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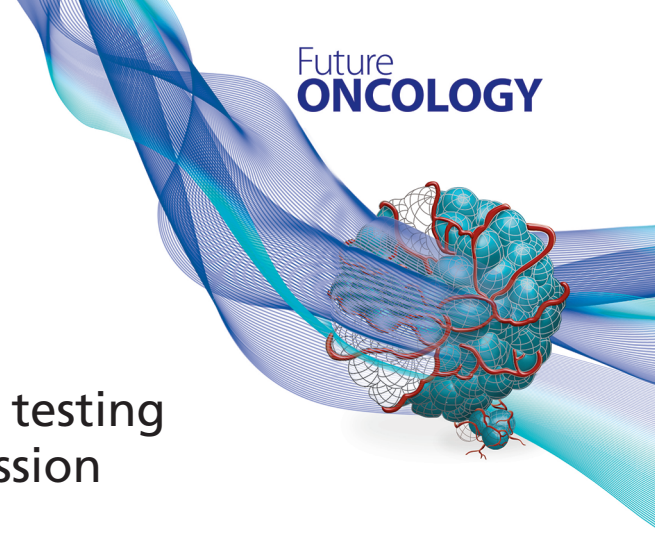

Blood-based *RAS* mutation testing: concordance with tissue-based *RAS* testing and mutational changes on progression

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Aim: To determine the concordance between plasma and tissue *RAS* mutation status in metastatic colorectal cancer patients to gauge whether blood-based testing is a viable alternative. We also evaluated the change in mutation status on progression. **Materials/methods:** *RAS* testing was performed on plasma from patients commencing first-line therapy (OncoBEAM™ RAS CEIVD kit). Results were then compared with formalin-fixed paraffin embedded tumor samples. **Results:** The overall percentage agreement (concordance) was 86.0% (86/100), which demonstrates that blood-based testing is an alternative to tissue-based testing. Reproducibility was 100% between three laboratories and 20% showed changes in their *RAS* mutational status on progression. **Conclusion:** These results show good concordance between tissue and plasma samples and suggest the need for longitudinal plasma testing during treatment to guide management decisions.

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Randomized controlled trials have demonstrated the benefit of anti-EGFR monoclonal antibodies such as cetuximab and panitumumab as single agents or in combination with other chemotherapeutic regimens in metastatic colorectal cancer (mCRC) patients [1–3]. Prior to initiation of anti-EGFR therapy, *RAS* (*KRAS*, Kirsten RAt Sarcoma virus; *NRAS*, Neuroblastoma RAt Sarcoma virus) genotyping is performed and mutations are detected in approximately 50% of patients with mCRC [4,5].

RAS mutation detection in routine clinical practice is usually performed using DNA extracted from formalin-fixed paraffin embedded (FFPE) tumor specimens [6]. Tissue processing, however, presents several practical challenges, including poor tissue quality, extensive workflow, lack of standardization of testing methodologies and delays in treatment. Results from Europe revealed that 48% of laboratories failed to complete *RAS* genotyping within 14 days [7]. From a biological perspective, accurate genotype determination can be obscured by tumor molecular heterogeneity [8]. A single tumor site may contain multiple clones with varying mutational status. A single tissue biopsy may therefore not represent true mutational burden, particularly in patients with tumor recurrence and multiple metastases [9]. Thus, for a variety of reasons, the aggregate of data suggests that although tissue has traditionally served as the primary material for *RAS* testing, it has limitations. In patients with metastatic disease, an accurate blood-based *RAS* assessment may overcome these limitations.

As tumor cells die, they release mutant tumor DNA fragments into the blood. Given that circulating tumor DNA (ctDNA) fragments are readily detected in patients with advanced cancers, detection of mutations in ctDNA provides an excellent biomarker to track patients with metastatic disease [10]. Recent studies also indicate that ctDNA testing provides rapid genotype results for mCRC patients that accurately reflect the mutation status of tumor tissue [11–14].

Among the various plasma ctDNA assays, the digital PCR-based BEAMing assay (beads, emulsions, amplification and magnetics) has been clinically validated across several tumor types including colorectal cancer (CRC). The OncoBEAM™ RAS CRC plasma assay (Sysmex Inostics), based on the BEAMing technology [15], is a CE-marked IVD liquid biopsy test that provides a systemic evaluation of *RAS* tumor mutation status from a blood sample in less than 7 days, enabling effective and timely evaluation of a patient's eligibility for anti-EGFR therapy.

The aim of the study was to demonstrate the utility of a standardized blood-based *RAS* genotyping test as an alternative to tissue-based *RAS* genotyping prior to treatment with anti-EGFR therapy. We also cross-checked the concordance of results between three independent laboratories and evaluated the longitudinal variation of *RAS* mutant allelic fraction (MAF) during progression to assess the prognostic value of serial plasma *RAS* analyses.

Materials & methods

Patient inclusion criteria

Approval was obtained from Manchester Cancer Research Biobank and eligible patients with untreated mCRC (could have had previous adjuvant chemotherapy) gave informed consent for blood to be drawn as part of this study.

Plasma tumor sampling

Approximately 30 ml of blood was collected in three Streck tubes and the plasma was prepared according to OncoBEAM RAS CRC kit instructions. Independent studies have previously demonstrated the stability and shipping requirements of collected blood and Sysmex provided a detailed protocol for plasma preparation. Plasma was stored in 2 ml aliquots at -80°C until analysis. Additional blood samples were obtained in cases where patients exhibited radiological and/or clinical disease progression. Although only 2 to 3 ml of plasma are required for *RAS* mutation testing using the OncoBEAM RAS IVD, several residual and replicate samples were made available for further accuracy and reproducibility studies performed in this study.

