BRAF and *NRAS* locus specific variants have different outcomes on survival to colorectal cancer

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STATEMENT OF TRANSLATIONAL RELEVANCE

Somatic mutation status at *KRAS*, *BRAF* and *NRAS* affects prognosis in patients with advanced colorectal cancer (aCRC) and it has been presumed that different variants in the same gene confer similar prognostic outcomes. Here, we studied inter- and intra-locus variant co-occurrence and variant-specific differences in survival and clinicopathology by analysing 2,157 patients with aCRC. We found significant differences between variants in *BRAF* (c.1781A>G [p.D594G] versus c.1799T>A [p.V600E]) and *NRAS* (mutant codons 12 and 13 versus codon 61) both in terms of co-occurrence with *KRAS* mutations and in their influence on survival. These data need to be considered in patient management and personalised therapy.

ABSTRACT

Purpose

Somatic mutation status at *KRAS*, *BRAF* and *NRAS* is associated with prognosis in patients with advanced colorectal cancer (aCRC); however, it remains unclear whether there are intra-locus, variant-specific differences in survival and other clinicopathological parameters.

Experimental design

We profiled 2,157 aCRCs for somatic mutations in *KRAS*, *BRAF* and *NRAS* and determined microsatellite instability status. We sought inter- and intra-locus correlations between mutations, and variant-specific associations with survival and clinicopathology.

Results

KRAS mutations were rarely found together and those in codons 12 and 13 conferred poor prognosis (HR 1.44, 95% CI 1.28-1.61, $p=6.4e^{-10}$ and HR 1.53, 95% CI 1.26-1.86, $p=1.5e^{-05}$, respectively). For *BRAF*, more c.1781A>G (p.D594G) CRCs carried *RAS* mutations (14% [3/21]) compared to c.1799T>A (p.V600E) CRCs (1% [2/178], $p=9.0e^{-03}$). c.1799T>A (p.V600E) was associated with poor prognosis (HR 2.60, 95% CI 2.06-3.28, $p=1.0e^{-15}$), whereas c.1781A>G (p.D594G) was not (HR 1.30, 95% CI 0.73-2.31, p=0.37); this intra-locus difference was significant (p=0.04). More c.1799T>A (p.V600E) CRCs were found in the right colon (47% [47/100]), compared to c.1781A>G (p.D594G) CRCs (7% [1/15], $p=3.7e^{-03}$). For *NRAS*, 5% (3/60) of codon 61 mutant CRCs had *KRAS* mutations compared to 44% (10/23) of codons 12 and 13 mutant CRCs ($p=7.9e^{-05}$). Codon 61 mutations conferred poor

prognosis (HR 1.47, 95% CI 1.09-1.99, p=0.01), whereas codons 12 and 13 mutations did not (HR 1.29, 95% CI 0.64-2.58, p=0.48).

Conclusions

Our data show considerable intra-locus variation in the outcomes of mutations in *BRAF* and *NRAS*. These data need to be considered in patient management and personalised cancer therapy.

INTRODUCTION

The only routinely used prognostic marker for survival after diagnosis of colorectal cancer (CRC) is clinical stage, which combines depth of tumour invasion, nodal status and distant metastasis (1). In stage 4 disease, Köhne's index based on performance status, white blood cell count, alkaline phosphatase levels and number of metastatic sites has been proposed (2). Other factors thought to influence survival include lifestyle (3,4), systemic inflammatory response to the tumour (5), tumour immunologic environment (6), and the germline (7) and somatic (8-11) molecular profiles. By studying patients with advanced CRC (aCRC) from the Medical Research Council (MRC) COIN trial, we previously showed that the somatic mutation status at *KRAS* and *BRAF*, and microsatellite instability (MSI), conferred poor prognosis irrespective of treatment: overall survival (OS, trial enrolment to death) *KRAS* mutant 14.4 months (12), *BRAF* mutant 8.8 months (12), MSI 9.3 months (13), all wild type 20.1 months (12). We also showed that neither individual somatic mutations, nor mutations grouped by codon or gene, affected response to cetuximab (13).

It remains unclear whether there are intra-locus, variant-specific differences in survival and this has been difficult to study for the less frequently mutated loci (such as c.1781A>G [p.D594G] in *BRAF*) due to the large numbers of samples required to make statistically robust associations. Here, we studied the influence of individual or codon specific somatic mutations in *KRAS*, *BRAF* and *NRAS* in 2,157 patients with aCRC from COIN (12) and COIN-B (14).

MATERIALS AND METHODS

Patients and samples

We prepared tumour DNA samples from unrelated patients with aCRC from the MRC clinical trials COIN (NCT00182715) (12) and COIN-B (NCT00640081) (14), as previously described (12,13). All patients had either previous or current histologically confirmed primary adenocarcinomas of the colon or rectum, together with clinical or radiological evidence of advanced and/or metastatic disease, or had histologically/cytologically confirmed metastatic adenocarcinomas, together with clinical and/or radiological evidence of a colorectal primary tumour. COIN patients were randomised 1:1:1 to receive continuous oxaliplatin and fluoropyrimidine chemotherapy, continuous chemotherapy plus cetuximab, or intermittent chemotherapy. COIN-B patients were randomised 1:1 to receive intermittent chemotherapy plus continuous cetuximab or intermittent chemotherapy plus intermittent cetuximab. All patients gave informed consent for their samples to be used for bowel cancer research (approved by REC [04/MRE06/60]).

