Mutation burden and other molecular markers of prognosis in the QUASAR2 clinical trial of colorectal cancer treated with curative intent

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ABSTRACT

Background

Several relatively large studies have assessed molecular indicators of colorectal cancer (CRC) prognosis, but most analyses have been restricted to a handful of markers.

Methods

In stage II/III CRCs from the QUASAR2 clinical trial and from an Australian community-based series, we assessed gene panels for somatic driver mutations and overall mutation burden. We determined molecular pathways of tumorigenesis, and analysed associations with treatment response and prognosis.

Findings

In QUASAR2 (N=511), *TP53*, *KRAS*, *BRAF* and *GNAS* mutations were independently associated with shorter relapse-free survival, whereas total somatic mutation burden was associated with longer survival, even after excluding mismatch repair-deficient (MSI+) and *POLE*-mutant tumours. We successfully validated these associations in the Australian sample set (N=296). In an extended analysis of 1,752 QUASAR2 and Australian CRCs for which *KRAS*, *BRAF* and MSI status was available, we found that *KRAS* and *BRAF* mutations were specifically associated with poor prognosis in MSI- cancers. This association was not present in MSI+ cancers, and MSI+ tumours with *KRAS* or *BRAF* mutation actually had better prognosis than MSI- cancers that were wildtype for *KRAS* or *BRAF*. New rare molecular pathways were also uncovered: mutations in the genes *NF1* and *NRAS* from the MAP kinase pathway co-occurred, mutations in *TP53* and *ATM* appeared to be alternative ways of inactivating the DNA damage response pathway.

Interpretation

A multi-gene panel has identified two previously unreported prognostic associations in CRC involving both *TP53* mutation and total mutation burden, and confirmed associations with *KRAS* and *BRAF.* We conclude that even a modest-sized gene panel can provide important information for use in clinical practice and out-perform MSI-based models.

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INTRODUCTION

It is increasingly recognised that treatment of the common cancers can be modified according to a patient's expected prognosis and/or response to therapy. For some of the newer molecularly guided therapies, there exist powerful biomarkers of response, often comprising mutations in the specific protein that is targeted. For more conventional cytotoxic therapies, however, predictive markers of response are rare. Given the relatively small survival benefits that such therapies provide for patients with the common solid malignancies, biomarkers of prognosis still have considerable potential clinical importance. Such markers can guide the use of more or less aggressive treatment regimens, balancing expected outcome against early and late therapeutic toxicities.

Biomarkers can be based on several different types of molecules, and recent high-profile work has highlighted the potential utility of mRNA profiling in identifying groups of colorectal cancers (CRCs) with varying prognosis[1]. Other biomarkers are based on DNA, which is generally easier to analyse given its stability. For CRCs treated with curative intent, the biomarker most consistently used in clinical practice is microsatellite instability (MSI), which usually results from defective DNA mismatch repair[2]. For stage II CRCs , MSI predicts relatively good survival, with hazard ratios as low as 0·6[3]. The association is less strong for stage III and in stage IV MSI+ CRCs may actually have a relatively poor prognosis[4].

Recently, the availability of a few large datasets (>500 participants) from clinical trials has begun to clarify the associations of certain somatic mutations with CRC prognosis However, most of these analyses have been restricted to *KRAS* mutations, and/or *BRAF* mutations and/or MSI; their results are summarised in Supp. Table 1. Overall, for CRCs treated with curative intent (generally stage II or III), there is support for an association between MSI and good prognosis, albeit possibly weaker in stage III, coupled with more limited evidence that *KRAS* and *BRAF* mutation, which are mutually exclusive, indicate poor prognosis in MSI- tumours[5-10]. MSI+ CRCs tend, however, to be *BRAF*-mutant and *KRAS*-wildtype so statistical interactions may exist between these prognostic biomarkers. It is, moreover, currently unclear whether combinations of other genetic biomarkers provide useful prognostic information.

