Review: *KRAS* mutations are influential in driving hepatic metastases and predicting outcome in colorectal cancer

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Abstract: Metastatic colorectal cancer (CRC) is a topic of intense research. KRAS mutations have emerged as aggressive drivers of disease. Here we discuss the role of KRAS mutations in metastatic progression of CRC. We describe how KRAS has become a useful biomarker in metastatic CRC and examine where future trials may look to target KRAS mutant tumors for therapeutic benefit.

Keywords: KRAS; metastatic; colorectal; cancer

Introduction

Colorectal cancer (CRC) remains a significant healthcare concern and remains the third most common cause of cancer-related death in the USA and is the 2nd leading cause of death (1). Death from CRC is usually due to metastatic disease, and how to best treat, stratify, and improve treatments for metastatic CRC (mCRC) patients using biomarkers to guide precise treatment decisions is a topic of intense study and interest. A better understanding of the biology of CRC has led to some advances in oncological therapies for patients. Cetuximab is an epidermal growth factor inhibitor which plays a role in treatment of mCRC. There have been numerous randomised controlled trials assessing the efficacy of the addition of anti-EGFR to current first-line chemotherapy with varying results. The greatest benefits have been seen in patients with *Kirsten Rat sarcoma viral oncogene* (*KRAS*) wild type tumors. *KRAS* mutations have been shown to exert a negative effect on efficacy of cetuximab when used with other chemotherapy agents (2).

Despite these advances, surgery remains the most effective therapy in the setting of oligometastatic disease and in particular, colorectal liver metastases (3). This review focuses on our understanding of the important role that *KRAS* mutations play in the progression of colorectal cancer. We describe the frequencies of *KRAS* mutations, their relevance to CRC in the metastatic setting, and proposed mechanisms by which these mutations may drive metastasis. We examine how future trials may look to target *KRAS* mutant tumors.

KRAS mutation in colorectal cancer

RAS proteins control signalling pathways that in the presence of normal homeostatic mechanisms lead to controlled cell growth and proliferation (4). Knockout mouse studies have shown that *Kras* deletion leads to synthetic lethality and, unlike closely related proteins *Hras* and *Nras*, is required for normal development (5). In the setting of cancer activating oncogenic mutations, usually via point mutations, there is chronic 'switching-on' of these pathways and resultant malignant potential (6). Activated RAS protein expression leads to dysregulated tumour cell growth, invasiveness and angiogenesis (7). *KRAS* mutations activate signal transduction via GTP binding. By binding
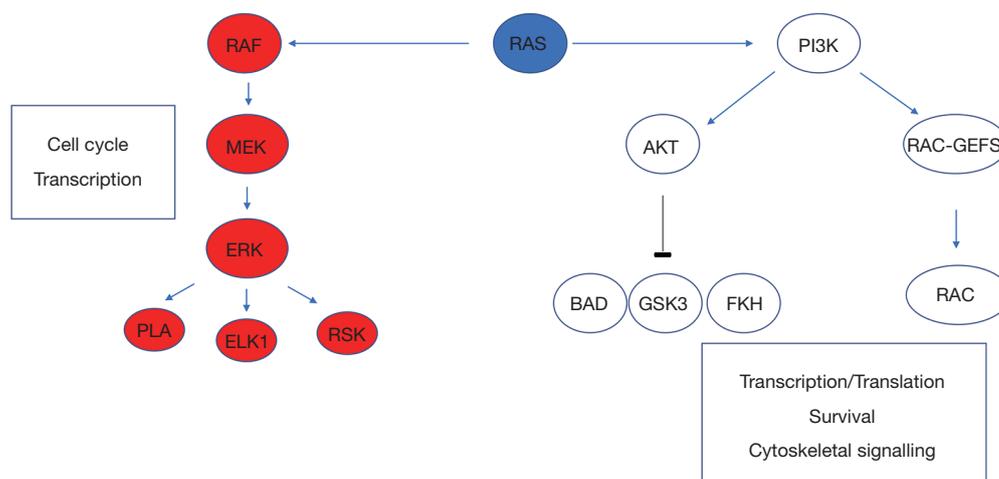
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Figure 1 Major RAS signalling pathways.

RAF, activated KRAS sets in motion a sequence of kinase activation that results in MEK then sequentially ERK kinase activation, resulting in ERK translocation to the nucleus and engagement of transcription mechanisms (*Figure 1*). Additionally, PI3-kinase activation may be engaged, resulting in control of a large number of downstream enzymes. It was recognised as early as 1987 by Bert Vogelstein's group that *KRAS* was mutated in approximately 30% of cases and mutations appeared most commonly at codon 12 (8). CRC was comprehensively molecularly characterised in 2012 (9). *KRAS* was mutated in 43% of non-hypermutated cases of CRC, with mutations of the oncogenic codons 12 and 13 of exon 2 predominating. Indeed, almost all RAS activity, in tumours from different tissues of origin, is due to activating mutations in codons 12, 13, and 61 (10). Data obtained from trials suggests chemotherapy resistance mechanisms may also be mediated by mutations in exon 4, codon 146, though the influence of codon 61 and 146 mutations is difficult to ascertain from studies due to their relative rarity. Interestingly, 55% of non-hypermutated patients had alterations in *KRAS*, *NRAS*, or *BRAF* with the majority of these being mutually exclusive. Furthermore, Poulin *et al*. (11) have recently shown by utilising a cross-disciplinary approach that tissue-specific phenotypes (i.e., those specific to the colon) result from the signalling properties of each *KRAS* allele. This work suggests that engagement of signalling mechanisms downstream of *KRAS* mutations is both tissue and context dependent.