Somatic analyses

We previously screened for somatic mutations in *KRAS* (codons 12, 13 and 61), *BRAF* (codons 594 and 600) and *NRAS* (codons 12, 13 and 61) using a combination of Pyrosequencing and Sequenom (13); for samples analysed by both technologies (n=1,612), genotype concordance in *KRAS* was 99% (8,642/8,719 calls were concordant). MSI status was determined using the markers BAT-25 and BAT-26 (13).

Mutation co-occurrence, survival and statistical analyses

We sought inter- and intra-locus correlations between somatic *KRAS*, *BRAF* and *NRAS* mutations and MSI status. Data was analysed using R (http://www.r-project.org). *Corrplot* was used to create a correlation matrix plot (*recode* from *car* was used to recode the data into binary format) and *Survfit, survdiff* and *coxph.test* from the OIsurv package and *ggsurv* from the GGally package were used to create and analyse the survival curves. To avoid potential confounding affects from other mutant loci, *KRAS* mutants (versus wild type) were analysed on a *BRAF* and *NRAS* wild type background; *BRAF* mutants (versus wild type) were analysed on a *KRAS* and *NRAS* (*RAS*) wild type and MSS background; *NRAS* mutants (versus wild type) were analysed on a *KRAS* and *BRAF* wild type background; and MSI (versus MSS) was analysed on a *RAS* and *BRAF* wild type background. We found no evidence of heterogeneity in OS between patients when analysed by trial (COIN versus COIN-B, p=0.49), trial arm (p=0.40 Cochran's Q Test: p=1.0 I^2 Test: p=0.74), type of chemotherapy received (OxMdG/XELOX) (p=0.60) or cetuximab use (p=0.41), so combined these groups for the survival analyses. We used Chi-square Tests or Fisher's Exact Test to study whether *KRAS*, *BRAF* and *NRAS* mutations and MSI status were associated with different clinicopathological findings. We corrected for multiple testing using Bonferroni correction ($p<1.7e^{-03}$ [n=30] for survival tests, $p<1.0e^{-04}$ [n=480] for somatic mutation cross-correlations, $p<1.3e^{-03}$ [n=39] for clinicopathological analyses of *KRAS*, *BRAF* and *NRAS* and $p<3.8e^{-03}$ [n=13] for clinicopathological analyses of MSI).

RESULTS

We screened for somatic *KRAS*, *BRAF* and *NRAS* mutations and for MSI status in aCRCs from 2,157 patients from the clinical trials COIN and COIN-B. In total, we

detected 14 *KRAS* mutations (c.34G>A [p.G12S], c.34G>C [p.G12R], c.34G>T [p.G12C], c.35G>A [p.G12D], c.35G>C [p.G12A], c.35G>T [p.G12V], c.37G>A [p.G13S], c.37G>C [p.G13R], c.37G>T [p.G13C], c.38G>A [p.G13D], c.38G>T [p.G13V], c.182A>G [p.Q61R], c.182A>T [p.Q61L] and c.183A>C [p.Q61H]) in 40% (858/2,157) aCRCs, 2 *BRAF* mutations (c.1781A>G [p.D594G] and c.1799T>A [p.V600E]) in 9% (199/2,097), 9 *NRAS* mutations (c.34G>T [p.G12C], c.35G>A [p.G12D], c.35G>T [p.G12V], c.37G>C [p.G13R], c.38G>A [p.G13D], c.181C>A [p.Q61K], c.182A>G [p.Q61R], c.182A>T [p.Q61L] and c.183A>C [p.Q61H]) in 4% (83/2,092) and MSI in 4% (66/1,567). Over 99% (2,152/2,157) of aCRCs harbouring *KRAS, BRAF* and *NRAS* mutations carried only a single variant allele at their respective loci (five CRCs carried two *KRAS* mutations; however, due to their rarity, these were likely to reflect mixed tumour populations).

Inter- and intra-genic mutation correlations

All mutations in *KRAS*, regardless of whether analysed individually or by codon, showed similar effects in terms of mutual exclusivity (Supplementary Figure). Codon 12 (4 of 627 mutant CRCs), 13 (4 of 161) and 61 (2 of 35) mutations were rarely found together.

Only specific mutations in *BRAF* (c.1799T>A [p.V600E]) and *NRAS* (codon 61 mutations) shared this characteristic. Only 1% (2/178) of *BRAF* c.1799T>A (p.V600E) CRCs had *RAS* mutations compared to 47% (894/1908) of *BRAF* wild type CRCs ($p<2.2e^{-16}$, $p<1.1e^{-13}$ after correction for multiple testing). In contrast, more *BRAF* c.1781A>G (p.D594G) mutations co-occurred with *RAS* mutations (14% [3/21]) as compared to c.1799T>A (p.V600E) ($p=9.0e^{-03}$); albeit less commonly than

found in *BRAF* wild-type CRCs ($p=3.0e^{-03}$). We noted one case of *KRAS* c.37G>A (p.G13S) which co-occurred with *BRAF* c.1799T>A (p.V600E) ($p=2.5e^{-03}$ as compared to other *KRAS* mutations [1/812 co-occurred]). For *NRAS*, only 5% (3/60) of codon 61 mutant CRCs had *KRAS* mutations compared to 43% (10/23) of codons 12 and 13 mutant CRCs ($p=7.9e^{-05}$, p=0.04 after correction); the latter being at similar level to that found in wild type CRCs (40% [808/2018], p=0.98).

