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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

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Background: Mutations affecting *RAS* genes are now established predictive markers of nonresponse to anti-EGFR antibodies in advanced CRC. This analysis assessed the prognostic and predictive impact of extended *RAS* and *PIK3CA* gene mutation status in patients receiving capecitabine plus or minus bevacizumab (\pm mitomycin C) in the randomised phase III MAX study.

Methods: DNA was extracted from archival macrodissected formalin-fixed paraffin-embedded tumour tissue. Mutation status was determined using pyrosequencing, confirmed with Sanger sequencing (for equivocal *RAS*) and correlated with efficacy outcomes. Predictive analyses were undertaken using a test for interaction involving both C vs CB + CBM.

Results: Of the available 280 of the 471 (59.4%) patients, mutations in *KRAS* exons 2, 3 and 4 and *NRAS* 2, 3 and 4 were as follows: 32%, 2.9%, 2.2%, 1.4%, 0.7% and 0% (total *RAS* MT 39%). The *PIK3CA* MT rate was 7.5% exon 9 and 3.6% exon 20. Extended *RAS* gene mutation status (WT vs MT) had no prognostic impact for PFS (HR 0.91 (0.71–1.17)) or OS (HR 0.95 (0.71–1.25)). The *RAS* gene mutation status was not predictive of the effectiveness of bevacizumab for PFS (HR 0.56 (0.37–0.85) for *RAS* MT and HR 0.69 (0.5–0.97) for *RAS* WT; *P* for interaction 0.50). The *PIK3CA* mutation was neither predictive for bevacizumab effect nor prognostic.

Conclusion: Of *KRAS* exon 2 WT patients, 10% had additional *RAS* mutations. Neither all *RAS* gene mutation status nor *PIK3CA* mutation status was prognostic for PFS or OS, or predictive of bevacizumab outcome in patients with advanced CRC.

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The management of advanced colorectal cancer has improved significantly over recent years. Targeted or biological agents provide additional benefit to standard chemotherapy. The most important targets for biological agents in metastatic colorectal cancer (mCRC) are the epithelial growth factor receptor (EGFR) and the vascular endothelial growth factor-A (VEGF-A). With targeted agents there was hope that better patient selection would follow and this has been the case for anti-EGFR therapy, but it remains elusive for therapy targeting VEGF.

The *KRAS* gene exon 2 mutation is predictive of nonresponse to EGFR-targeted monoclonal antibody (MAb) therapy across all treatment lines, either as a single agent or in combination with irinotecan- or oxaliplatin-based chemotherapy (Amado *et al*, 2008; Karapetis *et al*, 2008; Bokemeyer *et al*, 2011; Van Cutsem *et al*, 2011). More recent evidence assessing the impact of additional RAS mutations (*KRAS* exons 3 and 4 and *NRAS* exons 1–4) has further defined the group of patients who do not respond to anti-EGFR agent. Douillard *et al* (2013) and Peeters *et al* (2014) have reported that additional RAS mutations occur in 17–18% of patients previously classified as *KRAS* WT and that this group also do not respond to anti-EGFR therapy. Importantly, the recently published results from the PEAK trial reported extra RAS mutations in 23% of *KRAS* exon 2 WT patients, highlighting the potential for significant clinical impact (Schwartzberg *et al*, 2014). Determination of extended RAS status is therefore now recommended in advanced CRC patients being considered for anti-EGFR-targeted therapy and current evidence suggests that patients with a mutated RAS should not receive such therapy.

Phosphatidylinositol 3-kinases (PI3K) are a family of three lipid kinases. Class 1A molecules are heterodimers of regulatory (p85) and catalytic (p110) subunits and in CRC the p110 α isoform is encoded by *PIK3CA* and is mutated in 15–18% of patients (De Roock *et al*, 2011; Karapetis *et al*, 2014). More than 80% of these mutations occur in exon 9 or 20, with exon 9 and exon 20 making up 60–65% and 20–25%, respectively. *PIK3CA* mutations can co-occur with RAS mutations. Mutations of *PIK3CA* are thought to be potentially predictive of anti-EGFR effect, although prediction of cetuximab activity is thought to be primarily associated with exon 20 mutations (Sartore-Bianchi *et al*, 2009; De Roock *et al*, 2010). The low frequency of exon 20 mutations (3–5% in total) however makes assessment of these results difficult to interpret and the impact of *PIK3CA* mutations on prognosis is still uncertain (Day *et al*, 2013; Karapetis *et al*, 2014). More importantly, the question of whether *PIK3CA* may have some role in tumour angiogenesis and therefore anti-VEGF therapy has been postulated. In this setting, a difference based on type of mutation is also unclear (Jiang and Liu, 2009).