Two main reasons have limited large genetic biomarker studies to screening a small number of genes: sub-optimal sample quality or quantity; and the cost of mutation screening. Clearly, because somatic mutations tend to co-occur in molecular pathways of tumorigenesis, it would be highly desirable to screen many potentially prognostic mutations in the same data set and identify the primary determinants of tumour behaviour. However, the few studies to perform such analyses have lacked standardised recruitment and follow-up. The prime example is the exome or genome sequencing of over 600 CRCs by The Cancer Genome Atlas (TCGA) group[11]. This has provided an excellent data set for driver mutation discovery, but has limited use for biomarker discovery owing to the heterogeneous sample set and associated variability in the clinical data.

Our strategy in this exploratory study was to retain the advantages of a large clinical trial data set whilst assessing multiple prognostic biomarkers for CRC. To this end, we used a 82 gene panel to identify somatic mutations in all the major CRC driver genes in more than 500 tumours from the QUASAR2 clinical trial of stage II/III CRC. We also assessed MSI and the "ultramutator" phenotype from *POLE* mutations[12]. A larger QUASAR2 sample set was additionally tested for *KRAS*/*BRAF* mutations and MSI. Variables associated with survival in QUASAR2 were replication tested in an independent community-based cohort, and subjected to a combined analysis, resulting in the identification of four independent prognostic biomarkers: mutations in *KRAS, BRAF, TP53* and mutation burden.

MATERIALS AND METHODS

QUASAR2 was an international clinical trial comprising 1,952 patients with high-risk stage II or stage III CRC, randomised to capecitabine +/- bevacizumab without radiotherapy and median follow-up of 4.92 years[13]. Patients in the bevacizumab arm showed no benefit in overall or disease-free survival at three years follow-up; comparable results have also been reported in two similar trials[14,15]. Clinico-pathological data (Supp. Table 2) were obtained from the trial database. Some were converted to binary variables: sex; location (proximal vs distal);

depth of invasion (T4 vs T1/T2/T3); and lymph node metastasis (N2/N1 vs N0). Age and grade were assessed as continuous variables.

A total of 1,187 UK QUASAR2 CRCs was collected for molecular analysis, of which a subset of 598 tumours was analysed using an Ion Torrent sequencing gene panel (Supp. Table 3a). The remaining CRCs were analysed by Sanger sequencing for selected mutations and for MSI (Supp. Materials and Methods). 40um scrolls were cut from FFPE specimens of CRC that had >80% estimated purity, and from normal bowel; 10um sections were cut from the remaining CRCs and needle-microdissected to enrich for tumour using a haematoxylin and eosin (H&E) section as a guide. Peripheral blood samples were also available from most patients. DNA was extracted from FFPE tissue with the DNeasy kit (Qiagen) and from blood with the Maxwell 16 Blood DNA Purification kit (Promega).

The community-based series comprised 657 patients with stage II or III CRC treated at the Royal Melbourne Hospital, Western Hospital Footscray or St Vincent's Hospital, Sydney, Australia since 1993 (Supp. Table 2). Fresh-frozen tumour and matched normal specimens were retrieved from hospital tissue banks. Individuals with hereditary CRC syndromes were excluded. All patients received standard neo-adjuvant or adjuvant 5FU-based chemotherapy or concurrent chemoradiotherapy. 379 patients received adjuvant 5FU treatment, of whom 47 also received oxaliplatin and 38 had no data on oxaliplatin use. All patients were prospectively followed according to standard protocols, with a median follow-up of 60 months. All patients gave informed consent, and the study was approved by the medical ethics committees of all sites. Australian stage II patients were regarded as low-risk when they were T3/N0, otherwise as high-risk. A subset of 296 tumours was screened by targeted next-generation sequencing, the others by conventional PCR-based sequencing (Supp. Table 3b).

Individual driver gene mutations, combinations of mutations or global measures such as MSI or mutation burden (total number of non-synonymous mutations and coding indels) were tested for associations with relapse-free survival (RFS) in univariable and multivariable models, principally using Cox proportional hazards models, in accordance with published guidelines[16] (Supp. Table 4). Further details of patients and analytical methods are provided in Supp. Materials and Methods and Supp. Figure 1.