We also observed differences in the relationship between *BRAF* mutations and MSI status. *BRAF* c.1799T>A (p.V600E) was strongly associated with MSI (11% [20/178] c.1799T>A [p.V600E] CRCs had MSI compared to 2% [46/1,908] wild type CRCs, $p=5.3e^{-10}$, $p=2.5e^{-07}$ after correction), whereas *BRAF* c.1781A>G (p.D594G) and MSI did not co-occur (0/21).

Survival analyses

Five *KRAS* mutations (c.34G>A [p.G12S], c.35G>A [p.G12D], c.35G>C [p.G12A], c.35G>T [p.G12V] and c.38G>A [p.G13D]) individually showed significantly poorer prognosis with a median reduction in survival of 213, 111, 65, 160 and 165 days, respectively; four of these remained significant after correction for multiple testing (Table 1). When grouped by codons, both codon 12 and 13 mutations conferred poor prognosis (HR 1.44, 95% CI 1.28-1.61, $p=6.4e^{-10}$, $p=1.9e^{-08}$ after correction, and HR 1.53, 95% CI 1.26-1.86, $p=1.5e^{-05}$, $p=4.5e^{-04}$ after correction, respectively), whereas codon 61 mutations did not (HR 1.23, 95% CI 0.84-1.81, p=0.28) (Table 1); these intra-locus differences were not significant.

c.1799T>A (p.V600E) in *BRAF* was strongly associated with poor prognosis (HR 2.60, 95% CI 2.06-3.28, $p=1.0e^{-15}$, $p=3.0e^{-14}$ after correction, median reduction in survival 320 days) (Fig.1), whereas c.1781A>G (p.D594G) was not (HR 1.30, 95% CI 0.73-2.31, p=0.37); this intra-locus difference was significant (p=0.04) (Table 1).

Although individual *NRAS* mutations showed no differences in survival, when grouped by codon, codon 61 mutations conferred poor prognosis (HR 1.47, 95% CI 1.09-1.99, p=0.01, median reduction in survival 131 days) (Fig.1), whereas codons 12 and 13 mutations did not (HR 1.29, 95% CI 0.64-2.58, p=0.48); however, this intra-locus difference was not significant (p=0.73).

Patients with MSI CRCs had worse prognosis compared to those with stable tumours (HR 1.86, 95% CI 1.22-2.83, $p=4.0e^{-03}$), in agreement with our previous study (13).

For all analyses described *herein*, there were no significant differences measured using heterogeneity tests when the analyses were performed using date of diagnosis to death instead of OS (Supplementary Table S1) or when split by cetuximab use (Supplementary Table S2).

Clinicopathological analyses

KRAS

More *KRAS* mutant CRCs were found in the right colon (58% [182/314]) and caecum (70% [62/88]) as compared to the left colon (38% [123/326], $p=4.6e^{-07}$ and $8.4e^{-08}$,

respectively) and more were associated with metastases in the lung (50% [358/715]) as compared to liver only (37% [156/418], $p=4.2e^{-05}$) (Table 2).

In terms of codon specific mutations, more *KRAS* codon 12 and 13 mutant CRCs were found in the right colon (23% [173/760] versus 13% [132/1002], $p=1.9e^{-07}$) and caecum (8% [61/760] versus 3% [26/1002], $p=3.4e^{-07}$), less in the left colon (15% [117/760] versus 20% [203/1002], p=0.01) and sigmoid colon (5% [41/760] versus 11% [115/1002], $p=1.3e^{-05}$) and more were associated with metastases in the lung (45% [342/760] versus 36% [357/1002], $p=8.4e^{-05}$) and less in liver only (20% [152/760] versus 26% [262/1002], $p=3.1e^{-03}$), as compared to wild type CRCs; the correlations for right colon, caecum, sigmoid colon and lung remained significant after correction for multiple testing (Table 2). More *KRAS* codon 61 mutant patients had CRCs in the right colon (27% [9/33] versus 13% [132/1002], p=0.04) and more had peritoneal metastases (27% [9/33] versus 13% [133/1002], p=0.04) as compared to wild type patients. However, there were no significant differences in clinicopathology between *KRAS* codons 12 and 13 versus codon 61 mutant patients.

BRAF

More *BRAF* mutant CRCs were found in the right colon (38% [48/128]) as compared to the left colon (12% [18/146], $p=2.4e^{-05}$), and more were associated with metastases in the peritoneum (23% [25/107]) as compared to liver only (10% [22/214], $p=3.1e^{-03}$) (Table 3).

In terms of individual mutations, *BRAF* c.1781A>G (p.D594G) CRCs had similar clinicopathology to wild type CRCs (Table 3). In contrast, more *BRAF* c.1799T>A

(p.V600E) CRCs were found in the right colon (47% [47/100] versus 12% [80/693], $p<2.2e^{-16}$), and less in the rectum (11% [11/100] versus 34% [234/693], $p=7.1e^{-06}$) and sigmoid colon (2% [2/100] versus 11% [73/693], $p=3.2e^{-03}$) and more were associated with peritoneal metastases (24% [24/100] versus 12% [82/693], $p=1.5e^{-03}$) as compared to wild type CRCs; the correlations for right colon and rectum remained significant after correction for multiple testing (Table 3).

In terms of intra-locus differences, there was a significant difference between c.1781A>G (p.D594G) and c.1799T>A (p.V600E) CRCs in the location of the primary tumour ($p=9.3e^{-05}$, $p=3.6e^{-03}$ after correction), due to fewer c.1781A>G (p.D594G) CRCs in the right colon (7% [1/15] versus 47% [47/100], $p=3.7e^{-03}$), and more in the rectum (60% [9/15] versus 11% [11/100], $p=1.7e^{-05}$, $p=6.6e^{-04}$ after correction) (Table 3). There was no significant difference between the sites of metastases associated with these mutations.