Bevacizumab (anti-VEGF MAb) in combination with chemotherapy (single agent or combination) is an accepted standard of care for advanced colorectal cancer (Hurwitz *et al*, 2005; Saltz *et al*, 2008; Tebbutt *et al*, 2010). Unlike EGFR MAbs, there remains no reliable and reproducible predictive marker to assist in patient selection. In solid tumours, VEGF and EGFR pathways have been shown to interact effecting an increase in angiogenesis (Ellis, 2004; Ciardiello *et al*, 2006), although we have previously reported results from the MAX trial that failed to indicate any relationship between outcomes of anti-VEGF therapy and *KRAS* exon 2 and *BRAF* mutation status (Price *et al*, 2011). These results confirmed a previous but smaller retrospective analysis (Ince *et al*, 2005). Both of these analyses were based on *KRAS* exon 2 mutation status alone. With additional RAS mutation rates as high as 23%, there has been renewed interest in the potential interaction between the EGFR and VEGF pathway.

This analysis was performed to explore the rate of additional RAS mutations in a first-line population randomised within a trial focussing on anti-VEGF therapy and to further assess whether a change to an extended RAS mutation population has any impact

on the predictive and prognostic outcomes of extended RAS MT vs WT. In addition, we undertook an analysis of *PIK3CA* mutation status (exons 9 and 20 combined), exploring any interaction with progression-free survival (PFS), overall survival (OS) and response rate (RR) in patients receiving anti-VEGF therapy with bevacizumab when combined with capecitabine in the randomised phase III MAX study.

MATERIALS AND METHODS

Patients and study design. The MAX study design and eligibility criteria have been reported previously (Tebbutt *et al*, 2010). The primary objective of this phase III study was to evaluate the effect of adding bevacizumab with or without mitomycin to capecitabine on PFS among patients receiving first-line chemotherapy for their mCRC. Enrollment of patients in the original trial occurred between July 2005 and June 2007. Patients were randomly assigned to receive capecitabine (C); capecitabine and bevacizumab (CB); and capecitabine, bevacizumab and mitomycin C (CBM). All patients who participated in the MAX translational studies provided written informed consent at the time of study enrolment. Ethics approval for translational studies was obtained centrally.

Tumour collection and processing. Tissue consent and collection was performed at the time of trial commencement before this planned biomarker analysis. Formalin-fixed, paraffin-embedded samples of tumour tissue from archival specimens collected at the time of cancer diagnosis were retrieved from storage at hospital pathology departments. A histopathologist reviewed cases and if deemed to have <50% malignant crypts in the section, the tissue was macrodissected to ensure a high proportion (90%) of tumour cells. Assays of tissue samples for *KRAS* (exons 2, 3 and 4), *NRAS* (exons 2, 3 and 4) and *PIK3CA* (exons 9 and 20) mutations were performed by investigators who were blinded to patient outcomes. Patients were classified as mutated (MT) if a mutation was detected in any of the exons examined, or wild-type (WT) in the absence of any mutation, for RAS and *PIK3CA*, respectively.

Mutation analyses. Mutation analysis of RAS and *PIK3CA* genes was performed using genomic DNA extracted from formalin-fixed, paraffin-embedded tissue sections (QIAamp DNA FFPE Tissue Kit, Qiagen, Carlsbad, CA, USA) and pyrosequencing performed by the Perth node of the Australian Genome Research Facility (AGRF) using the PyroMark Q24 Pyrosequencing system (Qiagen). The sensitivity for this platform is 2% mutant to WT alleles; a cutoff of 10% was used to call mutations based on prior recommendations for pyrosequencing. Full details are described in Supplementary File online. Variant sequences were confirmed by Sanger sequencing using both forward and reverse primers (AGRF).

Statistical analysis. All randomly assigned patients for whom data on *KRAS*, *NRAS* and *PI3K* mutation status were available were included in this analysis. PFS, the primary end point, was defined as the time from randomisation until documented evidence of disease progression according to the Response Evaluation Criteria in Solid Tumours (RECIST) version 1.0, the occurrence of new disease or death from any cause. The secondary end points were OS, defined as the time from randomisation until death from any cause, and RR, defined according to RECIST version 1.0.

The PFS and OS of patients in each RAS group (MT vs WT) and treatment groups (C vs CB and CBM) were summarised using Kaplan–Meier curves, and the difference between these groups was compared using log-rank test, with the hazard ratio (HR) and its 95% confidence interval (CI) calculated from a Cox regression model with a single covariate. A Cox regression model with treatment covariate (C vs CB and CBM), RAS status and their

interaction was used to assess whether RAS was predictive of bevacizumab treatment efficacy. If no significant treatment interaction was detected, multivariate proportional hazards regression model was fitted to data for all patients, with RAS status and other protocol prespecified baseline covariates in the model to assess whether RAS was an independent prognostic factor.

