Intra-tumoral heterogeneity of \textit{KRAS} and \textit{BRAF} mutation status in patients with advanced colorectal cancer (aCRC) and cost-effectiveness of multiple sample testing

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Abstract. \textit{KRAS} mutation status is established as a predictive biomarker of benefit from anti-EGFr therapies. Mutations are normally assessed using DNA extracted from one formalin-fixed, paraffin-embedded (FFPE) tumor block. We assessed heterogeneity of \textit{KRAS} and \textit{BRAF} mutation status intra-tumorally (multiple blocks from the same primary tumor). We also investigated the utility and efficiency of genotyping a ‘DNA cocktail’ prepared from multiple blocks.

We studied 68 consenting patients in two randomized clinical trials. DNA was extracted, from \ensuremath{\geq}2 primary tumor FFPE blocks per patient. DNA was genotyped by pyrosequencing for \textit{KRAS} codons 12, 13 and 61 and \textit{BRAF} codon 600. In patients with heterogeneous mutation status, DNA cocktails were prepared and genotyped.

Among 69 primary tumors in 68 patients, 7 (10.1\%) showed intratumoral heterogeneity; 5 (7.2\%) at \textit{KRAS} codons 12, 13 and 2 (2.9\%) at \textit{BRAF} codon 600. In patients displaying heterogeneity, the relevant \textit{KRAS} or \textit{BRAF} mutation was also identified in ‘DNA cocktail’ samples when including DNA from mutant and wild-type blocks.

Heterogeneity is uncommon but not insignificant. Testing DNA from a single block will wrongly assign wild-type status to 10\% patients. Testing more than one block, or preferably preparation of a ‘DNA cocktail’ from two or more tumor blocks, improves mutation detection at minimal extra cost.

Keywords: KRAS, BRAF, heterogeneity, colorectal cancer

1. Introduction

The drug treatment of advanced colorectal cancer (aCRC) has seen recent advances; first newer cytotoxic drugs such as oxaliplatin (Ox) and irinotecan (Ir) [1–3], more recently, monoclonal antibody (mAb) therapies targeting vascular endothelial growth factor (VEGF) [4] and the epidermal growth factor receptor (EGFr) [5]. Anti-EGFr mAb therapies, cetuximab and panitumumab, have now been approved for the treatment of aCRC. Although beneficial to some patients, these drugs are costly [6], they cause significant toxicity [7], and if used unselectively may provide minimal or even negative [8, 9] net benefits. Therefore, attention has rightly focused on identifying predictive biomarkers for patient selection. The first of these, now included in amended drug licenses, is the \textit{KRAS} oncogene.
KRAS protein is involved in one of several EGFr signal transduction cascades. It is activated following ligand binding to the extracellular domain of EGFr, triggering downstream events including activation of the mitogen-activated protein kinases (MAPK). Mutation hotspots in KRAS codons 12, 13 and 61, lead to constitutively active KRAS protein, and thus EGFr-independent activation of the MAPK pathway [10–12]. BRAF, downstream from KRAS in the MAPK pathway, is subject to an activating mutation, in codon 600.

It is known that activated KRAS (KRAS-mut) confers clinical resistance to anti-EGFr-mAbs. Whilst patients with wild-type KRAS (KRAS-wt) cancers have, in some trials, benefited from cetuximab or panitumumab, those with KRAS-mut cancers have not, and in some trials have been harmed by them. Recent data suggest that activating mutations of NRAS or BRAF, also confer anti-EGFr-mAb resistance [13]. Their lower prevalence makes this harder to confirm within single trials, but in three recently-reported grouped series including over 500 patients receiving cetuximab or panitumumab-based treatments there were 35 patients with KRAS-wt, BRAF-mut tumors, none of whom responded to therapy [13]. Similarly, no responses have been seen in patients with NRAS-mut.