Tissue *RAS* mutation testing

Tissue *RAS* mutational analysis was performed as per standard of care using the QIAGEN Therascreen Pyro Kit, which detects mutations in exons 2 & 3 in *KRAS* and *NRAS* genes; a 2% mutant allele threshold was used as the cut-off for calling mutations. Additional testing to detect *RAS* mutations in exon 4 was performed using tissue BEAMing; for this analysis, a 1% mutant allele threshold was used as the cut-off for calling mutations. The 1% cut-off for tissue BEAMing was demonstrated in the Phase III CRYSTAL and OPUS studies [16,17]. With respect to the clinical relevance of the 1% cut-off for BEAMing, in a retrospective analysis of the CRYSTAL trial, patients with tumor *RAS* MAFs between 0.1 and <5% were more likely to benefit from the addition of cetuximab to FOLFIRI [16].

Concordance of ctDNA in three laboratories

Tumor-specific circulating DNA is distinguished from normal DNA by the occurrence of point mutations in tumor-derived DNA. The OncoBEAM RAS CRC IVD assay detects the MAF of ctDNA in a high background of normal DNA with high sensitivity and specificity. In this study, a panel of 34 frequent point mutations in the *KRAS* and *NRAS* genes are detected and used to identify and monitor the fraction of mutant DNA and normal DNA. Tumor-specific circulating DNA is measured by the BEAMing assay as a continuous variable and represented as a ratio between the mutant and wild-type allele evaluated. Plasma samples were analyzed for 34 mutations in *KRAS* and *NRAS* (codons 12, 13, 59, 61, 117 and 146) using the OncoBEAM RAS CRC CEIVD assay (Sysmex Inostics, Hamburg, Germany). A subset of plasma samples (replicates) were also tested independently at two other OncoBEAM testing centers, Genomic Diagnostics Laboratory (GDL, Manchester, UK) and IMP laboratory (Sysmex, Kobe, Japan), to examine the robustness of the quantitative values of the MAF obtained from *RAS*-mutation-positive CRC patients.

The OncoBEAM RAS CRC IVD kit is based on BEAMing, which is a flow cytometric emulsion digital PCR technique in which single molecule PCR reactions are performed on magnetic beads in water-in-oil emulsions [18]. BEAMing utilizes emulsion digital PCR performed on magnetic beads to amplify single DNA molecules. Individual beads are then hybridized to allele-specific fluorescently labeled probes complementary to the mutant and wild-type DNA sequences. Finally, the bead population is analyzed by flow cytometry to count and sort wild-type and mutant beads.

Although it has been shown that BEAMing can detect one mutant molecule in a background of 10,000 wild-type molecules [15], the setting of cut-offs for each of the 34 *RAS* mutations analyzed in the assay were determined to be between 0.02 and 0.04% MAF; this ensures that the limits of detection (LODs) for each of the 34 *RAS* mutations are well above background signals or limits of blank (LoBs) for each analyte to be detected in clinical samples. LODs were determined by probit regression analyses by spiking wild-type (non-*RAS* mutation-containing) plasma samples with each *RAS* analyte. Background signals (LoBs) were determined in DNA prepared from wild-type plasma samples lacking *RAS* mutations at low, medium and high concentrations of genomic DNA. Based on the results of these experiments, cut-offs of 0.02–0.04% were observed to be appropriate so as to obtain a 95% probability/CI of reporting a "mutation detected" result (OncoBEAM RAS CRC kit instructions for use, Sysmex Inostics GmbH, Hamburg, Germany). Between 2 and 3 ml of plasma from each sample were used for each assay.

RAS mutation results are reported as both the absolute quantity of mutant beads and the fractional abundance of mutant DNA alleles relative to wild-type DNA alleles. The result is reported as the fractional abundance of mutant DNA alleles relative to wild-type DNA alleles in a plasma sample. To generate the ratio of mutant to wild-type DNA alleles (MAF), $\sim 3 \times 10^6$ beads are interrogated in each BEAMing analysis (corresponding to ~90,000 beads per mutation). The absolute number of *RAS* mutant alleles are not reported by BEAMing, as the determination of mutant status is dependent on the total amount DNA in an individual sample. Total circulating DNA levels (both wild-type and mutant) are subject to interpatient variability, which may be directly related to tumor burden or other characteristics such as inflammation and immune response.

Statistical analysis of concordance

Concordance of *RAS* mutation status was determined by calculating the agreement of *RAS* mutant and wild-type cases between tissue and plasma specimens. For discordant cases, the mutational status of both tissue and blood samples was rechecked using BEAMing. The modified Wald method was used to determine boundaries for 95% CI for each of the following three proportions: overall percentage agreement (OPA), positive percentage agreement (PPA) and negative percentage agreement (NPA). The MAF values for newly diagnosed versus recurrent mCRC patients were evaluated by calculating mean MAF values with standard errors and compared with p-values derived using a Welch unequal variances t-test. All statistical tests were two-sided; the threshold for statistical significance was $p < 0.05$.

Results

Patients

In total, 104 patients (58 males and 46 females) with a median age of 65 years and PS 0–1 were entered into this study. However, only 100 patients were included in the concordance analysis; four cases were excluded because either the corresponding tissue samples were unavailable (n = 3) or the baseline plasma *RAS* result was not available (n = 1; Table 1).