We calculated the odds ratio (OR) and its 95% CI using univariate and multivariate logistic regression models to compute the difference in RR for the RAS group. A logistic regression model with treatment covariate, RAS status and their interaction was used to assess whether RAS was predictive of an effect of bevacizumab on RR.

The same methodologies were adopted in assessing the predictive and prognostic values of *PIK3CA*.

We also compared baseline characteristics of patients with and without tumour samples available for mutational analysis. Multivariate logistic regression model was also performed to identify predictors (if any) of tumour samples available for mutational analysis.

All reported *P*-values were two sided and were not adjusted for multiple testing.

RESULTS

Characteristics of the patients. Of the 471 patients who underwent randomisation, a total of 280 tumour specimens ($n=95$ from the C group, $n=99$ from the CB group and $n=86$ from the CBM group) were available and examined for RAS, accounting for 59.4% of the total study population (Figure 1). The median follow-up time of these patients was 30.2 months (range 0.4 to 42.4). Tumour specimens from the remaining patients could not be retrieved, were not suitable for mutational analysis or had insufficient DNA for mutational analysis to be performed for both *KRAS* and *NRAS*. The characteristics of patients with and without tissue sample available for mutational analysis were similar aside from a higher rate of primary resection and lower rate of residual local disease in the tissue available group (Supplementary Table 1 online), reflecting the amount of tissue available that will be greater in a resected primary specimen when compared with a colonoscopic or metastasis biopsy. In multivariate logistic regression of baseline

characteristics, prior resection of primary tumour (OR 6.98, $P<0.001$) and no prior radiotherapy (OR 0.47, $P<0.01$) were associated with having tumour specimens available for mutational analysis.

A total of 276 tumour specimens ($n=89$ from the C group, $n=97$ from the CB group and $n=90$ from the CBM group) were examined for *PIK3CA* mutation status, accounting for 58.6% of the total study population. The baseline characteristics were balanced between treatment groups in the tissue subpopulation (Supplementary Table 1).

The RAS mutation was detected in 108 (38.9%) patients. Mutation was detected in *KRAS* exon 2 ($n=89$), *KRAS* exon 3 ($n=8$), *KRAS* exon 4 ($n=5$), *NRAS* exon 2 ($n=4$), *NRAS* exon 3 ($n=2$) but none for *NRAS* exon 4. One patient had mutation in both *KRAS* exons 2 and 4. A total of 19 patients who were *KRAS* exon 2 WT had other RAS mutations (10%). *PIK3CA* mutation was rare and detected in only 30 patients (10.8%; exon 9, $n=20$; exon 20, $n=9$; exons 9 and 20, $n=1$).

Among the 262 patients with tumour specimens tested and results available for *KRAS*, *NRAS* and *PIK3CA* mutation status, 13 (5.0%) patients had both RAS and *PIK3CA* mutations, 16 (6.1%) RAS WT patients had *PIK3CA* mutation and 80 (30.5%) *PIK3CA* WT patients had RAS mutation.

Baseline characteristics by mutation status are summarised in Table 1. Clinical outcomes were comparable with no significant difference in primary or secondary clinical outcomes between the total study population and the patients who were evaluated for RAS mutations (Tables 2 and 3).

Progression-free survival. Among patients with RAS MT tumour, the median PFS was 6.2 months in the group receiving C and 8.8 months in the groups receiving CB or CBM. The HR for disease progression was 0.56 (95% CI, 0.37–0.86; $P=0.007$; Figure 2). Among patients with RAS WT tumour, the median PFS was 6.0 months in the group receiving C and 8.6 months in the groups receiving CB or CBM (HR 0.69; 95% CI, 0.49–0.97; $P=0.03$; Figure 2). The additional benefit of bevacizumab on PFS was not significantly greater among the patients with RAS WT tumours than among those with RAS MT tumours ($P=0.54$ for the interaction between RAS status and the assigned treatment).