KRAS and BRAF are also prognostic factors independent of treatment. We previously showed, in 711 aCRC patients treated without anti-EGFr therapy, that mutation in either oncogene is negatively prognostic for survival, with KRAS-mut giving a hazard ratio (HR) of 1.24 (95% CI 1.06–1.46; \( p = 0.008 \)) and BRAF-mut giving HR = 1.82 (95% CI 1.36–2.43; \( p < 0.0001 \)) [14]. In a recent report of 516 aCRC patients randomized to receive chemotherapy and bevacizumab with or without cetuximab, BRAF-mut was associated with inferior outcomes in both arms [15].

Currently, molecular testing usually involves DNA extraction from a single tumor tissue block, followed by a mutation-specific PCR-based assay or sequencing of the relevant codons. Sensitivity and errors of these assays in different laboratories may be significant, and are being addressed by international quality assurance programs [16]. Here we assess a second potential source of error: tumor heterogeneity. Using material from patients in randomized clinical trials, we ask how frequently KRAS or BRAF mutation status is heterogeneous within tumors, and therefore how representative is a single test as currently practiced. We also investigate the options of multiple testing, or of performing a single analysis using a ‘DNA cocktail’ extracted from multiple blocks per tumor. Based on heterogeneity levels in this study, we have estimated the clinical event cost consequences of these different approaches.

2. Methods

2.1. Patients

Material was available from aCRC patients in two large UK National Cancer Research Institute randomized clinical trials: FOCUS and PICCOLO. In each, separate consent was obtained for the use of surplus stored pathological material for research. From the trial biobank of over 2000 patients, patients were selected at random for this study provided they had at least two separate cancer-containing blocks from different areas of the primary tumor. Sample size was determined by the availability of material at the time of study.

FOCUS (Fluorouracil, Oxaliplatin and CPT11 [irinotecan]: Use & Sequencing) involved 2135 patients randomized at 60 centers between 2000 and 2003, comparing different sequences of first- and second-line chemotherapy for aCRC [17]. Formalin-fixed, paraffin-embedded (FFPE) tumor blocks were retrieved retrospectively. PICCOLO (Panitumumab, Irinotecan and Cyclosporin in Colorectal cancer therapy) opened in December 2006, comparing different second-line therapies. Unlike FOCUS, PICCOLO randomization differs according to KRAS status, so tumor samples are obtained prior to randomization.

2.2. Study plan

First, in line with standard practice, a single primary tumor sample from each patient was analyzed for mutations in KRAS codons 12, 13 and 61 and BRAF-codon 600. The rate of mutations at each codon was assessed. For each patient, all further available primary tumor blocks were sampled and analyzed in the same way as the first sample. These results were then compared with the initial result and with each other to assess the rate of intratumoral heterogeneity. As an internal control, one series of samples was duplicated, to assess intra-lab variability.

In patients identified as having intratumoral heterogeneity, a ‘DNA cocktail’ was produced and the
resulting mutation status was compared with the individual block results.

2.3. Laboratory methods

FFPE tumor blocks were retrieved, anonymized and sent to the research laboratory, where staff remained blind to the patients’ identity, treatment and outcomes. Tumor areas were identified on H&E-stained sections, then macrodissected from ten 5 μm whole-block sections.

Two DNA extraction protocols were used. For FOCUS trial material, DNA was extracted as previously described [14]. For PICCOLO, which requires prospective and hence urgent KRAS testing prior to randomization, the QIAGEN QIAamp DNA Micro kit was used, employing the standard manufacturer’s protocol. The DNA was resuspended in 20 μl water and stored at 4 °C. DNA was extracted from eight samples using both extraction methods, showing that either methodology could be used to obtain DNA of suitable quality and quantity for analysis.

To generate the ‘DNA cocktail’ samples, 5 μm sections from multiple individual blocks from the same tumor were macrodissected into a single tube, then the DNA extracted using the QIAGEN DNA Micro kit. Previous validation experiments in the laboratory determined the sensitivity of mutant allele detection to be 5% i.e. pyrosequencing will detect a mutant allele if it comprises as little as 5% of the extracted DNA.