49 patients had *RAS* wild-type (*RAS-WT*) tumors and 51 had mutations of the *RAS* gene (*mRAS*; Table 2). The median interval between obtaining a tissue sample and initiation of first-line treatment was 66.5 days. The median interval between obtaining a plasma sample and the start date of first-line treatment was 1 day (range: 0–104 days; mean: 4.13 days; Table 3).

RAS mutation analyses

The concordance of *RAS* status between matched plasma and tissue samples from each patient is summarized in Table 4. The *RAS* mutation status determined by OncoBEAM plasma testing versus the reference standard method performed on FFPE tissue samples was concordant in 86 out of 100 cases (86% OPA), with 86.2 PPA and 85.7% NPA. 14 discordant *RAS* mutation results were observed between plasma and tissue testing. Seven patients showed *mRAS* status in tissue but were *RAS-WT* in plasma (plasma false negatives). Conversely, plasma testing revealed *mRAS* status in seven patients whose tumors were determined *RAS-WT* by tissue testing (plasma false positives). For two out of three patients showing plasma false positives for which an additional plasma sample was available for testing at a later time point, the same *RAS* mutations were also detected. This suggests that low-frequency *RAS* mutations were likely to be present in the tumors of these patients below the limit of detection of the SOC tissue testing method. Recent data has suggested that low *RAS* frequency mutations may be a common occurrence in mCRC [19,20].

Table 1. Patient demographics and treatment.

Variable	n	%
Total number of patients	104[†]	100
Male	58	55.8
Female	46	44.2
Age (median, range)	65 (31-91)	
TNM staging at initial diagnosis:		
– Tx	23	22.1
– T1	1	1.0
– T2	4	3.8
– T3	40	38.5
– T4	36	34.6
– Nx	24	23.1
– N0	14	13.5
– N1	24	23.1
– N2	42	40.3
Metastatic lesions at trial entry:		
– Presented with metastatic colorectal cancer	70	67.3
– Relapsed after adjuvant treatment	34	32.7
Performance status:		
– PS0	45	43.3
– PS1	45	43.3
– PS2	12	11.5
– Unknown PS	2	1.9
First-line treatment:		
– IrMdG	20	19.2
– IrMdG + cetuximab	15	14.4
– Capecitabine	8	7.7
– OxCap	5	4.8
– OxMdG	33	31.7
– OxMdg + panitumumab	9	8.7
– Other treatments (IrMdG + masitinib, OxMdG + cetuximab, capecitabine + bevacizumab, irinotecan single agent, IrMdG + AZD8931, bevacizumab + trifluridine + tipiracil, cetuximab single agent)	13	12.5
– Unknown	1	1.0

[†]Four cases were excluded. In three cases, the tissue samples were not available for *RAS* status analysis and in one case the baseline plasma RAS result was not available. The italicized and underline terms shows the different demographic data collected with their explanations.

Table 2. Tissue sample site and mutational status.

Tissue sample	Patients (n)	%
Wild type	52	50.0
Mutant	52	50.0
Resection tissue	39	37.5
Biopsy tissue	61	58.7
Unknown tissue type	4	3.8
Sample from primary tumor	89	85.6
Sample from metastatic lesion	12	11.5
Unknown	3	2.9

Plasma mutant allele fraction analyses in three laboratories

A useful feature of ctDNA analysis using OncoBEAM is the ability to determine the MAF of cell-free mutant alleles as a proportion of the overall cell-free DNA content in circulation at the time of sampling. To examine the reproducibility of plasma *RAS* mutation results and to assure accuracy of *RAS* MAF values, replicate plasma samples

Table 3. Time from tissue/plasma to treatment.

Intervals	Patients (n)
Between plasma and treatment	
	Median: 1 day (range: 0–104 days; mean: 4.13 days)
1–4 days (%)	72 (69.2)
5–10 days (%)	12 (11.5)
>10 days (%)	12 (11.5)
After treatment (%)	3 (2.9)
NA (%)	5 (4.8)
Between tissue and treatment	
	Median: 66.5 days (range: 4–1990 days)

NA: Not applicable.

Table 4. Concordance of plasma and tissue *RAS* mutation results.

RAS	Plasma ctDNA *RAS* result		Total	Tumor-tissue *RAS* result		
	Mutant	WT		PPA (95% CI)	NPA (95% CI)	OPA (95% CI)
Mutant	44	7	51	100 × 44/51	100 × 42/49	100 × 86/100
WT	7	42	49	86.2%	85.7%	86.0%
Total	51	49	100	(74%–94%)	(73%–93%)	(78%–92%)

NPA: Negative percentage agreement; OPA: Overall percentage agreement; PPA: Positive percentage agreement; WT: Wild type.