For patients with *PIK3CA* MT tumours, the median PFS was 9.9 months in the group receiving C and 8.5 months in the groups

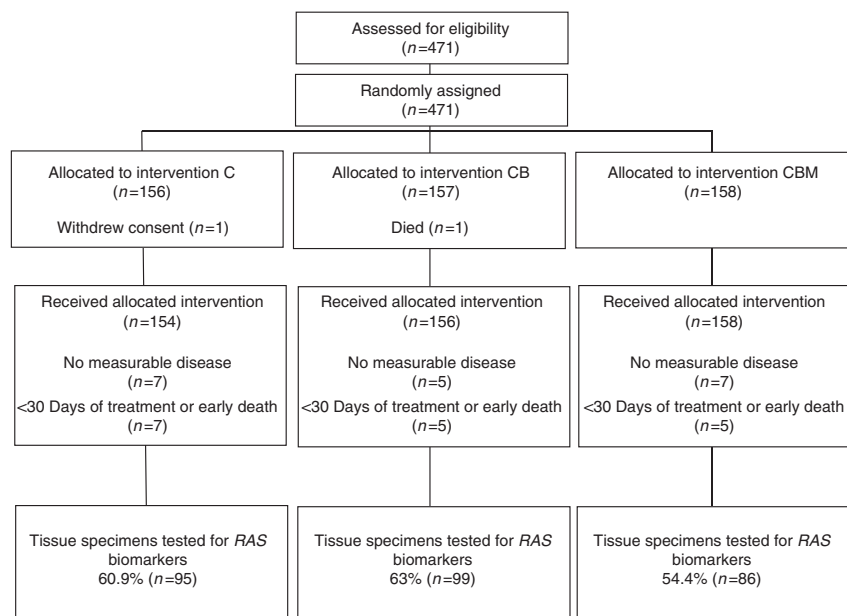


Figure 1. CONSORT diagram MAX AGITG TRIAL: RAS analysis.

Table 1. Patient demographic and clinical characteristics

Characteristics	% Of all patients (N = 471)	RAS		P-value ^a	PI3KCA		P-value ^a
		% WT (n = 171)	% MT (n = 109)		% WT (n = 246)	% MT (n = 30)	
Age, years							
Median	67	68	68	0.81	68	65	0.29
Range	32–86	36–85	37–85		32–85	41–85	
Male sex	63	64	58	0.31	62	63	1.00
ECOG performance status							
0	56	60	59	0.46	58	57	0.95
1	38	33	38		36	40	
2	6	7	4		6	3	
Capecitabine dosage, mg m⁻² per day							
2000	67	63	70	0.30	67	63	0.69
Disease-free interval > 12 months	27	30	28	0.79	32	17	0.14
Prior adjuvant chemotherapy	22	23	21	0.77	24	17	0.49
Prior radiotherapy	13	11	8	0.54	10	10	1.00
Primary site of cancer							
Caecum	10	8	14	0.11	9	13	0.51
Ascending colon	10	11	14	0.58	13	7	0.55
Transverse colon	6	7	6	0.80	7	3	0.70
Descending colon	3	4	4	1.00	3	3	1.00
Sigmoid colon	30	30	29	1.00	28	47	0.04
Rectosigmoid colon	11	15	9	0.20	13	13	1.00
Rectum	23	21	22	0.88	22	10	0.16
Primary tumour resected	79	93	88	0.20	91	100	0.14
Any metastases resected	10	9	7	0.66	7	17	0.09
Extent of disease at baseline							
Local disease in colon or rectum	36	27	32	0.42	28	20	0.39
Liver metastases	75	72	76	0.49	72	83	0.20
Lymph node metastases	47	49	45	0.54	46	57	0.34
Lung metastases	39	37	45	0.21	41	47	0.56
Bone metastases	4	4	5	0.76	3	7	0.25
Peritoneal metastases	18	18	15	0.51	19	3	0.04
Other metastases	10	12	12	1.00	12	13	0.77
BRAF mutation ^b	11	15	1	<0.001	11	7	0.46

Abbreviations: ECOG = Eastern Cooperative Oncology Group; MT = mutated; WT = wild type.
^aP-value is for the comparison between MT and WT within the RAS and PI3KCA cohorts respectively.
^bTotal number of patients with BRAF assessment was 313, with only 278 also having RAS testing and only 274 also having PI3KCA testing.

Table 2. Progression-free survival in the total study population and the patients evaluated for RAS mutation

Treatment	Total study population (N = 471)					Patients evaluated for RAS (n = 280)					Patients without tissue (n = 191)				
	N	Median PFS (months)	HR	95% CI	P-value	N	Median PFS (months)	HR	95% CI	P-value	N	Median PFS (months)	HR	95% CI	P-value
C	156	5.7	1.00			95	6.1	1.00			61	5.1	1.00		
CB	157	8.5	0.62	0.50–0.79	<.0001	99	9.1	0.63	0.47–0.85	0.002	58	7.4	0.59	0.40–0.85	0.005
CBM	158	8.4	0.59	0.47–0.75	<.0001	86	8.4	0.66	0.49–0.90	0.009	72	8.3	0.48	0.34–0.70	<0.001
CB + CBM	315	8.4	0.61	0.50–0.74	<.0001	185	8.6	0.65	0.50–0.84	0.001	191	8.2	0.53	0.38–0.73	<0.001

Abbreviations: C = capecitabine; CB = capecitabine and bevacizumab; CBM = capecitabine, bevacizumab and mitomycin; CI = confidence interval; HR = hazard ratio; PFS = progression-free survival.