NRAS

There was no difference between the frequency of *NRAS* mutant and wild type CRCs in the site of the primary tumour (Table 4). However, more *NRAS* mutant CRCs were associated with metastases in the lung (11% [43/400]) as compared to liver only (4% [10/272], $p=1.4e^{-03}$).

In terms of individual codons, codon 12 and 13 mutant CRCs showed similar clinicopathology to wild type CRCs (Table 4). Codon 61 mutant CRCs had similar primary tumour distributions but significantly fewer liver only (12% [7/57] versus 26% [262/1002], p=0.03) and more lung metastases (68% [39/57] versus 36% [357/1002],

p=1.3e^{-06}, p=5.1e^{-05} after correction) as compared to wild type CRCs (Table 4). There were no significant differences in clinicopathology between codons 12 and 13 versus codon 61 mutant CRCs.

MSI

More MSI CRCs were found in the right colon (41% [12/29] versus 12% [80/693], p=9.2e^{-06}, p=1.2e^{-04} after correction) and less in the rectosigmoid junction (3% [1/29] versus 18% [126/693], p=0.04) and less were associated with liver metastases (48% [14/29] versus 77% [536/693], p=7.3e^{-04}, p=9.5e^{-03} after correction) as compared to MSS CRCs (Table 5).

DISCUSSION

Variants in *BRAF* and *NRAS* have been presumed to confer similar oncogenic and prognostic outcomes; however, here we demonstrate clear intra-locus differences. For *BRAF*, c.1799T>A (p.V600E) was almost mutually exclusive of *RAS* mutations and was associated with poor prognosis. In contrast, c.1781A>G (p.D594G) was more often associated with *RAS* mutations and had no apparent influence on survival. However, c.1781A>G (p.D594G) is unlikely to be benign and more likely to be hypomorphic, as it had significantly fewer co-occurrences with *RAS* mutations as compared to *BRAF* wild type CRCs. Interestingly, our data are consistent with a recent report showing that patients with codon 594 or 596 mutated tumours had longer OS compared to those with c.1799T>A (p.V600E) CRCs (15). There are clear biological differences between these mutant codons to support our observed pathological differences; p.V600E increased extracellular signal-regulated kinase (ERK) and nuclear factor kappaB (NFκB) signalling and the transformation of

NIH3T3 cells, whereas p.D594V failed to activate ERK (16) and did not affect NFκB signalling nor NIH3T3 transforming activity (17).

Others have reported that *NRAS* mutant patients have shorter OS as compared to wild type patients (HR 1.91, 95% CI 1.39-3.86; $p=1.0e^{-03}$) (18). Here, we noted a more complex relationship; *NRAS* codon 61 mutations, which were rarely associated with *KRAS* mutations, conferred a poor prognosis, but codons 12 and 13 mutations, which co-occurred with *KRAS* mutations at similar frequencies to wild type CRCs, had little influence on survival. Together, our data suggest that *NRAS* codons 12 and 13 mutations may have a minor role in colorectal tumourigenesis. Interestingly, using mouse models others have shown that endogenous levels of *Nras* p.Q61R, but not *Nras* p.G12D, were able to efficiently drive *in vivo* melanomagenesis (19), supporting their differing biological effects.

We have also shown that different mutant loci are associated with differences in the clinicopathology of the primary tumours and/or their sites of metastases. For example, in agreement with two recent reports (20, 21), we observed more *KRAS* mutant CRCs in the caecum (70%) and, to a lesser extent, in the right colon (58%), as compared to the left colon (38%). It has been suggested that different somatic profiles are associated with different clinicopathology, by influencing the tumour's biological behaviour (22). Here, we focussed on intra-locus differences and found a significant difference between c.1781A>G (p.D594G) and c.1799T>A (p.V600E) in *BRAF* in the location of the primary tumour providing additional support for these variants having different biological effects.

In conclusion, our study shows considerable intra-locus variations in survival, particularly in the outcomes of mutations in *BRAF* and *NRAS*. These data need to be considered in patient management.

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Table 1. Prognostic outcomes of individual mutations, or mutations grouped by codon or gene on OS.