The tumor content (%) of the macrodissected region was assessed for each H&E section. Each was given a value within one of the categories; <25%, 25–50%, 50–75% or >75%. No tumor displayed a tumor content of <10% tumor cells within the macrodissected region.

Mutations were assessed by pyrosequencing as previously described [14]. See Table 1 for reaction conditions. Assessment was carried out blind to any previous results obtained for each patient.

External quality assurance of our KRAS genotyping assays is performed within a European Quality Assurance Program and furthermore, we act as a reference lab for UKNEQAS. To date, we have successfully completed each with a score of 100%.

3. Results

3.1. KRAS/BRAF mutation status rates

Mutation rates as determined in the first tumor block assessed per patient were as follows; mutations were found in KRAS codons 12, 13 in 32/69 (46.4%) tumors. Mutations in KRAS codon 61 were detected in 2/69 (2.9%), and 8/69 (11.6%) tumors carried a mutation in BRAF codon 600. All mutations were amino-acid altering, activating mutations as described on the COSMIC database.

3.2. Intratumoral heterogeneity

A total of 69 primary tumors in 68 patients (one with two synchronous primaries) had two or more FFPE blocks assessed for mutation status at KRAS codons 12, 13. Of these, 64 (92.8%) were homogeneous and 5 (7.2%) heterogeneous. All 5 discordant cases were in tumors with two available blocks. In three, the first test was KRAS-wt and the second KRAS-mut; in one, the first test was KRAS-mut with the second test KRAS-wt; in one, different mutations were seen in the two samples. No heterogeneity was seen at KRAS codon 61.

| Region of interest | Primer sequences and PCR conditions for amplification and analysis of KRAS and BRAF | Pyrosequencing primer (5′ → 3′) | PCR amplicon length |
|--------------------|---------------------------------------------------------------------------------|---------------------------------|---------------------|
| KRAS codons 12/13* | Fwd: GDCCTCTGCTGAAAATGACTGA | AACTCTGCTGGTGGTGA | 80 bp |
| Rev: AGCTGTATCGTCAAGGCACTCT | AAACCTGCTGGTGGTGA | |
| KRAS codon 61 | Fwd: AATTGATGGAGAAACCTGTCTCTT | GGAATCTGCTGACACGCA | 86 bp |
| Rev: TCCTCA4GTCCTGGCTCTCCTCA | TCCCTCA4GTCCTGGCTCTCCTCA | |
| BRAF codon 600 | Fwd: TGAAGACCTCACAGTAAAAATAGGT | TGATTTCTGCTGACACGCA | 91 bp |
| Rev: CTGCTGCTGACACGCA | TGGCTGCTGACACGCA | |

Abbreviations: PCR, polymerase chain reaction; Fwd, forward; Rev, reverse; bp, base pairs; * KRAS codons 12 and 13 amplify together as a single amplicon; Thermal cycling conditions for all three amplicons were as follows: 95 °C for 12 minutes followed by 40 cycles of 95 °C for 10 seconds, 55 °C for 20 seconds and 72 °C for 20 seconds; PCR reaction conditions for all three amplicons were as follows: primer concentration 200 nM, MgCl₂ concentration 2 mM, 20 ng of genomic DNA and 12.5 μl of Qagen HotStar mastermix in a final reaction volume of 25 μl.
All 69 primary tumors were assessable for BRAF codon 600, and 2 (2.9%) were discordant. In one case, three FFPE tumor blocks were tested, with the first and third tests BRAF-mut and the second test being BRAF-wt. In the second case, two tumor blocks were tested, with the first giving a BRAF-mut result and the second a BRAF-wt result (see Table 2 below).