Table 3. Overall survival in the total study population and the patients evaluated for RAS (and PIK3A) mutation

Treatment	Total study population (N = 471)					Patients evaluated for RAS(n = 280)				
	N	Median OS (months)	HR	95% CI	P-value	N	Median OS (months)	HR	95% CI	P-value
C	156	18.9	1.00			95	21.4	1.00		
CB	157	18.9	0.86	0.66–1.11	0.24	99	20.4	0.90	0.65–1.26	0.55
CBM	158	16.4	1.00	0.78–1.29	0.98	86	17.3	1.02	0.73–1.44	0.90
CB + CBM	315	17.3	0.93	0.75–1.16	0.51	185	19.4	0.96	0.71–1.28	0.77

Abbreviations: C = capecitabine; CB = capecitabine _ bevacizumab; CBM = capecitabine, bevacizumab and mitomycin; CI = confidence interval; HR = hazard ratio; OS = overall survival.

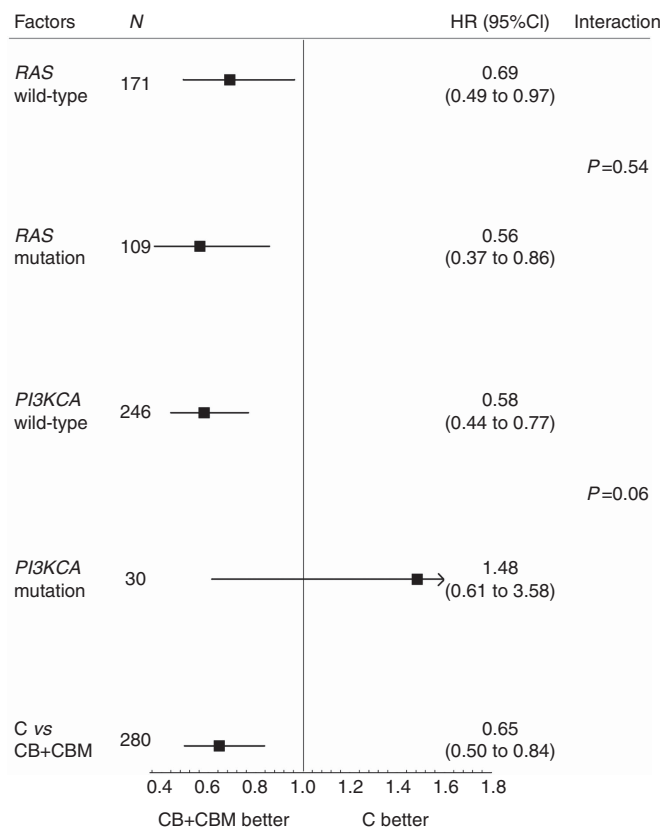


Figure 2. Hazard ratios for progression-free survival according to RAS and PI3KCA. Forest plot to demonstrate hazard ratios for progression-free survival in subgroup analyses by RAS and PI3KCA status.

receiving CB or CBM (HR, 1.48; 95% CI, 0.61–3.58; $P=0.38$; Figure 2). Among patients with PI3KCA WT tumours, the median PFS was 5.9 months in the group receiving C and 8.4 months in the groups receiving CB or CBM (HR, 0.58; 95% CI, 0.44–0.77; $P<0.001$; Figure 2). The additional benefit of bevacizumab on PFS appears greater among the patients with PI3KCA WT tumours than among those with PI3KCA MT tumours but does not reach statistical significance ($P=0.06$ for the interaction between PI3KCA status and the assigned treatment).

Overall survival. Among patients with RAS MT tumours, there was no statistical difference in OS, and the median OS was 22.8 months in the group receiving C and 20.4 months in the groups receiving CB or CBM (HR, 0.91; 95% CI, 0.58–1.44; $P=0.70$). Among patients with RAS WT tumours, the median OS was 20.6 months in the group receiving C and 18.9 months in the groups receiving CB or CBM (HR, 0.99; 95% CI, 0.67–1.45; $P=0.95$). The effect of the addition of bevacizumab on OS was not significantly greater among the patients with RAS WT tumours than among those with RAS MT tumours ($P=0.73$ for the interaction between RAS status and the assigned treatment).