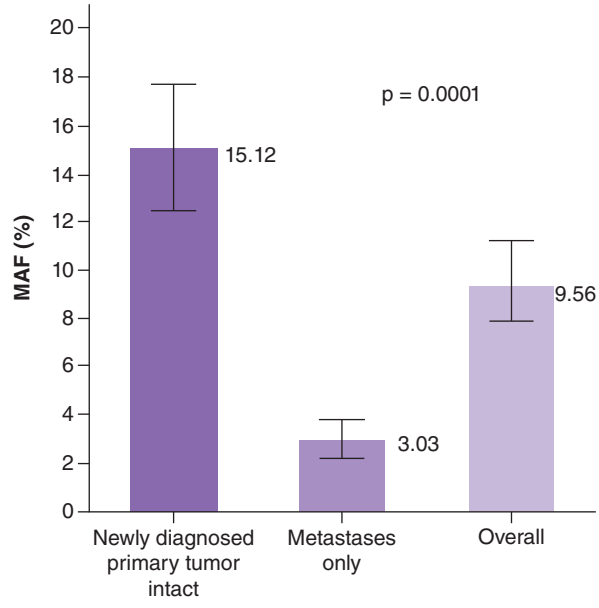

Figure 1. Bar chart showing the average plasma DNA-mutant fractions detected in ctDNA of patients with newly diagnosed compared with recurrent disease and the overall cohort of patients with *RAS* mutations detected in the plasma by BEAMing. The average value and standard error (SE) for *RAS* MAF for all patients (n = 48; overall) in which *RAS* mutations were detected by OncoBEAM RAS CRC assay is represented by the lilac bar on the right (mean: 9.56 ± 1.70 SE). The average values and SEs for *RAS* MAF from newly diagnosed mCRC patients with primary tumor intact (prior to surgery; n = 26) and patients with primary tumor removed (post-surgery) and or patients with recurrent disease/metastases only (n = 22) are represented by the violet bar on the left (mean: 15.12 ± 2.61 SE) and the medium purple bar in the middle (mean MAF: 3.03 ± 0.79 SE), respectively. p = 0.0001 for MAF% in newly diagnosed patients compared with those presenting with recurrent disease/metastases only. The p-values were derived from a Welch unequal variances t-test.
MAF: Mutant allelic fraction.

were evaluated and compared at three separate laboratories (UK, Germany, Japan). As shown in Table 5, results from testing of 76 replicate plasma samples from 27 mCRC patients at the three different laboratories showed 100% agreement, with only one of the tests (1.3%) giving an invalid result. To further evaluate the accuracy of MAF values, 38 replicates from 13 *RAS*-mutation-positive patients were examined in the three separate laboratories. As shown in Table 5, the average variance in MAF values was no more than +/-13.4% (95 CI; 9.1–17.8%). This low variance was consistent for MAF values over three orders of magnitude of ctDNA concentration present in the samples.

For 51 patients with *RAS* mutations, the average MAF value was 9.56% (Figure 1). Since the frequency of circulating mutant alleles might be related to overall tumor burden or extent of metastatic invasion, the mean MAF

Table 5. Comparison of mutant allelic fraction percentages obtained in replicate sample testing using the OncoBEAM RAS CRC assay across three different laboratories.

Patient ID	Tissue result	GDL plasma result	GDL MAF%	IMP plasma result(s)†	IMP MAF (%)	SI plasma result	SI MAF (%)	Mean MAF
PT2163	*RAS-WT*			*RAS-WT*		*RAS-WT*		
PT2654	KR12+	KR12+	42.435	KR12+	48.011, 45.194	KR12+	51.889	46.88
PT2679	*RAS-WT*			*RAS-WT*		*RAS-WT*		
PT2716	KR12+			KR12+	21.09, 19.403	KR12+	27.62	22.7
PT2732	*RAS-WT*			*RAS-WT*		*RAS-WT*		
PT2733	KR12+	KR12+	6.756	KR12+	8.645, 8.466	KR12+	11.69	8.89
PT2738	*RAS-WT*			*RAS-WT*		*RAS-WT*		
PT2794	KR12+	KR12+	0.809	KR12+	0.692	KR12+	0.6549	0.7186
PT2814	No result	KR12+	11.743	KR12+	1.792	KR12+	8.9684	7.5011
PT2815	*RAS-WT*	*RAS-WT*		*RAS-WT*		*RAS-WT*		
PT2817	*RAS-WT*			*RAS-WT*		*RAS-WT*		
PT2821	*RAS-WT*	*RAS-WT*		*RAS-WT*		*RAS-WT*		
PT3269	KR12+	KR12+	9.564	KR12+	10.65	KR12+	8.771	9.662
PT3272	*RAS-WT*	*RAS-WT*		*RAS-WT*		*RAS-WT*		
PT3273	No result	KR13+	14.674	KR13+	15.589	KR13+	16.036	15.433
PT3274	*RAS-WT*	*RAS-WT*		*RAS-WT*		*RAS-WT*		
PT3275	KRASG13D	KR13+	0.232	KR13	0.28	KR13+	0.187	0.233
PT3276	*RAS-WT*			*RAS-WT*		*RAS-WT*		
PT3277	KRASG12D	KR12+	11.446	KR12+	13.905, 12.294	KR12+	14.092	12.923
PT3278	*RAS-WT*	*RAS-WT*		*RAS-WT*, ***RAS-WT***		*RAS-WT*		
PT3279	*RAS-WT*	*RAS-WT*		*RAS-WT*, ***RAS-WT***		*RAS-WT*		
PT3280	*RAS-WT*	*RAS-WT*		*RAS-WT*, **invalid**‡		*RAS-WT*		
PT3281	*RAS-WT*	*RAS-WT*				*RAS-WT*		
PT3282	No result	KR12+	11.088			KR12+	10.001	10.5445
PT3283	KRASG13D	KR13+	6.236			KR13+	5.615	5.926
PT3284	*RAS-WT*	KR12+	1.553			KR12+	1.606	1.5795
PT3285	KRASG12V	KR12+	23.79			KR12+	19.804	21.797

Replicate aliquots of plasma samples that were centrally prepared were tested in either the GDL at the Christie Hospital (Manchester, UK), IMP laboratory (Kobe, Japan), or at the SI Research and Development laboratory (Hamburg, Germany).