RESULTS

QUASAR2 mutation profiling using a custom gene panel

598 QUASAR2 tumours were sequenced for 82 genes by Ion Torrent technology. We eliminated mutations with a high probability of being artefacts and cancers with high levels of artefactual hypermutation owing to *ex vivo* cytosine deamination (Supp. Figures 2-5, Supp. Tables 6-9). 511 tumours remained for further analysis (Supp. Figure 1).

We identified all likely driver mutations (see Materials and Methods; Supp. Table 9) and selected the 13 most commonly mutated genes (mutated in ≥8 tumours) for further analysis to identify mutations tending to occur together in genetic pathways (Supp. Tables 10, 11; Supp. Figure 6). In addition to known associations, new findings included a negative association between *TP53* and *GNAS,* and a positive association of *NF1* with *PTEN* and *NRAS*.

Since several mutations co-varied, we searched for primary associations by multivariable regression (Supp. Table 13), hierarchical clustering and Bayesian networks (Supp. Figures 7, 8). Interestingly, all three analytical methods found that mutations in *NF1*, a gene involved in the pathogenesis of neuromas and a negative regulator of the Ras pathway, were positively associated with *NRAS* mutations, but not with mutations in *KRAS* or *BRAF*. *SMAD4* mutations were associated with *BRAF* mutations, but not with *KRAS* or *NRAS* changes, suggesting possible synergy between *BRAF* and the TGFβ or BMP pathways. In addition, logistic regression and Bayesian network analyses showed a strong negative association between driver mutations in *TP53* and *ATM*, two key mediators in the DNA damage response (DDR) , suggesting that these mutations were alternatives DDR inactivators. Finally, clustering and Bayesian network analysis suggested a positive association between *ATM* and *PTEN* mutations. It has recently been shown than PTEN is phosphorylated by ATM in response to DNA-damaging agents, thus inducing autophagy[17]. Regression analysis between molecular and clinical variables showed that *KRAS* mutations were associated with female sex, similarly

to *BRAF* mutations[11,18]. In addition, mutations in *FBXW7* and *CTNNB1* were associated with high grade, the latter suggesting that activation of the Wnt pathway through beta-catenin rather than *APC* mutation might predispose to poorly differentiated CRCs.

Sub-clonal mutations may be clinically important, for example by driving drug resistance, but can be difficult to identify. Our high depth sequencing allowed us to identify 58 tumours (11%) carrying somatic mutations at significantly reduced allele frequency, suggesting sub-clonal status (see Materials and Methods). Of the 13 most commonly mutated genes, *PIK3CA*, *ATM* and *SMAD4* had lower driver mutation allele frequencies than the other genes (P=0·001, 0·002 and 0·05 respectively), suggesting they were more often sub-clonal (Supp. Table 14).

Mutation burden, clonal diversity (presence of any identified mutation at low allele frequency) and driver mutations in the 13 genes were tested for prediction of bevacizumab treatment response, with no significant associations found (data not shown).

Markers of prognosis in gene panel analysis of QUASAR2 and the community cohort

In QUASAR2, overall mutation burden and mutations in 4 specific genes (*TP53*, *KRAS*, *BRAF* and *GNAS*) showed promising individual associations with relapse-free survival (RFS) (pre-defined P<0·10) and were therefore selected for multivariable analysis, together with T stage, N stage, treatment arm (as bevacizumab had previously been associated with poorer prognosis in the analysis of our patent sub-group although not the whole trial), and MSI (as it co-varied with mutation burden and is probably the best established prognostic factor for CRC) (Table 1a; Supp. Tables 15, 16). We found mutation burden (HR=0·81; 95%CI=0·68-0·96; P=0·014), mutations in *TP53*, *KRAS*, *BRAF* and *GNAS*, T/N stage and use of bevacizumab were all independently associated with poor prognosis (P<0·05), but MSI was not (HR=1·12; 95%CI=0·57-2·19; P=0·75) (Table 1a). To test whether the prognostic effect of mutation burden was only due to hypermutation, the same model was run in the sub-set of tumours without MSI or pathogenic *POLE* mutations. Mutation burden retained a borderline significant association with outcome (HR=0·85, 95%CI=0·73-1·00, P=0·051), with the other variables showing results similar to those previously found (Table 1a).