Among patients with PI3KCA MT tumours, the median OS was 18.9 months in the group receiving C and 19.2 months in the groups receiving CB or CBM (HR, 1.18; 95% CI, 0.46–3.06; $P=0.73$). Among patients with PI3KCA WT tumours, the median OS was 20.6 months in the group receiving C and 19.8 months in the groups receiving CB or CBM (HR, 0.89; 95% CI, 0.65–1.22; $P=0.47$). The effect of the addition of bevacizumab on OS was not significantly greater among the patients with PI3KCA WT tumours than among those with PI3KCA MT tumours ($P=0.49$ for the interaction between PI3KCA status and the assigned treatment).

Table 4. Response rate by RAS and PI3KCA status

Treatment	RAS			PI3KCA		
	WT (%)	MT (%)	P-value ^a	WT (%)	MT (%)	P-value ^a
C	28.6	48.7	0.04 ^b	34.2	60.0	0.26
CB	39.3	26.3		37.2	36.4	
CBM	46.3	50.0		46.9	55.6	

Abbreviations: C = capecitabine; CB = capecitabine and bevacizumab; CBM = capecitabine, bevacizumab and mitomycin; MT = mutated; WT = wild type.
^aP-value for interaction between biomarker status and the assigned treatment (C vs CB or CBM).
^bResults could not be confirmed when additional analysis comparing C with CBM only was undertaken ($P=0.25$ for the interaction between RAS status and the assigned treatment).

Response to treatment. The response rate based on RAS and PI3KCA mutation status is summarised in Table 4 and Supplementary Table 2 online. Patients with RAS WT tumour appeared to have a borderline greater likelihood of response with the addition of bevacizumab ($P=0.04$ for the interaction between RAS status and the assigned treatment). The effect of the addition of bevacizumab on response was similar among the patients with PI3KCA WT tumours compared with those with PI3KCA MT tumours ($P=0.26$ for the interaction between PI3KCA status and the assigned treatment).

Prognostic value of RAS and PI3KCA mutation. There was no significant difference in PFS between patients with RAS MT tumours and those with RAS WT tumours. The median PFS was 7.7 months among the patients with RAS MT tumours as compared with 7.6 months among those with RAS WT tumours (HR (RAS WT vs MT), 0.92; 95% CI, 0.71–1.17; $P=0.49$). Similarly, RAS has no prognostic impact on OS (Figure 3A), with median OS of 20.4 months for RAS MT tumours compared with 19.7 months for RAS WT tumours (HR (RAS WT vs MT), 0.94; 95% CI, 0.71–1.24; $P=0.65$). Multivariate analysis did not change these results significantly (results not shown).

There was also no significant difference in PFS and OS between patients with PI3KCA MT tumours and those with PI3KCA WT tumours. The median PFS was 8.8 months among the patients with PI3KCA MT tumours as compared with 7.5 months among those with PI3KCA WT tumours (HR (PI3KCA WT vs MT), 0.90; 95% CI, 0.60–1.35; $P=0.61$). The median OS was 19.2 months among patients with PI3KCA MT tumours as compared with 20.4 months among those with PI3KCA WT tumours (HR (PI3KCA WT vs MT), 1.06; 95% CI, 0.68–1.66; $P=0.77$; Figure 3B). Multivariate analysis did not change these results significantly (results not shown).

Sensitivity analysis. As there was higher rate of primary resection and lower rate of residual local disease in the patients who were evaluated for RAS and PI3KCA mutations as compared with those without tissue, we perform sensitivity analysis by adjusting for these baseline factors. With adjustment, RAS remained nonpredictive of benefit for bevacizumab (PFS: $P=0.62$, OS: $P=0.95$ for the interaction between RAS status and the assigned treatment). PI3KCA also remained nonpredictive of benefit for bevacizumab (PFS: $P=0.06$, OS: $P=0.47$ for the interaction between PI3KCA status and the assigned treatment).

DISCUSSION

We have previously shown that KRAS exon 2 mutation status is not predictive of bevacizumab effect despite the proposed cross-talk between the EGFR and VEGF signalling pathways (Ellis, 2004). Given the recently reported data on the frequency and