Gene/event	Mutation/codon	No of events[1]	HR	95% CIs	p-value
KRAS	c.34G>A (p.G12S)	35	1.78	1.27-2.50	$9.2e^{-04}$ (0.03)
	c.34G>C (p.G12R)	10	0.95	0.51-1.78	0.88
	c.34G>T (p.G12C)	52	1.21	0.91-1.60	0.18
	c.35G>A (p.G12D)	187	1.48	1.26-1.74	$1.6e^{-06}$ ($4.8e^{-05}$)
	c.35G>C (p.G12A)	41	1.43	1.04-1.96	0.03
	c.35G>T (p.G12V)	161	1.48	1.25-1.76	$7.5e^{-06}$ ($2.3e^{-04}$)
	c.37G>T (p.G13C)	6	1.36	0.61-3.03	0.46
	c.38G>A (p.G13D)	116	1.53	1.26-1.87	$2.2e^{-05}$ ($6.6e^{-04}$)
	c.38G>T (p.G13V)	1	-	-	-
	c.182A>G (p.Q61R)	6	1.41	0.63-3.15	0.41
	c.182A>T (p.Q61L)	6	1.27	0.57-2.84	0.56
	c.183A>C (p.Q61H)	15	1.17	0.70-1.95	0.56
	Codon 12	486	1.44	1.28-1.61	$6.4e^{-10}$ ($1.9e^{-08}$)
	Codon 13	123	1.53	1.26-1.86	$1.5e^{-05}$ ($4.5e^{-04}$)
	Codon 61	27	1.23	0.84-1.81	0.28
	Any *KRAS* mutation	632	1.45	1.30-1.61	$1.9e^{-11}$ ($5.7e^{-10}$)
BRAF	c.1781A>G (p.D594G)	12	1.30	0.73-2.31	0.37
	c.1799T>A (p.V600E)	87	2.60	2.06-3.28	$1.0e^{-15}$ ($3.0e^{-14}$)
	Any *BRAF* mutation	99	2.31	1.85-2.87	$7.8e^{-14}$ ($2.3e^{-12}$)
NRAS	c.34G>T (p.G12C)	5	1.42	0.59-3.43	0.43
	c.35G>A (p.G12D)	2	-	-	-
	c.35G>T (p.G12V)	1	-	-	-
	c.181C>A (p.Q61K)	21	1.43	0.96-2.21	0.11
	c.182A>G (p.Q61R)	13	1.58	0.91-2.73	0.11
	c.182A>T (p.Q61L)	11	1.51	0.83-2.73	0.18
	Codons 12 and 13	8	1.29	0.64-2.58	0.48
	Codon 61	45	1.47	1.09-1.99	0.01
	Any *NRAS* mutation	53	1.44	1.09-1.90	0.01
MSI	MSI	23	1.86	1.22-2.83	$4.0e^{-03}$
	MSS	476	1.00	ref.	ref.

KRAS mutants (versus wild type) were analysed on a *BRAF* and *NRAS* wild type background; *BRAF* mutants (versus wild type) were analysed on a *RAS* wild type and MSS background; *NRAS* mutants (versus wild type) were analysed on a *KRAS* and *BRAF* wild type background; and MSI (versus MSS) was analysed on a *RAS* and *BRAF* wild type background. Number of events, HR, CIs and p-values are shown (except for cases where

number of events ≤2). [1]Mutations not listed when number of events=0. p-values that remained significant after correction for multiple testing are shown in *parentheses*.

Table 2: Clinicopathology according to *KRAS* mutation status

Characteristics		Frequency of *KRAS* mutations[1]	codons 12 and 13 (n= 760)	codon 61 (n= 33)	wild type (n= 1002)	p (codons 12 and 13 vs. wild type)	p (codon 61 vs. wild type)	p (codons 12 and 13 vs. codon 61)
Sex	Female	289/593 (49)	275 (36)	14 (42)	304 (30)	0.01	0.20	0.59
	Male	503/1201 (42)	485 (64)	19 (58)	698 (70)	0.01	0.20	0.59
Age	Mean	NA	63	61	63	NA	NA	NA
Primary Tumour Site	Right Colon	182/314 (58)	173 (23)	9 (27)	132 (13)	$1.9e^{-07}$ [$7.4e^{-06}$]	0.04	0.70
	Caecum	62/88 (70)	61 (8)	1 (3)	26 (3)	$3.4e^{-07}$ [$1.3e^{-05}$]	0.59	0.51
	Transverse Colon	14/35 (40)	14 (2)	0 (0)	21 (2)	0.84	1.0	1.0
	Left Colon	123/326 (38)	117 (15)	6 (18)	203 (20)	0.01	0.94	0.85
	Sigmoid Colon	44/159 (28)	41 (5)	3 (9)	115 (11)	$1.3e^{-05}$ [$5.1e^{-04}$]	1.0	0.42
	Rectosigmoid Junction	108/269 (40)	105 (14)	3 (9)	161 (16)	0.22	0.34	0.61
	Rectum	251/577 (44)	241 (32)	10 (30)	326 (33)	0.75	0.94	1.0
Site of Metastases[2]	Liver Only	156/418 (37)	152 (20)	4 (12)	262 (26)	$3.1e^{-03}$	0.07	0.37
	Liver	598/1356 (44)	577 (76)	22 (67)	758 (76)	0.94	0.33	0.32
	Nodal	359/832 (43)	345 (45)	15 (45)	473 (47)	0.48	0.98	1.0
	Lung	358/715 (50)	342 (45)	16 (48)	357 (36)	$8.4e^{-05}$ [$3.3e^{-03}$]	0.18	0.83
	Peritoneum	126/259 (49)	117 (15)	9 (27)	133 (13)	0.23	0.04	0.11

Mutations were analysed on an *NRAS* and *BRAF* wild type background. [1]There was a significant difference between *KRAS* mutant CRCs in the location of the primary tumour ($p=6.4e^{-14}$) and in the sites of metastases ($p=4.6e^{-04}$) as compared to wild type CRCs. Percentages are shown in *regular parentheses* ([2]some patients had multiple metastases so percentages do not add up to 100%). p-values that remained significant after correction for multiple testing are shown in *square parentheses*. NA - not applicable. Discrepancies in column totals are due to patients with multiple mutations or due to missing data.