To replication test our prognostic markers, we used an independent data set from an Australian community-based cohort of stage II and III CRC patients (N=296) (Supp. Table 2; Supp. Figures 9, 10) in which all prognostic markers identified in QUASAR2, except *GNAS* mutations, had been assessed (see Materials and Methods). A multivariable analysis incorporating the same clinical and molecular variables and co-variables showed that, in agreement with the QUASAR2 analysis, *BRAF* mutation, *TP53* mutation, and mutation burden were associated (P<0·05) with RFS, whereas MSI was not (Table 1b). *KRAS* mutation also showed a similar prognostic association in the Australian patients to that present in QUASAR2, although formal significance was not reached. Exclusion of MSI+ and ultramutator tumours from the Australian analysis made little difference to the associations, although *KRAS* became formally associated with prognosis and *BRAF* mutation lost that status (Table 1b).

A combined analysis of the QUASAR2 and Australian cohorts confirmed that mutations in *KRAS*, *BRAF* and *TP53*, together with lower mutation burden, were all independently associated with poor prognosis, whereas MSI was not (Figure 1; Table 1c; Supp. Table 17). Exclusion of MSI+ and ultramutator cancers did not alter our findings. There was no significant heterogeneity between cohorts and our model persisted in Australian patients treated with chemotherapy (details not shown).

We compared a prognostic model based on the current gold standard of clinico-pathological variables and MSI with our new model incorporating clinical variables, mutation burden and driver mutations in *KRAS, BRAF* and *TP53.* In both QUASAR2 and the Australian cohort, the new model performed significantly better ($P=4x10^{-5}$ and P=0·0057 respectively, likelihood ratio test). A 10% leave-out cross-validation analysis showed these analyses to be robust (see Supp. Material and Methods).

We then explored the prognostic model separately in stage II (N=266) and stage III (N=499). The model was only formally significant ($P=7.3x10^{-8}$) in the latter case (Supp. Table 18), but hazard ratios were very similar in both stages, suggesting the lack of formal significance was the result of lower power in the smaller stage II set. Correspondingly, despite inherently reduced power, an analysis by tumour location (proximal colon, distal colon, rectum) showed

similar hazard ratios for all biomarkers across sites, even after exclusion of hypermutated tumours (Supp. Table 19). In addition, formal assessment of interactions between individual biomarkers and stage or tumour location found no evidence of significant deviation from a log-additive model (details not shown).

Patient outcome in relation to combinations of MSI, KRAS and BRAF status in enlarged cohorts

Based on previous reports[5-10], we investigated the prognostic associations of *KRAS* and *BRAF* mutations in relation to MSI status. We pooled data from the QUASAR2 gene panel, the Australian validation set, and an additional 676 QUASAR2 and 362 stage II/III Australian CRCs that had been analysed for MSI and by Sanger sequencing for *KRAS*/*BRAF* mutations (Supp. Table 2) for an extended set of 1,732 patients. In a multivariable analysis we found MSI was associated with good prognosis (HR=0·45, 95%CI=0·31-0·64, P=1x10^{-5}), and *KRAS* and *BRAF* mutations were both associated with poor prognosis (HR=1·22, 95%CI=1·01-1·48, P=0·035; HR=1·53, 95%CI=1·14-2·04, P=0·004 respectively; Supp. Table 20). Since the strong co-variation of these biomarkers could potentially have confounded or obscured prognostic effects, we added to the multivariable model multiplicative interaction terms between MSI and mutations in *KRAS* and *BRAF*. We found both of these interactions to be significant (P=0·003 and P=0·023 respectively) suggesting differential prognostic effects.

Accordingly, we explored different combinations of MSI, *KRAS* mutation and *BRAF* mutation. Compared with "triple negative" (MSI-, *KRAS*-wildtype, *BRAF* wildtype) cancers, MSI- tumours with *KRAS* or *BRAF* mutations had a worse prognosis (respectively HR=1·35, 95%CI=1·11-1·64, P=0·003 and HR=2·02, 95%CI=1·47-2·77, P=1·19x10^{-5}; Table 2, Figure 2). By contrast, and explaining the statistical interactions detected, MSI+ CRCs with *KRAS* or *BRAF* mutation had a significantly better prognosis than the triple negatives (respectively HR=0·28, 95%CI=0·09-0·89, P=0·03 and HR=0·55, 95%CI=0·34-0·90, P=0·017; Table 2), although the difference was not significant compared with MSI+ CRCs without *KRAS/BRAF* mutation. The 6 MSI/*KRAS*/*BRAF* sub-groups showed consistent effects between the QUASAR2 and Australian cohorts (details not shown).