†Dual replicates for certain patient samples were run at the IMP laboratory; these are indicated as two separate results to report both the qualitative *RAS-WT* or *mRAS* status for each replicate, as well as the corresponding MAF values obtained in each replicate testing. Results obtained for patients 3278 and 3279, indicated in boldface type are replicates that were retested after carry-over contamination was noted in samples containing high MAF in adjacent samples; retesting confirmed that the *RAS* status for these two patients were indeed *RAS-WT*.

‡ For patient 3280, testing of the second replicate gave invalid results for certain codons; therefore, the overall result from this replicate was deemed invalid.

GDL: Genetics Diagnostics Laboratory; MAF: Mutant allelic fraction; *RAS-WT*: *RAS* wild-type; SI: Sysmex Inostics.

values in plasma were compared between newly diagnosed mCRC patients with intact primary tumor/metastases prior to surgery (n = 26) versus patients having either their primary tumors removed and/or experiencing recurrent disease, presenting with metastases (n = 22). A statistically significant relationship was observed between the patient clinical diagnosis status and mean proportion of mutant *RAS* alleles in circulation. In stage IV newly diagnosed patients with intact primary tumors, the MAF was fivefold higher (15.12%) than in patients presenting with recurrent disease after removal of their primary tumors (3.03%; p = 0.0001; Figure 1).

RAS mutational analysis in plasma in mCRC patients at progression

To examine changes in *RAS* mutational status occurring in mCRC patients during treatment, ctDNA analyses were performed on blood samples obtained from patients with radiologically confirmed disease progression. Among 49 patients showing tumor tissue *RAS-WT* status prior to chemotherapy, ctDNA analyses were performed on 24 patients (49%) at disease progression. One sample was also taken from a patient who had a tissue *NRAS* mutation

at baseline. 15 patients (30.6%) having *RAS-WT* status died before a progression sample could be obtained and 10 patients (20.4%) remained progression free and continued on first-line treatment. As shown in Table 6, a comparison of baseline plasma *RAS* mutational status indicated that 23 out of 25 ctDNA results were in agreement (92.0% concordance; two patients showed a *RAS* mutation in plasma but not in tissue). At progression, five out of these 25 patients (20%) showed a change in mutational status in plasma as compared with the tissue and plasma *RAS* test result obtained at baseline.

Plasma *RAS* mutation testing revealed that 13 out of 16 patients receiving first-line anti-EGFR therapy (81.2%) retained a *RAS-WT* status at the time of progression and 3 of these patients (18.8%) showed a change from *RAS-WT* to mutated *RAS* status at progression (Table 6). Two of the patients with *RAS-WT* status at baseline (2822 and 2817) received cetuximab + FOLFIRI and showed KRAS12 (MAF 2.5146) and KRAS13 mutations (MAF 0.3318), respectively, in plasma on progression. One patient (3274; Table 6) received Panitumumab + FOLFOX and showed a KRAS12 mutation in plasma (MAF 0.06%) on progression, with an elevation in KRAS12 MAF to 0.4488% later during progression, along with evidence of two additional *RAS* mutations, KRAS61 (MAF 0.1031%) and NRAS61 (MAF 0.356%). These observations are consistent with the notion that failure of EGFR treatment in these patients likely resulted from expansion of multiple independent RAS-resistant clones at different metastatic sites. One patient with *RAS-WT* status at baseline treated with capecitabine showed a KRAS61 mutation in plasma at progression, indicating that a component of the resistance to treatment might involve *RAS*-mediated resistance.

Representative examples of the flow cytometry data from patients 2822 and 3274 showing quantitative readouts of *RAS*-mutation-positive versus wild-type molecular fractions detected by the OncoBEAM assay and emergence of *RAS* mutations at progression are shown in Figure 2. These data show clearly the absence of *RAS* mutations in baseline samples obtained from both patients prior to anti-EGFR therapy treatments, as well as dramatic rises in *RAS* mutant ctDNA molecules in plasma samples taken at progression.

Discussion

We have demonstrated that ctDNA *RAS* testing is a potential alternative to the current standard of mutation testing of FFPE material. This is supported by our results showing a high level of concordance (OPA 86%) upon comparison of *RAS* mutation testing on prospectively collected plasma samples using the OncoBEAM RAS CRC assay versus those obtained using FFPE tumor samples. These results are in accord with findings from previous studies [12–14,19]. This is the first prospective study to examine both OncoBEAM RAS CEIVD assay performance in the UK, as well as to monitor mCRC patients for changes in ctDNA *RAS* mutational status at progression during first-line anti-EGFR therapy treatment.

The use of an extremely sensitive method to detect *RAS* mutations in ctDNA from plasma provides several advantages over the standard tissue-based approach. Although primary tumor biopsy samples are routinely used for therapy selection, the use of such samples is subject to several technical and logistical limitations. For instance, a single-site tissue sample obtained at diagnosis may not fully represent disease heterogeneity and may not be informative of *RAS* mutation status in those patients that relapse later with distant metastases. Intratumor heterogeneity is not insignificant: reports have demonstrated that testing DNA from a single colorectal tumor tissue block wrongly assigns *KRAS* wild-type status in 8–11.6% of patients [20–22] Moreover, studies evaluating intertumor heterogeneity between primaries and metastases have also revealed mutational discordance in 3.6–32.4% of cases [23–29]. Both inter- and intra-tumor heterogeneity are inherent features of metastatic disease [30–32] prudence would call fora systemic evaluation of *RAS* status across metastatic sites to better inform the accurate prescription of anti-EGFR therapy. Indeed, results support the utility of a liquid biopsy to overcome sampling bias associated with single tumor site-specific sampling [33].