Table 3: Clinicopathology according to *BRAF* mutation status

Characteristics		Frequency of *BRAF* mutations[1]	c.1781A>G (p.D594G) (n = 15)	c.1799T>A (p.V600E) (n = 100)	wild type (n = 693)	p (c.1781A>G [p.D594G] vs. wild type)	p (c.1799T>A [p.V600E] vs. wild type)	p (c.1781A>G [p.D594G] vs. c.1799T>A [p.V600E))
Sex	Female	55/249 (22)	7 (47)	48 (48)	194 (28)	0.20	$8.0e^{-05}$ [$3.1e^{-03}$]	1.0
	Male	60/559 (11)	8 (53)	52 (52)	499 (72)	0.20	$8.0e^{-05}$ [$3.1e^{-03}$]	1.0
Age	Mean	NA	67	63	63	NA	NA	NA
Primary Tumour Site	Right Colon	48/128 (38)	1 (7)	47 (47)	80 (12)	1.0	$<2.2e^{-16}$ [$<8.6e^{-15}$]	$3.7e^{-03}$
	Caecum	4/24 (17)	0 (0)	4 (4)	20 (3)	1.0	0.53	1.0
	Transverse Colon	4/20 (20)	0 (0)	4 (4)	16 (2)	1.0	0.30	1.0
	Left Colon	18/146 (12)	1 (7)	17 (17)	128 (18)	0.34	0.83	0.46
	Sigmoid Colon	4/77 (5)	2 (13)	2 (2)	73 (11)	0.67	$3.2e^{-03}$	0.08
	Rectosigmoid Junction	15/141 (11)	2 (13)	13 (13)	126 (18)	1.0	0.26	1.0
	Rectum	20/254 (8)	9 (60)	11 (11)	234 (34)	0.07	$7.1e^{-06}$ [$2.8e^{-04}$]	$1.7e^{-05}$ [$6.6e^{-04}$]
Site of Metastases[2]	Liver Only	22/214 (10)	3 (20)	19 (19)	192 (28)	0.77	0.09	1.0
	Liver	83/619 (13)	13 (87)	70 (70)	536 (77)	0.54	0.14	0.23
	Nodal	53/368 (14)	7 (47)	46 (46)	315 (45)	1.0	1.0	1.0
	Lung	35/272 (13)	6 (40)	29 (29)	237 (34)	0.85	0.36	0.57
	Peritoneum	25/107 (23)	1 (7)	24 (24)	82 (12)	1.0	$1.5e^{-03}$	0.19

Mutations analysed on a *RAS* wild type and MSS background. [1]There was a significant difference between *BRAF* mutant CRCs in the location of the primary tumour ($p=1.2e^{-13}$) and in the sites of metastases (p=0.03) as compared to wild type CRCs. Percentages are shown in *regular parentheses* ([2]some patients had multiple metastases so percentages do not add up to 100%). p-values that remained significant after correction for multiple testing are shown in *square parentheses*. NA - not applicable. Discrepancies in column totals are due to patients with multiple mutations or due to missing data.

Table 4: Clinicopathology according to *NRAS* mutation status

Characteristics		Frequency of *NRAS* mutations[1]	codons 12 and 13 (n = 11)	codon 61 (n = 57)	wild type (n = 1002)	p (codons 12 and 13 vs. wild type)	p (codon 61 vs. wild type)	p (codons 12 and 13 vs. codon 61)
Sex	Female	20/324 (6)	2 (18)	18 (32)	304 (30)	0.52	0.96	0.49
	Male	48/746 (6)	9 (82)	39 (68)	698 (70)	0.52	0.96	0.49
Age	Mean	NA	59	62	63	NA	NA	NA
Primary Tumour Site	Right Colon	5/137 (4)	2 (18)	3 (5)	132 (13)	0.65	0.10	0.18
	Caecum	4/30 (13)	0 (0)	4 (7)	26 (3)	1.0	0.07	1.0
	Transverse Colon	3/24 (13)	0 (0)	3 (5)	21 (2)	1.0	0.13	1.0
	Left Colon	12/215 (6)	1 (9)	11 (19)	203 (20)	0.70	1.0	0.67
	Sigmoid Colon	11/126 (9)	2 (18)	9 (16)	115 (11)	0.37	0.44	1.0
	Rectosigmoid Junction	10/171 (6)	0 (0)	10 (18)	161 (16)	0.23	0.91	0.20
	Rectum	19/345 (6)	6 (55)	13 (23)	326 (33)	0.22	0.17	0.08
Site of Metastases[2]	Liver Only	10/272 (4)	3 (27)	7 (12)	262 (26)	1.0	0.03	0.35
	Liver	52/810 (6)	8 (73)	44 (77)	758 (76)	0.74	0.92	0.71
	Nodal	35/508 (7)	3 (27)	32 (56)	473 (47)	0.23	0.24	0.11
	Lung	43/400 (11)	4 (36)	39 (68)	357 (36)	1.0	$1.3e^{-06}$ [$5.1e^{-05}$]	0.08
	Peritoneum	5/138 (4)	1 (9)	4 (7)	133 (13)	1.0	0.22	1.0

Mutations analysed on a *KRAS* and *BRAF* wild type background. [1]There was a significant difference between *NRAS* mutant CRCs in the sites of metastases ($p=2.5e^{-03}$) as compared to wild type CRCs. Percentages are shown in *regular parentheses* ([2]some patients had multiple metastases so percentages do not add up to 100%). p-values that remained significant after correction for multiple testing are shown in *square parentheses*. NA - not applicable. Discrepancies in column totals are due to patients with multiple mutations or due to missing data.

