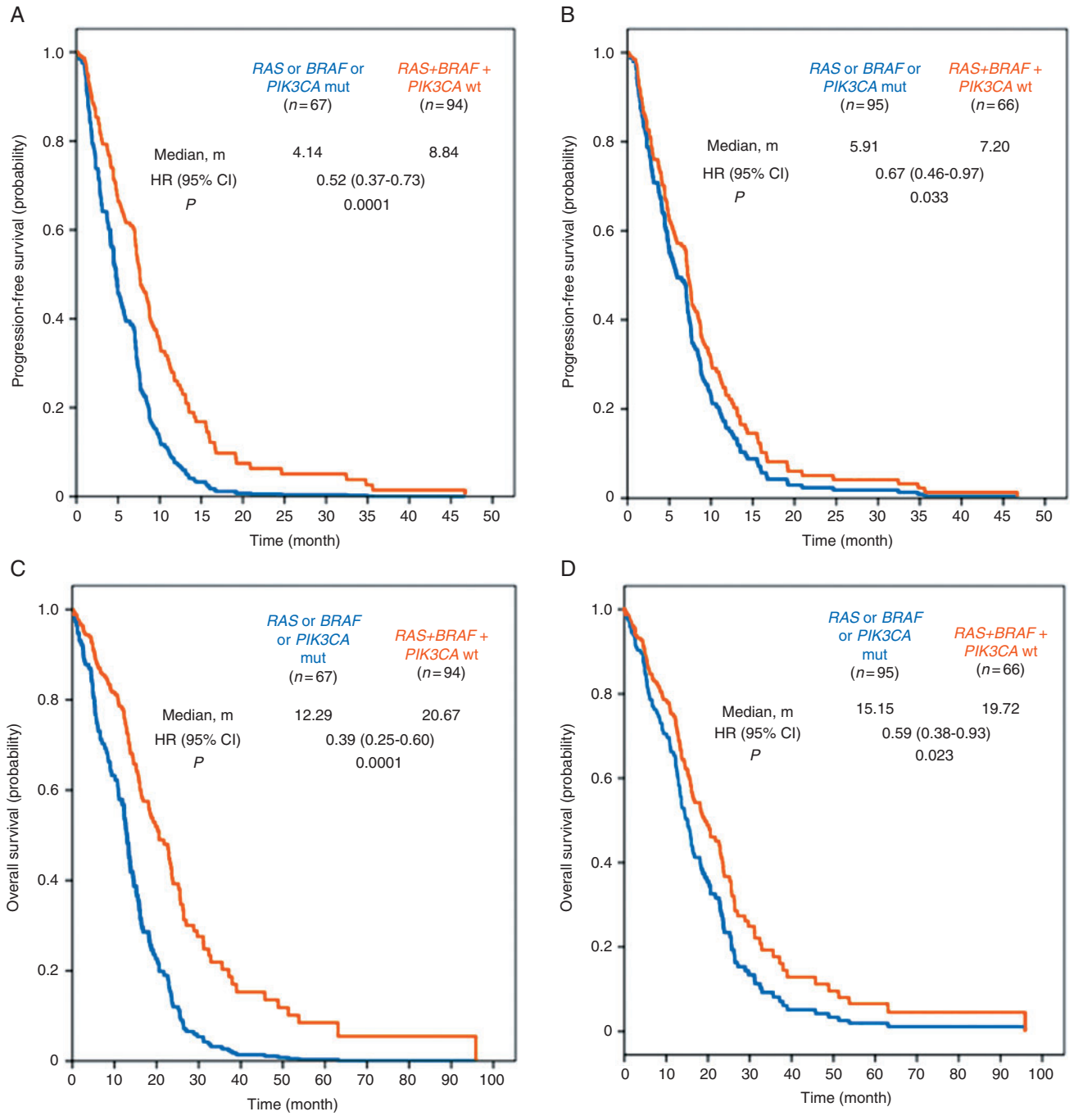

Figure 2. Progression-free survival (PFS) and overall survival (OS) in patients treated with anti-EGFR according to mutational status analysed by therascreen pyro and NGS ($N = 161$). Kaplan–Meier analysis for PFS and OS of patients treated with anti-EGFR therapy adjusted for age, gender and chemotherapy line. (A) PFS in wild-type versus *RAS/BRAF*^{V600E}*/PIK3CA*-mutated populations analysed by therascreen pyro. (B) PFS in wild-type versus *RAS/BRAF*^{V600E}*/PIK3CA*-mutated populations analysed by NGS. (C) Overall survival in wild-type versus *RAS/BRAF*^{V600E}*/PIK3CA*-mutated populations analysed by therascreen pyro. (D) Overall survival in wild-type versus *RAS/BRAF*^{V600E}*/PIK3CA*-mutated populations analysed by NGS. CI, confidence interval; HR, hazard ratio; mut, mutated; wt, wild-type.