In the PICCOLO trial samples where two blocks were available, the second sample was re-tested to assess intra-lab variability. All KRAS and BRAF statuses were in agreement with the original result.

Thus, 7/109 primary tumors—10.1% of those tested—showed intratumoral heterogeneity of KRAS or BRAF status in this study.

3.3. DNA cocktail study

Of the 7 patients identified as having intratumoral heterogeneity, full sets of tumor blocks were available in all cases. Sections were cut from each block and pooled. “DNA cocktails” were produced from the mixed tumor material. Testing of this DNA correctly identified the mutation previously detected in the individual samples (see Table 3). In the tumor where two different KRAS codon 12 mutation had been detected, only one of the two mutations was identified in the DNA cocktail sample.

4. Discussion

The licensing of anti-EGFR-mAb therapies, and the inclusion of a molecular selection strategy, is impacting greatly upon aCRC management.

Whilst many patients benefit from this progress, the use of anti-EGFR-mAb therapy comes at a significant cost, both clinically and financially. Patients must accept significant toxicity risks, attributable directly to the mAb and, when used in combination therapy, enhanced chemotherapy side-effects [7]. The financial burden on health services is higher than many other interventions. Perhaps most importantly, several trials have now demonstrated that for some patients the effect of adding an anti-EGFR-mAb will be to hasten cancer progression and death. This has been observed most consistently in patients with KRAS-mut tumors [9]. It is imperative to make every effort to confine the use...

### Table 2

| Codon analyzed       | Total no. of cases assessed | No. of tumors displaying homogeneity | No. of tumors displaying heterogeneity |
|----------------------|----------------------------|--------------------------------------|---------------------------------------|
| KRAS codons 12 & 13  | 68                        | 64*                                 | 5 (7.2%)                              |
| KRAS codon 61        | 68                        | 66*                                 | 0                                     |
| BRAF codon 600       | 68                        | 67*                                 | 2 (2.9%)                              |

*3 samples from one patient displayed a c.38G>A mutation, whereas the remaining 5 samples carried a c.34G>T mutation. It was determined from the pathology report that the patient had 2 synchronous tumors.

### Table 3

| Sample ID | Tumor content (% of block) | 1st KRAS result (KRAS 12/13) | 2nd KRAS result (KRAS 12/13) | DNA Cocktail |
|-----------|---------------------------|-----------------------------|-----------------------------|--------------|
| P17       | c.25                      | c.35G>A                     | 25–50                       | WT           |
| P33       | c.25                      | c.35G>A                     | 25–50                       | WT           |
| P46       | 30–70                     | WT                           | 25–50                       | c.35G>A      |
| P932      | c.25                      | WT                           | 25–50                       | c.35G>A      |
| P964      | c.25                      | c.35G>A                     | 25–50                       | c.35G>A      |

| Sample ID | Tumor content (% of block) | 1st BRAF result (BRAF) | 2nd BRAF result (BRAF) | Tumor content (% of block) | DNA Cocktail (BRAF) |
|-----------|---------------------------|------------------------|------------------------|----------------------------|---------------------|
| P662      | 25–50                     | Mutant                 | <25                    | WT                         | >75                 |
| P710      | <25                       | Mutant                 | <25                    | WT                         | n/a                 |

n/a: Not applicable.
of anti-EGFR-mAbs to patients who can gain benefit, and avoid the futile and harmful treatment of patients with detectable mutations. Current UK license for cetuximab and panitumumab specify their use in patients with KRAS-wt tumors, as determined “by an experienced laboratory using a validated test method”. Usually this entails a single test from one tumor block. The data presented here suggest that the simple expedient of testing a second block in patients whose first test is KRAS-wt (or, if separately validated with larger numbers, performing a single test using a cocktail of DNA from two or more tumor blocks in the genotyping assay) will detect mutations in an additional 10% of patients. We have also shown that the heterogeneity is unlikely to be due to the testing of areas of differing tumor content because, despite the percentage of tumor cells in the heterogeneous samples often falling below 25%, in none of the tumors was the level less than 10%, which is still well above the threshold of 5% for detecting mutant alleles. Duplication of the analysis of the DNA from the second block of the 50 PICCOLO samples revealed that all samples gave identical results to the original analysis, showing that heterogeneity is not caused by intra-laboratory reproducibility. Indeed, during validation experiments, reproducibility was determined to be >99%.