Table 5: Clinicopathology according to MSI status

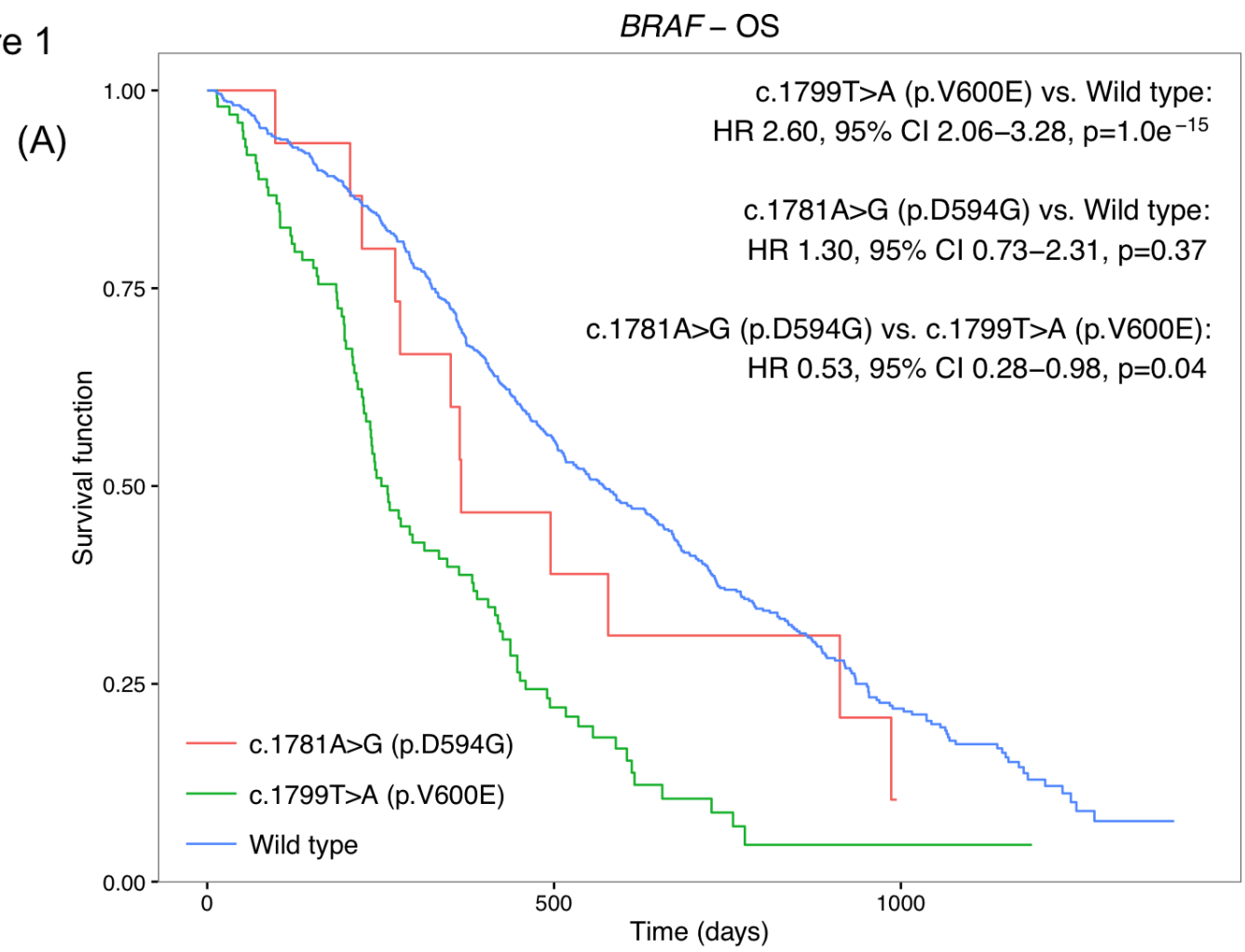
Characteristics		Frequency of MSI[1]	MSI (n=29)	MSS (n=693)	p (MSI vs. MSS)
Sex	Female	11/205 (5)	11 (38)	194 (28)	0.34
	Male	18/517 (3)	18 (62)	499 (72)	0.34
Age	Mean	NA	58	63	NA
Primary Tumour Site	Right Colon	12/92 (13)	12 (41)	80 (12)	$9.2e^{-06}$ [$1.2e^{-04}$]
	Caecum	1/21 (5)	1 (3)	20 (3)	0.58
	Transverse Colon	1/17 (6)	1 (3)	16 (2)	0.51
	Left Colon	7/135 (5)	7 (24)	128 (18)	0.60
	Sigmoid Colon	1/76 (1)	1 (3)	73 (11)	0.35
	Rectosigmoid Junction	1/127 (1)	1 (3)	126 (18)	0.04
	Rectum	6/240 (3)	6 (21)	234 (34)	0.21
Site of Metastases[2]	Liver Only	3/195 (2)	3 (10)	192 (28)	0.05
	Liver	14/550 (3)	14 (48)	536 (77)	$7.3e^{-04}$ [$9.5e^{-03}$]
	Nodal	17/332 (5)	17 (59)	315 (45)	0.23
	Lung	6/243 (2)	6 (21)	237 (34)	0.19
	Peritoneum	7/89 (8)	7 (24)	82 (12)	0.09