In addition, locating appropriate tissue specimens for mutational testing at the time of therapy selection can present challenges and delay timely administration of a therapy, whereas the rapid turn-around time of plasma *RAS* mutational testing enables timely initiation of first-line therapy.

There are obviously limitations in the use of ctDNA. First, the concentration of circulating-free DNA varies considerably between patients and the proportion of ctDNA within this is small (0.1–10%) [34]. There is also a lack of standardization for ctDNA detection and analysis together with a lack of broadly accepted standard operating procedures [35]. In our study, analysis was duplicated in three different laboratories showing a high degree of consistency. Nonetheless, there are several clinical applications of liquid biopsies. These include genotyping for mutations, detecting minimal residual disease and helping to predict an early response to systemic chemotherapy for metastatic disease [34,36–38]. Furthermore, next-generation sequencing is able to rapidly and efficiently sequence

Table 6. Comparison of plasma ctDNA *RAS* mutational status of metastatic colorectal cancer patients on first-line therapy at disease progression with baseline tissue and plasma *RAS* mutation testing results.

Patient number	Stage at diagnosis	Diagnosed newly at stage IV or recurrent disease	Treatment	Baseline tissue *RAS* result	Baseline plasma *RAS* result	Progression plasma *RAS* result	Date of baseline sample collection	Date of blood sample at progression	Date of radiological progression	Time interval: baseline and progression blood sample	Time interval: baseline to radiological progression (wk)
PT2818	TxNxM1	R	Capecitabine	*RAS-WT*	*RAS-WT*	*RAS-WT*	08/03/2015	01/02/2016	18/01/2016	47	45
PT2679	T2N0M0	R	OxMdG	*RAS-WT*	*RAS-WT*	*RAS-WT*	21/05/2015	16/08/2016	19/07/2016	65	61
PT3124	T3N2M1	N	IrMdG + cetuximab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	28/09/2015	20/10/2016	26/09/2016	55	52
PT3127	TxNxM1	R	IrMdG + cetuximab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	10/08/2015	05/12/2016	09/11/2016	69	65
PT3229	TxNxM1	R	OxMdG + panitumumab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	19/11/2015	21/09/2016	25/08/2016	44	40
PT3232	TxNxM1	R	OxMdG + panitumumab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	27/11/2015	18/08/2016	11/08/2016	38	37
PT3265	TxNxM1	R	OxMdG + panitumumab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	15/12/2015	04/07/2016	23/05/2016	29	23
PT3274	TxNxM1	R	OxMdG + panitumumab†	*RAS-WT*	*RAS-WT*‡	KRAS12 (0.0583)§	15/02/2016	10/02/2017	09/01/2017	52	47
PT3292	TxNxM1	R	IrMdG + cetuximab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	26/07/2016	31/01/2017	18/01/2017	27	25
PT3297	T4N2M1	N	IrMdG + cetuximab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	16/09/2016	08/02/2017	17/01/2017	21	18
PT2163	TxNxM1	R	Capecitabine	*RAS-WT*	*RAS-WT*‡	KRAS61 (0.0123)§	27/07/2015	08/02/2016	19/12/2015	28	21
PT2820	TxNxM1	R	Capecitabine	*RAS-WT*	NR12 (0.1854)	NRAS12 (0.14)	27/08/2015	18/02/2016	23/01/2016	25	21
PT2892	T3N2M1	N	IrMdG	*RAS-WT*	*RAS-WT*	*RAS-WT*	23/07/2015	05/02/2016	30/12/2015	28	23
PT4108	T3N1M0	R	IrMdG + cetuximab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	10/10/2016	15/06/2017	10/06/2017	35	35
PT2819	TxNxM1	R	IrMdG + cetuximab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	20/08/2015	27/03/2017	11/03/2017	84	81
PT3289	T4N2M1	N	OxMdG + panitumumab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	04/07/2016	06/07/2017	30/06/2017	52	52
PT2821	TxNxM1	R	IrMdG + cetuximab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	14/09/2015	18/09/2017	11/08/2017	105	100
PT2822	T3N1M1	N	IrMdG + cetuximab†	*RAS-WT*	*RAS-WT*‡	KRAS12 (2.5146)§	16/09/2015	04/10/2017	26/09/2017	107	106
PT3299	T3N2M0	R	IrMdG + AZD8931	*RAS-WT*	*RAS-WT*	*RAS-WT*	29/09/2016	09/10/2017	14/09/2017	54	50
PT3288	T2N1M0	R	IrMdG + cetuximab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	11/07/2016	15/10/2017	13/09/2017	66	61
PT3284	T4bN1M1	N	OxMdG	*RAS-WT*	KR12 (1.606)	KRAS12 (0.1311)	10/05/2016	18/10/2017	13/10/2017	75	74
PT2817	T4N1M1	N	IrMdG + cetuximab†	*RAS-WT*	*RAS-WT*‡	KRAS13 (0.3318)§	08/03/2016	30/10/2017	09/09/2017	86	79
PT3272	TxNxM1	R	IrMdG + cetuximab†	*RAS-WT*	*RAS-WT*	*RAS-WT*	08/02/2016	13/11/2017	07/07/2017	92	74
PT3296	T4N2M1	N	OxMdG	*RAS-WT*	*RAS-WT*	*RAS-WT*	07/09/2016	13/12/2017	21/10/2017	66	58
PT3129	T4N2M1	N	IrmdG	NR12	NR12 (0.74)§	*RAS-WT*‡	20/10/2015	01/08/2016	09/06/2016	41	33

†The 16 patients receiving anti-EGFR therapy.
‡Baseline sample.
§Patients differing in progression plasma sample ctDNA mutational status versus the baseline sample.
25 patients were examined for *RAS* mutation status using OncoBEAM RAS IVDct DNA testing on progression.
N: Diagnosed newly at stage IV; R: Recurrent disease; *RAS-WT*: *RAS* wild type.