Of note, the benefit of anti-EGFR treatment was lower than previously reported in the pivotal studies of first-line chemotherapy plus panitumumab or cetuximab [11, 12]. A potential explanation is that only 44.1% of patients in the current study received anti-EGFR mAbs in a first-line setting. Moreover, 13 frail elderly patients treated with single-agent panitumumab were included.

It has been previously reported that bevacizumab efficacy is independent of *RAS* and *PIK3CA* mutations [15, 16]. We confirmed that the mutational status of *RAS*, *BRAF* or *PIK3CA* is not a predictor of response to bevacizumab, but is a prognostic factor with an HR for OS of 0.58 [95% CI, (0.41–0.82), $P = 0.002$] in patients with quadruple wild-type tumours.

Table 3. Progression-free survival and overall survival in patients treated with anti-EGFR therapy ($N = 161$)

			Therascreen pyro		NGS 454 GS junior	
			WT	MUT	WT	MUT
KRAS 2	PFS months	*N*	144	17	112	49
		Median	7.10	4.14	5.82	7.92
		HR (95% CI)	0.78 (0.45–1.33)		1.13 (0.77–1.66)	
		P	0.354		0.540	
	OS months	*N*	144	17	112	49
		Median	16.30	12.59	15.15	20.24
		HR (95% CI)	0.69 (0.38–1.25)		0.97 (0.62–1.52)	
		P	0.224		0.902	
All RAS	PFS months	*N*	112	49	79	82
		Median	7.20	4.44	6.97	7.03
		HR (95% CI)	**0.59** (0.40–0.87)		0.83 (0.58–1.19)	
		P	0.007		0.315	
	OS months	*N*	112	49	79	82
		Median	18.40	12.59	16.30	15.74
		HR (95% CI)	**0.52** (0.33–0.82)		0.69 (0.45–1.05)	
		P	0.005		0.085	
All RAS or *BRAF*	PFS months	*N*	101	60	74	87
		Median	7.92	4.11	7.03	6.97
		HR (95% CI)	**0.48** (0.33–0.69)		0.79 (0.55–1.14)	
		P	<0.001		0.204	
	OS months	*N*	101	60	74	87
		Median	20.24	12.29	16.43	15.18
		HR (95% CI)	**0.45** (0.29–0.70)		0.74 (0.48–1.31)	
		P	0.0001		0.163	
All RAS or *BRAF* or *PiK3CA*	PFS months	*N*	94	67	66	95
		Median	8.84	4.14	7.20	5.91
		HR (95% CI)	**0.52** (0.37–0.73)		**0.67** (0.46–0.97)	
		P	0.0001		0.033	
	OS months	*N*	94	67	66	95
		Median	22.67	12.29	19.72	15.15
		HR (95% CI)	**0.39** (0.25–0.60)		**0.59** (0.38–0.93)	
		P	0.0001		0.023	

Note: Adjusted for age, gender and chemotherapy line.
HR, hazard ratio; mut, mutated; *N*, number of patients for each subgroup; OS, overall survival; PFS, progression-free survival; wt, wild-type.
In bold HR for OS and PFS statistically significant.

We also aimed to explore $EGFR^{S492R}$ mutations as a predictive biomarker for anti-EGFR response. *EGFR ECD* mutations have been described as an acquired mechanism of resistance to anti-EGFR treatment [6, 7], and there is clinical evidence that molecular factors driving secondary resistance may emerge from a selection of a pre-existing sub-population of mutant cells [17]. This study confirms previous results that $EGFR^{S492R}$ mutation is not detected in the tumour before treatment at least when employing 1% sensitivity [6, 8].

Overall, the results of this study show that increasing the threshold of $RAS/BRAF^{V600E}$ testing up to 1% by NGS technique does not improve patient selection for anti-EGFR therapy in mCRC. These data support the use of a 5% MAF cut-off in tumour tissue samples as the standard-of-care in mCRC.