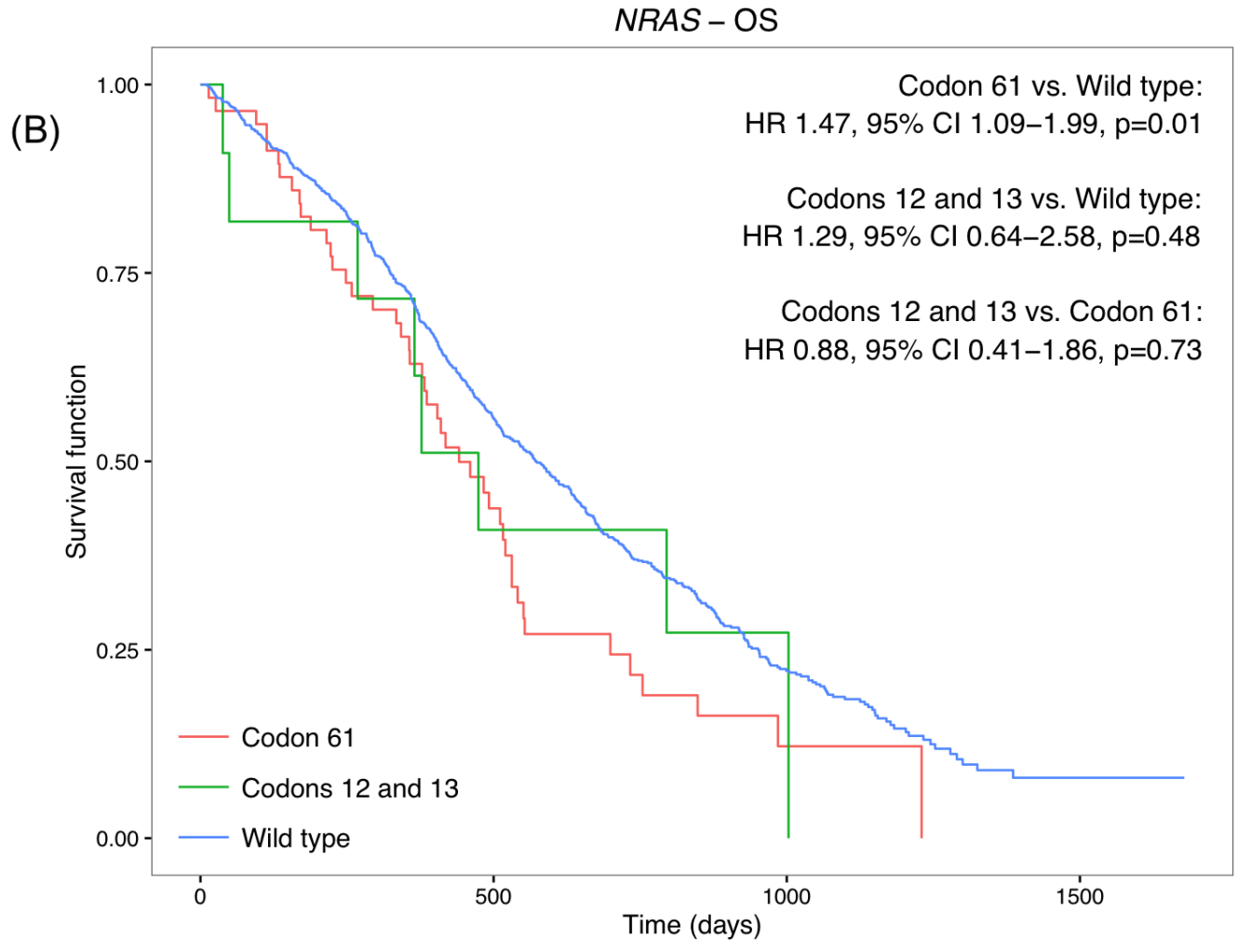
MSI status was analysed on an *RAS* and *BRAF* wild type background. [1]There was a significant difference between MSI CRCs in the location of the primary tumour ($p=2.5e^{-04}$) and in the sites of metastases (p=0.02) as compared to MSS CRCs. Percentages are shown in *regular parentheses* ([2]some patients had multiple metastases so percentages do not add up to 100%). p-values that remained significant after correction for multiple testing are shown in *square parentheses*. NA - not applicable. Discrepancies in column totals are due to patients with multiple mutations or due to missing data.

LEGEND TO FIGURE

Figure 1. Kaplan-Meier plots showing the prognostic outcome of **(A)** c.1781A>G (p.D594G) and c.1799T>A (p.V600E) in *BRAF*, and **(B)** codons 12 and 13 and codon 61 mutations in *NRAS*.

Figure 1


(A)
BRAF – OS
c.1799T>A (p.V600E) vs. Wild type:
HR 2.60, 95% CI 2.06–3.28, p=1.0e−15
c.1781A>G (p.D594G) vs. Wild type:
HR 1.30, 95% CI 0.73–2.31, p=0.37
c.1781A>G (p.D594G) vs. c.1799T>A (p.V600E):
HR 0.53, 95% CI 0.28–0.98, p=0.04
Survival function
1.00
0.75
0.50
0.25
0.00
0
500
1000
Time (days)
c.1781A>G (p.D594G)
c.1799T>A (p.V600E)
Wild type


(B)
NRAS – OS
Codon 61 vs. Wild type:
HR 1.47, 95% CI 1.09–1.99, p=0.01
Codons 12 and 13 vs. Wild type:
HR 1.29, 95% CI 0.64–2.58, p=0.48
Codons 12 and 13 vs. Codon 61:
HR 0.88, 95% CI 0.41–1.86, p=0.73
Survival function
1.00
0.75
0.50
0.25
0.00
0
500
1000
1500
Time (days)
Codon 61
Codons 12 and 13
Wild type