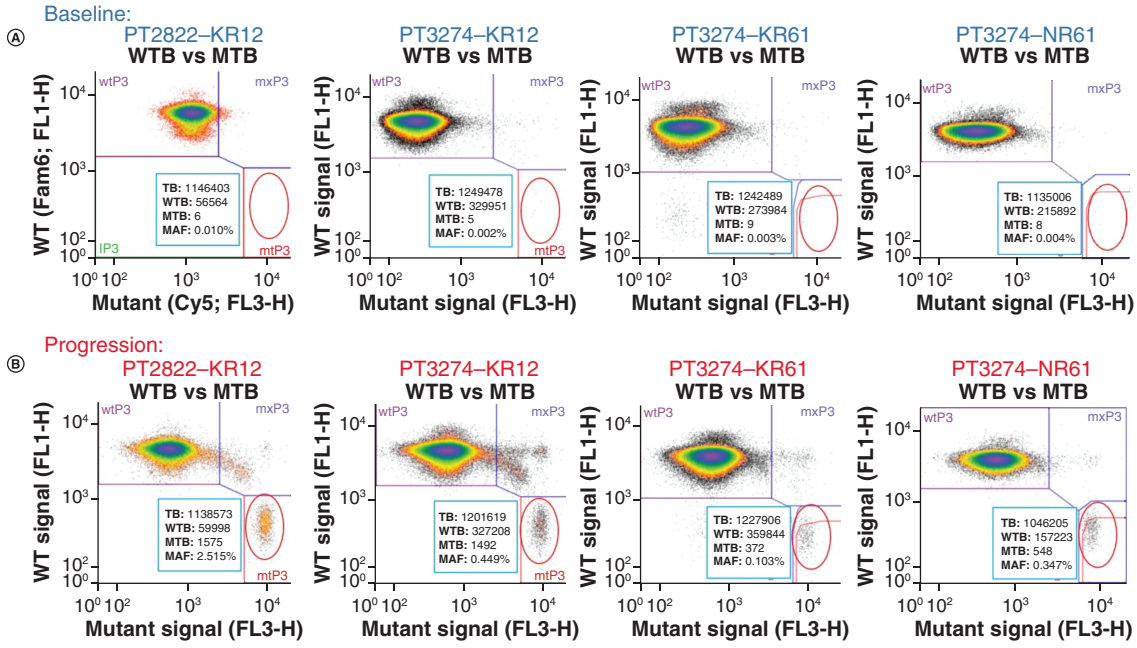

Figure 2. Quantitative measurement of *RAS*-mutation-positive versus wild-type ctDNA molecules in plasma samples obtained from patients 2822 and 3274 at baseline and disease progression. Comparison of *RAS* mutant (x-axis) versus WT (y-axis) flow cytometry analyses of ctDNA molecules detected on fluorescent beads using the OncoBEAM RAS IVD assay. Plots show the absolute numbers of TB, WTB, MTB and MAF percentages; these values from flow cytometric measurements are presented for each plot (see values in inset boxes within graphs). The eight plots presented are from data obtained in four different pairs (upper vs lower in a column) of ctDNA analyses of two different metastatic colorectal cancer patients (PT2822 and PT3274). PT2822 shows emergence of KRAS12 mutation at progression, whereas PT3274 shows emergence of three separate *RAS* mutations (KRAS12, KRAS61 and NRAS61) at progression. Plasma samples from these two patients measured at baseline are provided in panels shown in the top row denoted by **(A)**, and samples measured at progression are provided in panels shown in the bottom row denoted by **(B)**. Patterns of emerging *RAS* resistance of KR12, KR61 and NR61 mutations versus WT ctDNA in progression versus baseline measurements in these patients are visualized by comparing the patterns found in the lower with the upper plots, which also show ctDNA quantities in each of the four columns shown. Fluorescent signals from *RAS* mutant ctDNA molecules are observed as clustered signals in the lower right quadrants of each plot, where each cluster of ctDNA molecules is seen within the boundaries of the red ovals drawn in each plot. In contrast, the predominant pool of ctDNAs visualized and quantitated in each assay consist of WTBs; these are located in the upper left quadrants of each plot. For each assay, at least 1×10^6 beads are queried. Qualitative calls of WT (no mutation detected) or *RAS* mutation positive are determined by dividing the MTB counts (MB) by the WTB counts. The resulting ratio or MAF must exceed a cut-off value that has been derived for each of the codons detected in the assay. This concept is made clear with the data shown in the plots where the quantities of mutant molecules in baseline samples (plots in top row) are in the single digits (compare low MTB–MTB numbers to the number of WT molecules). Accordingly, the MAF values observed in baseline samples are below the cut-off thresholds set for each analyte. This pattern is in contrast to the *RAS*-positive calls made for the progression samples, where MTB counts are in the several hundred/thousands in comparison to similar levels of WT beads.
MAF: Mutant allelic fraction; MTB: Mutant beads; TB: Total beads; WTB: Wild-type beads.

millions of DNA reads using extremely low amounts of nucleic acids. This can lead to the identification of novel mutations or genomic rearrangements together with the possible evaluation of therapy response. Next-generation sequencing has also been used in the analysis of ctDNA for molecular profiling and tracking of minimal residual disease [39]. As part of the management of patients with CRC, it is important to know the mutational status of another gene, *BRAF*. In the EXPERT-C trial, 97 patients were assessable for the analysis of *KRAS*/*BRAF* mutations in ctDNA. Only three patients were found to have *BRAF* mutant tumors in the tissue samples, two of which were found to have mutations in the paired ctDNA samples [40].