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Disclosure

JV is an advisory board member for Amgen, Merck-Serono and is a speaker for Amgen, Merck-Serono and Sanofi. EE has received payment for advisory role from Amgen, Merck-Serono, Sanofi, Roche and Servier, research grant from Merck-Serono, travel grant from Roche, Sanofi, Merck-Serono and Sanofi. FL has received honoraria for advisory role from Amgen, Merck y Sanofi. MVA is an advisory board member for Amgen, Merck-Serono, Sanofi, and is a speaker for Amgen, Roche, Merck-Serono, Sanofi, Servier, Bristol-Myers, and has received commercial research grant from Roche. JMV has received payment for advisory role from Roche, Amgen, Bayer, Shire and Servier, honoraria from Shire and Servier and research funding form Roche and Amgen. JG has received payment for advisory role from Amgen and Merck, honoraria from Amgen and other remuneration from Amgen. JT reports personal financial interest in form of scientific consultancy role for Array Biopharma, AstraZeneca, Bayer, BeiGene, Boehringer Ingelheim, Chugai, Genentech Inc., Genmab A/S, Halozyme, Imugene Limited, Inflection Biosciences Limited, Ipsen, Kura Oncology, Lilly, MSD, Menarini, Merck Serono, Merrimack, Merus, Molecular Partners, Novartis, Peptomyc, Pfizer, Pharmacyclics, ProteoDesign SL, Rafael Pharmaceuticals, F. Hoffmann-La Roche Ltd, Sanofi, SeaGen, Seattle Genetics, Servier, Symphogen, Taiho, VCN Biosciences, Biocartis, Foundation Medicine, HalioDX SAS and Roche Diagnostics. EA has received honoraria for advisory role from Amgen, Bayer, Celgene, Merck, Roche, Sanofi. CM is an advisory board member for Amgen, Merck-Serono, Sanofi, Symphogen, is a speaker for Amgen and Merck-Serono, has received commercial research grant from Amgen, Merck-Serono and Symphogen.

All remaining authors have declared no conflicts of interest.

References

1. Van Cutsem E, Cervantes A, Adam R et al. ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. Ann Oncol 2016; 27(8): 1386–1422.
2. 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.
3. Sartore-Bianchi A, Martini M, Molinari F et al. PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. Cancer Res 2009; 69(5): 1851–1857.
4. De Roock W, Claes B, Bernasconi D et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 2010; 11(8): 753–762.
5. Tian S, Simon I, Moreno V et al. Combined oncogenic pathway signature of BRAF, KRAS and PI3KCA mutation improves colorectal cancer classification and cetuximab treatment prediction. Gut 2013; 62(4): 540–549.
6. Montagut C, Dalmases A, Bellosillo B et al. Identification of a mutation in the extracellular domain of the epidermal growth factor receptor conferring cetuximab resistance in colorectal cancer. Nat Med 2012; 18(2): 221–223.
7. Arena S, Bellosillo B, Siravegna G et al. Emergence of multiple EGFR extracellular mutations during cetuximab treatment in colorectal cancer. Clin Cancer Res 2015; 21(9): 2157–2166.
8. Esposito C, Rachiglio AM, La Porta ML et al. The S492R EGFR ectodomain mutation is never detected in KRAS wild-type colorectal carcinoma before exposure to EGFR monoclonal antibodies. Cancer Biol Ther 2013; 14(12): 1143–1146.
9. Tougeron D, Lecomte T, Pagès JC et al. Effect of low-frequency KRAS mutations on the response to anti-EGFR therapy in metastatic colorectal cancer. Ann Oncol 2013; 24(5): 1267–1273.
10. T. C. G. A. Network et al. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012; 487(7407): 330–337.
11. Douillard J-Y, Oliner KS, Siena S et al. Panitumumab–FOLFOX4 treatment and *RAS* mutations in colorectal cancer. N Engl J Med 2013; 369(11): 1023–1034.
12. Van Cutsem E, Lenz H-J, Köhne C-H et al. Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. J Clin Oncol 2015; 33(7): 692–700.
13. 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(5): 1087–1097.
14. 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.
15. Hurwitz HI, Yi J, Ince W et al. The clinical benefit of bevacizumab in metastatic colorectal cancer is independent of K-ras mutation status: analysis of a phase III study of bevacizumab with chemotherapy in previously untreated metastatic colorectal cancer. Oncologist 2009; 14(1): 22–28.
16. Price TJ, Bruhn MA, Lee CK et al. Correlation of extended RAS and PIK3CA gene mutation status with outcomes from the phase III AGITG MAX STUDY involving capecitabine alone or in combination with bevacizumab plus or minus mitomycin C in advanced colorectal cancer. Br J Cancer 2015; 112(6): 963–970.
17. Diaz LA, Williams RT, Wu J et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 2012; 486 (7404): 537–540.