TP53-based prognostic sub-sets in MSI- CRCs

Although MSI was not an independent prognostic marker when mutation burden was also assessed, it was prognostic in the absence of that information (Supp. Table 20). We therefore explored whether new prognostic groups within the larger MSI- subset could be identified using *KRAS, BRAF* and *TP53*, given that *TP53* mutation remained an independent prognostic marker when MSI+ and ultramutator CRCs were excluded from our main analysis based on gene panels (Table 1). We used our extended QUASAR2 and Australian cohorts and derived *TP53* status from either NGS or Sanger sequencing. Within the MSI- CRC set (N=991), tumours with *BRAF* and *TP53* mutations had a particularly poor prognosis (HR=3·08, 95%CI=1·88-5·03, $P=7{\cdot}12x10^{-6}$; Supp. Table 21; Figure 3), with a suggestive, but non-significant, interaction between these markers (HR=2·21, P=0·058), but no evidence of interaction between *TP53* and *KRAS* (HR=1·13, P=0·62). Overall, therefore, we convincingly detected only independent prognostic effects of these three driver genes.

DISCUSSION

The use of prognostic molecular markers in the management of solid tumours is still not widespread. In part, this reflects a lack of validated markers and in part, differences between studies that have led to inconsistency in the recommended markers to use and their estimated effect sizes. For CRC, whilst several relatively large studies have assessed molecular indicators of CRC prognosis, most have been restricted to a handful of markers. In this study, we have used overlapping cancer gene mutation panels to analyse a high quality clinical trial of CRCs treated with curative intent and a validation cohort. In multivariable analysis incorporating known clinico-pathological prognostic factors, we have shown that low overall mutation burden and mutations in *KRAS, BRAF* and *TP53* are independently associated with poorer RFS from CRC treated with curative intent. All these findings were also present in our Australian validation set, even though the patients in that study were from a community-based collection rather than a clinical trial. The fact that we found no molecular marker of bevacizumab response in QUASAR2 or chemotherapy response in the Australian cohorts

(details not shown) suggested that the markers we have found are prognostic, although formally demonstrating this is difficult given that most of our patients received 5FU-based chemotherapy.

The complexity of associations between mutations and CRC prognosis is arguably reflected in the generally stronger associations of markers in multivariable than univariable analyses. Furthermore, MSI was generally not prognostic in these analyses, because its effects were captured by mutation burden (somatic single nucleotide variants and small indels): however, mutation burden not only strongly co-varied with MSI and *POLE,* but also provided prognostic information in MSI- CRCs. Although high mutation burden has been associated with good CRC prognosis in the context of MSI and *POLE* proofreading deficiency[12], this has not previously been shown for CRCs without those forms of genomic instability. Similar data from other tumour types are limited[19-21], although in other cancers with generally high mutation burdens but without specific forms of genomic instability, such as lung carcinoma and melanoma, mutation burden has predicted response to immune checkpoint inhibitors[22,23]. It remains possible in our study that undetected hyper/ultramutator cancers contributed to the mutation burden association, although the frequencies of MSI and *POLE* mutations that we found were typical of other studies[12] and we found a monotonic relationship between mutation burden quartile and RFS in our data. A further potential cause of the mutation burden association was non-excluded deamination artefacts if they happened to be associated with an unknown factor correlated with good prognosis; however, we made strenuous efforts to exclude those artefacts, no plausible explanatory causes such as tumour age were detectable within QUASAR2 (details not shown), and the Australian validation cohort was from fresh frozen tissue which is unlikely to have deamination. We note that the new observed association with mutation burden is sufficiently strong that even a modestly sized gene panel can pick it up, as it may be representative of mutation burden in the exome[24]. The underlying reason for the association between mutation burden and prognosis remains unclear, although anti-tumour immune responses are evidently the prime candidate[19-21].