Despite the significant therapeutic advances made by targeting EGFR pathways, most clinical benefit from anti-EGFR treatment is often short-lived due to the inevitable emergence of drug resistance. In *RAS-WT* mCRC patients receiving anti-EGFR therapy, the detection of *RAS* mutations during therapy has been associated with

disease progression [41–43]. In fact, using a liquid biopsy approach, independent research has demonstrated that *RAS* mutant tumor DNA is detectable in plasma several months in advance of radiological progression [11,41,43]. Furthermore, mutated *KRAS* clones that emerge in blood during treatment with EGFR inhibitors decline on withdrawal of these antibodies [11,43]. Therefore, longitudinal ctDNA sampling for *RAS* status can help to refine subsequent treatments. The ability to draw serial blood samples and perform ctDNA analyses from patients receiving anti-EGFR therapy provides signal opportunities to identify emerging *RAS* mutant clones at early stages of treatment; this provides better visualization of treatment responses/failures that can further personalize therapy approaches for mCRC patients. In the present study, we showed that out of 25 progression samples taken, 5 (20%) had a change in mutational status, with 3 out of 16 patients receiving anti-EGFR therapy showing *RAS* resistance to first-line treatment (18.8%). These observations further underscore the importance of performing plasma testing in a longitudinal fashion during anti-EGFR therapy. Plasma *RAS* mutation monitoring would likely add benefit to the outcome/cost effectiveness of management of anti-EGFR treatment of CRC patients by providing a relatively inexpensive diagnostic tool to gauge therapy efficacy, switch therapy or schedule a timely treatment hiatus, particularly in patients who show early signs of *RAS* resistance. The aggregated evidence therefore supports use of baseline and longitudinal ctDNA-based *RAS* mutation testing as an alternative to standard testing of FFPE material. This approach is likely to reduce unnecessary toxicity, cost and inconvenience, as well as to increase survival benefits for CRC patients receiving systemic therapy.

Conclusion

An accurate blood-based *RAS* mutation assay would benefit routine clinical management of mCRC patients to assist in guiding decisions to administer systemic therapy independent of the availability of tumor tissue samples. Here we used the OncoBEAM RAS IVD kit to show good concordance (86%) of *RAS* status between tissue and plasma. We also showed that 20% of patients showed a change in their *RAS* mutational status during treatment. These results demonstrate that blood-based *RAS* mutation testing is a viable alternative to tissue-based *RAS* testing and that there is a need for longitudinal plasma testing during treatment to guide management decisions and EGFR inhibitor administration.

Author contributions

T Germetaki, M Braun, J Rogan, DL Edelstein, FS Jones and MP Saunders were responsible for trial design. T. Germetaki, C Nicholls, RA Adams, M Braun, J Rogan, S Moghadam and MP Saunders were responsible for sample acquisition. E Lenfert, A Lukas, DL Edelstein and FS Jones were responsible for sample analysis. T Germetaki, C Nicholls, RA Adams, M Braun, J Rogan, S Moghadam, E Lenfert, A Lukas, DL Edelstein, FS Jones and MP Saunders were responsible for data interpretation and manuscript drafting. Final approval was given by T Germetaki, C Nicholls, RA Adams, M Braun, J Rogan, S Moghadam, E Lenfert, A Lukas, DL Edelstein, FS Jones and MP Saunders. All agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Summary points

- Prior to initiation of anti-EGFR therapy, *RAS* (*KRAS* and *NRAS*) genotyping is performed and mutations are detected in approximately 50% of patients with metastatic colorectal cancer.
- Tissue processing presents several practical challenges, including poor tissue quality and from a biological perspective, accurate genotype determination can be obscured by tumor molecular heterogeneity.
- Performing ctDNA *RAS* testing is a potential alternative to the current standard of mutation testing of formalin-fixed paraffin embedded material.
- The OncoBEAM™ RAS CRC plasma assay, based on the BEAMing technology [15], is liquid biopsy test that provides a systemic evaluation of *RAS* tumor mutation status from a blood sample in less than 7 days.
- The positive percentage agreement was 86.2% and the negative percentage agreement was 85.7%.
- Our study showed a high overall percentage concordance (86%) when comparing *RAS* mutation testing on prospectively collected plasma samples using the OncoBEAM RAS CRC assay with formalin-fixed paraffin embedded tumor samples.
- Reproducibility studies showed that OncoBEAM testing results had 100% agreement between three independent laboratories.
- 20% of patients showed changes in their *RAS* mutational status during treatment.
- Overall, these results show good concordance between tissue and plasma samples and suggest the need for longitudinal plasma testing during treatment to guide management decisions during anti-EGFR therapy.

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Ethical conduct of research

The authors state that they have obtained appropriate approval from Manchester Cancer Research Biobank. For investigations involving human subjects, informed consent has been obtained from the participants involved.

Data sharing statement

This manuscript presents original results from sample analysis collected via the MCRC as described above in the acknowledgments.

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