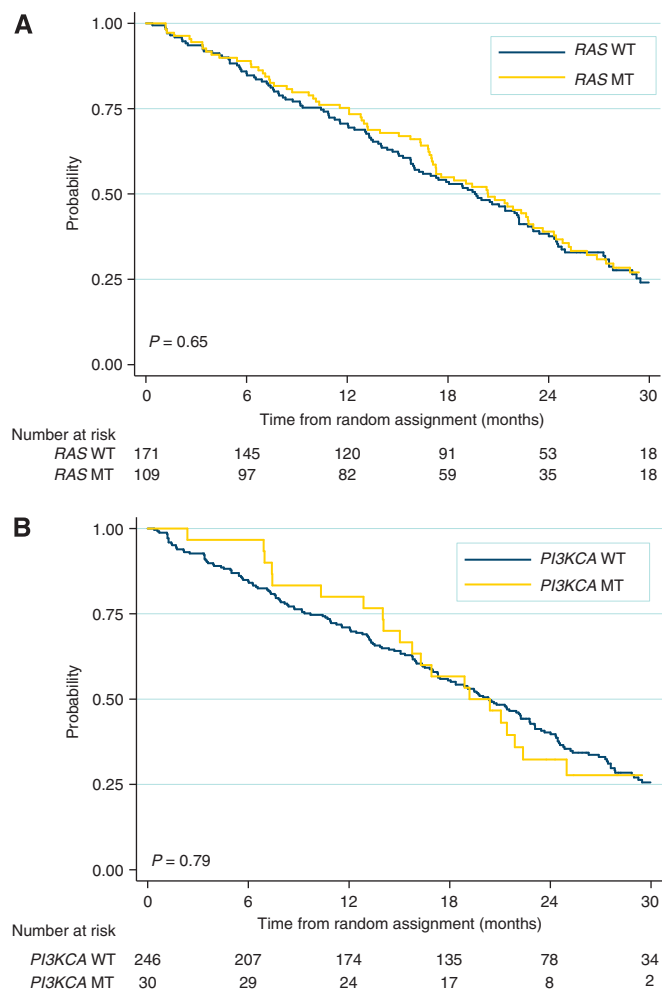


Figure 3. Kaplan–Meier curves for overall survival according to RAS and PIK3CA status. A comparison of the overall survival of all patients according to their (A) RAS and (B) PIK3CA status

relevance of additional RAS mutations on prediction for anti-EGFR therapy, we aimed to assess the mutation rate in the MAX trial as an extension of our prior analysis (Price *et al*, 2011). In our population we now show that 1 in 10 patients (10%) considered KRAS WT have an additional RAS mutation. Despite these additional mutations, there was still no predictive effect on the outcome of bevacizumab, and RAS mutation status remained nonprognostic.

The MAX trial reported improved PFS with the addition of bevacizumab to capecitabine when compared with capecitabine alone. The potential impact of KRAS mutation status on outcomes with bevacizumab had been questioned and this led to a number of investigators to undertake retrospective analysis of bevacizumab trials, our group being one of them. The finding that up to 23% of KRAS exon 2 WT patients may have additional RAS mutation led us to re-analyse our data set with extended RAS results. Of the patients, 59% had available tissue for RAS assessment. Outcomes for PFS and OS were consistent between the intention-to-treat (ITT) and tissue population (Tables 2 and 3). Consistent with the primary analysis, PFS was improved by a similar magnitude and there was no apparent impact of the extended RAS status (HR, 0.56 RAS MT and 0.69 RAS WT, *P* for interaction between RAS status and assigned treatment = 0.54). These data for extended RAS are consistent with the retrospective analyses of the MAX and the pivotal Hurwitz study (IFL ± bevacizumab) for exon 2 KRAS MT (Ince *et al*, 2005; Hurwitz *et al*, 2009). In contrast to the study of

Hurwitz *et al* (2009) where only 31.3% of patient's tissue was assessable, our data set has 60% of the ITT population available for analysis and thus we are able to confirm that changes in RAS MT rates has not altered our prior conclusions.

We detected extended RAS mutations in 39% of the patient group with available tissue. Importantly, 10% of patients previously labelled KRAS (exon 2) WT harboured an additional RAS mutation. The proportion of exon 2 WT patients with an additional RAS mutation is less than reported in the published series with panitumumab and cetuximab (PRIME 17%, FIRE-3 16% and PEAK 23%) (Douillard *et al*, 2013; Heinemann *et al*, 2014; Schwartzberg *et al*, 2014). Subsequently, investigators from the CRYSTAL and OPUS studies have reported in abstract form additional RAS MT rates of 15% and 26%, respectively (Bokemeyer *et al*, 2014; Ciardiello *et al*, 2014). The difference may be explained partly as the MAX trial represented an older population considered suitable for bevacizumab and single-agent fluorouracil. Although there is no definite evidence that KRAS exon 2 MT rate may differ with age, additional mutations may vary and Russo *et al* (2014) recently reported different rates of NRAS MT based on age. Thus, subtle differences in these patient populations and patient characteristics may explain the lower rate of additional RAS mutations seen here. It is also likely that the method chosen to detect mutations will have differing sensitivity and recommended cutoff percentages, resulting in variation in reported rates of mutation (Whitehall *et al*, 2009; Pinto *et al*, 2011). Here, for example, we used pyrosequencing that differs with the method used by the PRIME and PEAK investigators who chose bidirectional sanger sequencing and WAVE-based surveyor kits. The investigators from the CRYSTAL and OPUS studies used the BEAMing technique. Although this is one of the most sensitive techniques reported, a 5% sensitivity cutoff was chosen for these analyses to be consistent with other literature. Variation between the technique chosen is further highlighted by the NRAS exon 4 results that appear only detectable when using BEAMing. Only the FIRE-3 investigators used pyrosequencing and their extended RAS MT rate was one of the lowest reported. Ultimately, differences in patient populations within trials and the sensitivity of the methods used will lead to a range of RAS mutations rates. The exact frequency will become clearer as additional studies are analysed and this will help define the optimal technique and sensitivity cutoff for clinical practice.