The interplay between *KRAS, BRAF* and *TP53* mutations, MSI and mutation burden in our data set is intriguing. These mutations co-vary strongly (Supp. Table 12), and are additionally associated with other molecular variables. Deciphering primary associations is therefore

extremely challenging. Nevertheless, our study strongly supports the reported poor prognosis of MSI- CRCs with *KRAS* or *BRAF* mutations[5-10] compared with MSI- CRCs wildtype for these genes and unselected MSI+ CRCs; in addition, we found that *KRAS* or *BRAF* mutation may be associated with improved prognosis in MSI+ CRCs. *TP53* has not previously been consistently reported as a prognostic marker for CRC in the curative setting, but very few large studies have undertaken a sufficiently comprehensive molecular analysis to include *KRAS, BRAF, TP53* and MSI. Notably, addition of these four prognostic markers improved outcome prediction compared with current clinical guidelines based on MSI.

The strengths of our study are multiple potential biomarkers screened in a large, high quality clinical trial and a comparable, community-based cohort. We have very carefully performed quality control analysis to derive high-quality mutation calls. For mutation burden, the study is arguably limited by the size of the gene panels used, and a larger panel or exome/genome sequencing might detect even stronger associations with prognosis. In addition, the lower numbers of stage II patients in the sample set means that the utility of our model in such patients remains formally unproven. Moreover, we cannot formally distinguish between the model being prognostic, or predictive for 5FU response. Another potential weakness is the different treatment regimens used in each cohort, although regimen was incorporated as a co-variable into the analyses. Finally, our study may have sub-optimal power to draw firm conclusions about outcomes in small patient groups or sub-groups, such as those with combinations of several molecular variables.

Advances in molecular testing hold considerable promise for the delivery of precision cancer medicine, but their clinical use to date has largely been limited to the analysis of small numbers of actionable variants. In CRC, these include *KRAS* and *NRAS* mutation testing for prediction of resistance to anti-EGFR therapies[25], or MSI, which identifies stage II tumours with excellent prognosis[26] and stage IV tumours likely to respond to immune checkpoint inhibition[27]. Our findings show that the use of even a modest-sized gene panel can provide clinically useful information beyond individual driver mutations. In particular, tumour mutation burden displaced MSI/*POLE* as a marker of prognosis in multivariable analysis, thus extending the group of good-prognosis CRCs to include those with high mutation burden in the absence of a specific underlying mutator phenotype. While we were unable to test

whether mutational load is predictive for immunotherapy response in CRC, this correlation is well documented in other tumour types, including melanoma, lung and ovarian cancers[28]. Accordingly, our results suggest that the use of tumour mutation burden as a prognostic and predictive marker in CRC is worthy of further exploration, beyond tumours with MSI or *POLE* mutation. It is likely that other genome-wide molecular phenotypes, such as mutational signatures[29], will come to play a role in cancer management in the future.

CONTRIBUTORS

ED, JCT and IT designed the study. RLW, JS, OS, JCT and IT acquired funding. RLW, NJH, PG, DK, RK and OS provided resources. ED, CC, PJK, MJP, MMP, SM, MP, RLW, NJH, PG, HA, DO, HW, JW, ET, YB, KK, EJ, CP, DNC, MN, HD and OS collected the data. ED, RLW and OS curated the data. ED, DM, MMP, OS and IT analysed the data. ED, OS, JCT and IT interpreted the data. ED and IT wrote the paper. All authors read and approved the manuscript.

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Table 1. Associations between clinico-pathological-molecular variables and relapse-free survival.

Cox proportional hazards analysis was performed. The "univariable" analyses are adjusted by T stage, N stage and treatment arm (or 2 of these if the adjustment variable itself is being assessed). Multivariable analysis is based on all variables shown. Mutation burden is derived here from coding mutations , since these are most likely to be functionally relevant, but similar results are obtained when other somatic variants are also included (Supp. Figure 11). *POLE* proofreading mutation is not shown as a prognostic variable owing to the low frequency of those cancers (Supp. Table 11).