Furthermore, we would like to suggest that heterogeneity is not a result of tumor differentiation status as all tumors with heterogeneity were moderately differentiated, like the vast majority of all those tested. Tumor size also does not appear to be an influencing factor, as all the tumors tested were from primary resection blocks, reducing the size difference between tumors. This is just one of several relatively simple approaches that, together, could greatly refine the selection of patients for anti-EGFR-mAb therapy. The commonly-used TheraScreen assay (DSx, Manchester, UK) detects the 7 most common KRAS mutations, giving typically a 40% KRAS-mut detection from a single test. We previously demonstrated that pyrosequencing of KRAS codons 12, 13 and 61 detects a further 4% less common activating mutations, and others have recently characterized a common activating mutation at codon 146, present in 5% colon cancers [18]. NRAS codon 12–13 mutations are found in around 4% tumors, and BRAF codon 600 mutations in 8%, mutually exclusive of KRAS mutation.

Combining these data, we estimate that a screening approach using DNA from 2 or more tumor blocks and sequencing these 6 hotspots (KRAS c.12, 13, 61, c.146, NRAS c.12, 13, c.61; BRAF c.600) would detect activating mutations in approximately 65% of patients. The clinical consequences of more refined patient selection could be profound. To date, the clinical impact of anti-EGFR-mAb therapies, especially in first-line combination, has been inconsistent. Even in KRAS-wt subpopulations, some trials have shown no benefit or even significant detriment. However, if 40% patients included in these “KRAS-wt” subpopulations have undetected mutations in KRAS, NRAS or BRAF, it is possible that negative impacts in these patients are abrogating the benefits in truly EGFr-driven tumors – for whom the benefits may be substantially greater than is currently apparent.

Given the high costs of therapy, the approaches discussed above are likely to be economically and clinically beneficial. Molecular analysis costs vary internationally and between commercial and academic sectors, but in our UK laboratory the full economic cost of DNA extraction and KRAS pyrosequencing, including sample preparation and overheads, is around £150 ($249; €166) per block. The average UK cost of cetuximab treatment, assessed by NICE (www.nice.org.uk), is approximately £22,000 ($37,000; €24,000). At the most basic level, restricting analysis to KRAS, testing a second block in patients with an initial KRAS-wt result would be expected to give a mutation pick-up rate of 10%, saving £139,000–£205,000 ($231,000–$341,000; €154,000–€227,000) per 100 patients re-tested. Alternatively, using the ‘DNA cocktail’ approach for the first test in all patients, including DNA from two or more tumor blocks, the cost of testing approximates to one single test, further increasing cost-savings and avoiding delay in sample processing. However, it is difficult to speculate as to the optimal number of block to be tested in a ‘cocktail’. The concern would be that, in a heterogeneous tumor, the more blocks that were tested, the more likely it would become for the heterogeneous nature to be masked by dilution. Further work is needed to validate this approach on a larger sample size to confirm that heterogeneously-expressed mutations can be consistently detected.

Adding detection of other activating mutations in the MAPK pathway (KRAS c.146, NRAS c.12, 13, c.61, BRAF c.600) will add modest but variable unit costs depending on the assay technique employed. Given
that overall some 15–20% patients may have an acti-
vating mutation at one of these sites, there is clearly a
high imperative, both clinically and economically, to
confirm initial data suggesting that they carry the same
negative predictive value for anti-EGFR-mAb therapies
as KRAS-mut.

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