The PI3K signalling is considered to regulate tumour growth and angiogenesis by activating AKT and other targets, as well as inducing VEGF and HIF-1 expression (Jiang and Liu, 2009; Zhang *et al*, 2012). Furthermore, Soler *et al* (2013) have reported in a mouse model that inactivation of PIK3CA leads to increased vascular density, reduced vessel size and altered pericyte coverage, and thus PIK3CA has a role in regulation of vessel formation. As there is evidence for a potential interaction of PIK3CA and angiogenesis, and importantly as a biomarker for guiding antiangiogenic therapy remains elusive, we decided to explore whether there was any signal for an association between PIK3CA mutation status and outcome. In the MAX patients PIK3CA mutation was seen in 11.1% of patients (7.5% exon 9 and 3.6% exon 20). We chose to combine PIK3CA MT (exons 9 and 20) for this analysis. In contrast to prior studies assessing MT status and activity of anti-EGFR therapy (De Roock *et al*, 2010, 2011) where only exon 20 is relevant for predicting outcome, there is no suggestion of a similar relationship with a specific mutation when considering a potential interaction with the VEGF pathway (Zhang *et al*, 2012). Furthermore, the small numbers of each PIK3CA exon MT would make results difficult to interpret. Despite theoretical relationships between PIK3CA and angiogenesis and VEGF activation, we found no predictive effect. The borderline trend to a reduced effect of bevacizumab in the PIK3CA MT patients is likely because of small numbers, although ultimately larger data

sets or prospective analysis will be required to assess whether this outcome is real. Furthermore, differences in response rate are also likely to be a random phenomenon based on the small sample size. That said, and although not directly comparable, patients with *PIK3CA* MT treated with the combination of bevacizumab, temsirolimus and liposomal doxorubicin had the opposite outcome with improved response rates when compared with those with WT *PIK3CA*, potentially supporting some interaction of VEGF pathway (Moroney *et al*, 2012), and further analysis of larger data sets may therefore be appropriate. Finally, in keeping with a number of publications, we found no indication that *PIK3CA* MT was prognostic. When considering whether investigators should assess *PIK3CA* based on individual exon or combined, evidence from Liao *et al* (2012) recently found that neither exon 9 nor 20 was prognostic when assessed independently, supporting a combined analysis particularly given the small numbers.

It is important to comment on the potential limitations of a subset analysis. This study is restricted to 59.4% of the tissue that was available for mutation analysis and hence not necessarily generalisable to the broader population. However, the results of our study still demonstrate good internal validity. There is no strong evidence to support that either *RAS* or *PIK3CA* is a predictive factor for bevacizumab benefit. The available tissue was obtained at the time of study enrolment and not *post hoc* after the primary trial result became available. Although there is an imbalance between the tissue population and ITT population in terms of primary *in situ*, this is balanced in each analysis. Thus, although this may be prognostic, the outcomes for bevacizumab benefit are unaltered. Although there might be differences in patients' baseline characteristics between those with and without tissue (Supplementary Table 1), such differences might exist for patients not enrolled in the MAX trial. Furthermore, sensitivity analysis performed to adjust for these different baseline characteristics does not change the overall conclusion of this study.

In conclusion, our analysis of extended *RAS* and *PIK3CA* mutation status from the MAX trial tissue population did not find any therapeutic relevance of mutation status of either *RAS* or *PIK3CA* when bevacizumab is given in combination with capecitabine chemotherapy. The *RAS* and *PIK3CA* status was not prognostic for metastatic CRC for PFS or OS. Finally, we confirm that a clinically relevant proportion of patients (10%) considered *KRAS* exon 2 WT have an additional mutation in the *RAS* pathway.

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CONFLICT OF INTEREST

TJ Price: advisory board Roche, Merck, AMGEN; G Van Hazel: advisory board Roche; D Cunningham: research funding Roche; NC Tebbutt, advisory board Roche. The other authors declare no conflict of interest.

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