(a) QUASAR2. N=511 (all cases, univariable and multivariable); N=443 (MSI-/Non-pathogenic POLE).

	All cases univariable			All cases multivariable			MSI- & Non-pathogenic POLE multivariable		
	HR	**95% CI**	**P**	**HR**	**95% CI**	**P**	**HR**	**95% CI**	**P**
KRAS mutation	1·48	1·07-2·05	0·018	1·99	1·37-2·91	$3{\cdot}44x10^{-4}$	2·25	1·51-3·35	$6{\cdot}07x10^{-5}$
BRAF mutation	1·42	0·94-2·13	0·093	2·46	1·51-4·03	$3{\cdot}31x10^{-4}$	2·88	1·70-4·85	$7{\cdot}50x10^{-5}$
TP53 mutation	1·53	1·08-2·18	0·018	1·63	1·12-2·38	0·011	1·61	1·09-2·38	0·025
GNAS mutation	2·19	0·89-5·35	0·087	2·76	1·08-7·04	0·034	4·00	1·42-11.3	0·009
Mutation burden (quartiles)	0·87	0·75-1·00	0·055	0·81	0·68-0·96	0·014	0·85	0·73-1·00	0·051
MSI	0·73	0·42-1·28	0·271	1·12	0·57-2·19	0·75	-		
Chemotherapy (bevcap vs cap)	1·37	0·98-1·92	0·065	1·43	1·02-2·00	0·039	1·55	1·09-2·22	0·015
T4 v T123	2·11	1·52-2·94	$8{\cdot}59x10^{-6}$	2·10	1·50-2·93	$1{\cdot}36x10^{-5}$	2·29	1·61-3·25	$3{\cdot}66x10^{-6}$
N+ v N0	1·80	1·22-2·63	0·003	1·85	1·25-2·73	0·002	2·03	1·33-3·09	0·001

(b) Australian. N=296 (all cases without missing data, univariable); N=253 (all cases, multivariable); N=209 (MSI-/Non-pathogenic POLE). Note that *BRAF* was only tested for the common V600E variant and that *GNAS* was not tested.

	All cases univariable			All cases multivariable			MSI- & Non-pathogenic POLE multivariable		
	HR	**95%CI**	**P**	**HR**	**95%CI**	**P**	**HR**	**95%CI**	**P**
KRAS mutation	1·31	0·92-1·87	0·136	1·51	0·97-2·38	0·066	1·61	1·02-2·59	0·040
BRAF mutation	0·91	0·52-1·64	0·780	2·18	1·08-4·56	0·029	1·79	0·73-4·24	0·204
TP53 mutation	1·19	0·83-1·71	0·334	1·82	1·12-2·73	0·014	1·81	1·09-2·82	0·020
Mutation burden (quartiles)	0·72	0·62-0·85	$8{\cdot}62x10^{-5}$	0·78	0·63-0·95	0·014	0·82	0·64-0·93	0·008
MSI	0·39	0·18-0·71	0·003	0·62	0·24-1·44	0·247	-	-	-
Chemotherapy (yes vs no)	1·01	0·71-1·44	0·946	0·60	0·34-0·91	0·019	0·51	0·18-0·90	0·018
Radiotherapy (yes vs no)	1·21	0·50-3·02	0·653	1·33	0·53-3·32	0·546	1·29	0·51-3·20	0·603
T4 v T123	2·19	1·54-3·22	$2{\cdot}01x10^{-5}$	2·38	1·57-3·75	$6{\cdot}34x10^{-5}$	2·67	1·73-4·21	$1{\cdot}62x10^{-5}$
N+ v N0	1·4	0·97-2·08	0·070	1·21	0·71-2·04	0·493	1·19	0·66-2·05	0·597

(c) Combined

For this analysis, N=807 (all cases without missing data, univariable); N=764 (all cases, multivariable); N=652 (MSI-/Non-pathogenic POLE). Mutation burden quartile was derived separately for the QUASAR2 and Australian cohorts owing to the different content of the two panels. Note that the cohort/treatment variables are categorical.

	All cases univariable			All cases multivariable			MSI- & Non-pathogenic POLE multivariable		
	HR	**95%CI**	**P**	**HR**	**95%CI**	**P**	**HR**	**95%CI**	**P**
KRAS mutation	1·40	1·10-1·78	0·006	1·74	1·31-2·29	$1·21x10^{-4}$	1·88	1·40-2·51	$2·11x10^{-5}$
BRAF mutation	1·23	0·88-1·72	0·231	2·21	1·47-3·29	$1·02x10^{-4}$	2·32	1·50-3·58	$1·49x10^{-4}$
TP53 mutation	1·30	1·01-1·67	0·039	1·65	1·24-2·19	$4·67x10^{-4}$	1·68	1·24-2·26	0·001
Mutation burden (quartiles)	0·82	0·74-0·92	$5·1x10^{-4}$	0·8	0·70-0·91	0·001	0·84	0·74-0·94	0·004
MSI	0·58	0·38-0·89	0·012	0·8	0·46-1·35	0·399	-	-	-
Cohort/treatment Q2 cap	Ref.			Ref.			Ref.		
Cohort/treatment Q2 bev+cap	1·45	1·04-2·03	0·029	1·44	1·02-2·01	0·034	1·53	1·07-2·18	0·019
Cohort/treatment Australia no chemo	2·04	1·4-2·98	$2·2x10^{-4}$	3·48	2·28-5·30	$7·04x10^{-9}$	4·05	2·58-6·34	$9·96x10^{-10}$
Cohort/treatment Australia chemo	2·06	1·45-2·93	$5·61x10^{-6}$	1·75	1·18-2·58	0·005	1·88	1·25-2·83	0·002
Radiotherapy (yes vs no)	1·56	0·64-3·78	0·326	1·37	0·54-3·41	0·503	1·3	0·51-3·24	0·579
T4 v T123	1·81	1·42-2·29	$1·30x10^{-6}$	2·19	1·68-2·83	$3·03x10^{-9}$	2·36	1·80-3·09	$4·38x10^{-10}$
N+ v N0	1·45	1·11-1·89	0·006	1·63	1·21-2·20	0·001	1·68	1·21-2·30	0·002

Table 2. Prognosis associated with sub-groups by *KRAS* mutation, V600E *BRAF* mutation and MSI in all cohorts (N=1,732).

P for interaction between MSI and *BRAF* and *KRAS* is 0·003 and 0·023 respectively. Results are from multivariable analysis adjusted by cohort arms as shown in Table 1(c). Six patients in very rare subgroups are not shown.

All cohorts (N=1,732)	HR	95%CI	P
KRASwt/BRAFwt/MSI-	Ref.		
KRASmut/BRAFwt/MSI-	1·35	1·11-1·64	0·003
KRASwt/BRAFmut/MSI-	2·02	1·47-2·76	$1{\cdot}20x10^{-5}$
KRASwt/BRAFwt/MSI+	0·90	0·56-1·45	0·670
KRASmut/BRAFwt/MSI+	0·28	0·09-0·89	0·028
KRASwt/BRAFmut/MSI+	0·55	0·35-0·90	0·017
T4 v T123	2·26	1·88-2·71	$3{\cdot}32x10^{-18}$
N+ v N0	2·07	1·65-2·59	$2{\cdot}62x10^{-10}$

FIGURE LEGENDS

Figure 1. Relapse-free survival in combined QUASAR2 and Australian cohorts by mutation burden from gene panel analysis (N=672). Burden data are shown by quartile (highest burden in Q4). Cancers that were MSI+ or carried pathogenic *POLE* mutations were excluded. Cox proportional hazards model results are also shown for univariable and multivariable analyses with Q1-4 as a continuous variable and other co-variables as per Table 1c. Note that the numbers in each quartile are not equal owing to ties in mutation burden.

Figure 2. Relapse-free survival by combinations of MSI and mutations in *KRAS* and *BRAF* in the combined extended QUASAR2 and Australian cohorts. Cancers that carried pathogenic *POLE* mutations were excluded.

Figure 3. Relapse-free survival by combinations of mutations in *KRAS, BRAF* and *TP53* in the combined extended QUASAR2 and Australian cohorts. Cancers that were MSI+ or carried pathogenic *POLE* mutations were excluded.