Concordance in *KRAS* mutations between primary and metastatic sites in colorectal cancer

As described, mutant KRAS functions as a transducer of growth signals downstream of EGFR activation following constitutive activation of G12D/G13 codons, resulting in the essential neoplastic function of *KRAS* mutations, and subsequent resistance of tumor cells to EGFR inhibitors including cetuximab (12). Therefore, *KRAS* status of tumors has played an important role in determining subsequent sensitivity to EGFR inhibitors. Likewise, microsatellite instability (MSI) and mismatch repair protein expression segregates a subset of patients that may respond favourably to T cell directed checkpoint inhibition including PD1 inhibitors (13,14). Tissue is required for assessment of mutation status. Studies have assessed correlation between primary and metastatic sites, analysing if primary tissue is sufficient to guide treatment. Fujiyoshi *et al*. enrolled 457 patients with primary and metastatic tumours (499 synchronous and 57 metachronous metastases) and performed analysis of *KRAS* and *BRAF* mutation and MSI status (15). Concordance was very high between primary and metastatic site and remained high across all mutations (>90%). *KRAS* status between primary and metastatic site showed discordance in 16 cases. Most of these cases were discordant between lymph node metastases and may be due to intratumoral heterogeneity. Liver metastases showed high concordance, in keeping with Knijn *et al*. (16). These data

confirm *KRAS* mutations are an early event in colorectal carcinogenesis. There was no difference observed between synchronous and metachronous metastasis formation.

Bhullar *et al*. published a meta-analysis of biomarker concordance between primary and metastatic sites in CRC (17). They confirmed across all studies *KRAS* concordance was 93.7%. These findings are in keeping with Vakiani *et al*. who found very high concordance between primary and secondary sites for *KRAS* mutations but not *TP53* and *BRAF* mutations (18). These data confirm that sampling of either primary or metastatic site is adequate for assessment of *KRAS* mutations that are key in treatment decision making and in helping understand the process of metastasis in CRC. Few genetic changes are thought to occur in progression from primary to metastatic disease, suggesting that epigenetic and microenvironmental factors are likely to play a key role in the process of metastasis in CRC.

Clinical relevance: KRAS influence on prognosis following liver resection

Liver resection remains the only option for cure for patients with CRLM (19,20). Extent of disease involvement is the primary measure of concern when making treatment decisions to ensure limitation of postoperative morbidity and maximisation of survival benefit (21). Prediction of early recurrence remains difficult. Modern treatment regimens now dictate that preoperative chemotherapy is standard of care prior to CRLM resection. Medical oncologists use *KRAS* status to select patients for EGFR-directed chemotherapy in more advanced, irresectable disease. However, *KRAS* mutation status represents a useful prognostic marker in response to therapy for patients undergoing liver resection and can be incorporated into modern risk scores to help inform treatment decision making. Brudvik *et al*. undertook a meta-analysis of studies examining the influence of *KRAS* mutations on outcome following CRLM resection. In the 14 studies that met inclusion criteria, 8 studies associated *KRAS* status with overall survival (22). KRAS mutations were adversely associated with overall survival following liver resection in each of these studies, and with recurrence free survival in 7 of these. These findings are entirely independent of EGFR inhibitor utilisation. These data demonstrate in a clinical context that the relevance of *KRAS* mutations in CRC extends beyond response to EGFR inhibitors and has an important role to play in progression of advanced disease. Indeed, this study identified the propensity for higher rates

of *KRAS* mutation in widespread metastatic disease, with patients with resectable oligometastatic disease harbouring fewer *KRAS* mutations than most studies of all-comers with metastatic disease. These findings must be considered in light of findings by certain authors who have found traditional clinical risk scores limited in their usefulness when assessing response to resection for CRLM (23). The main advantage of *KRAS* mutation status in this context is as a single biomarker, an early event in colorectal cancer pathogenesis, and an event unperturbed by chemotherapy. Importantly though, recent work by Kawaguchi *et al*. has demonstrated that *KRAS* status alone carries less prognostic power than *KRAS* mutations coexisting with mutations in *TP53* and *SMAD4* (24). In future, personalised therapies will look to target complex interactions between multiple genetic aberrations in colorectal cancer.

The relationship between KRAS expression, mutation location and outcome following resection of colorectal liver metastases

It is accepted that *KRAS* and *BRAF* mutations are associated with poor prognosis following resection for CRLM. Interestingly, using a temporal assessment formula, Margonis *et al*. have shown that as time passes from resection, surgeon dependent factors including margin status and resected extrahepatic disease predominate after the first year (25). By using a meta-analysis approach, Dr. Pawlik and his group have shown that *KRAS* is a poor prognostic factor following CRLM resection, with *BRAF* mutant patients having even poorer prognosis (26). Additionally, in response to preoperative chemotherapy and low CRLM tumor burden, 5-year survival for *KRAS/NRAS/BRAF* WT patients was 55%, *KRAS/NRAS* mutant patients was 35%, and for *BRAF* mutant patients was 0%. Therefore, genetic status, tumor burden and subsequent change in tumor burden should influence pre- and post-operative chemotherapy decision making in the setting of CRLM resection (27).

Given that resistance to chemotherapy regimens may be mediated by genetic heterogeneity within tumors, investigators have begun to assess the impact of mutation location on outcome following resection for CRLM. Frankel *et al*. studied the location of *KRAS* mutation in context of survival following resection for CRLM (28). Whole genome analysis of 211 patients revealed that 43% had either a *KRAS* or *NRAS* mutation with the majority found in *KRAS*. Most mutations were in codons 12 and 13
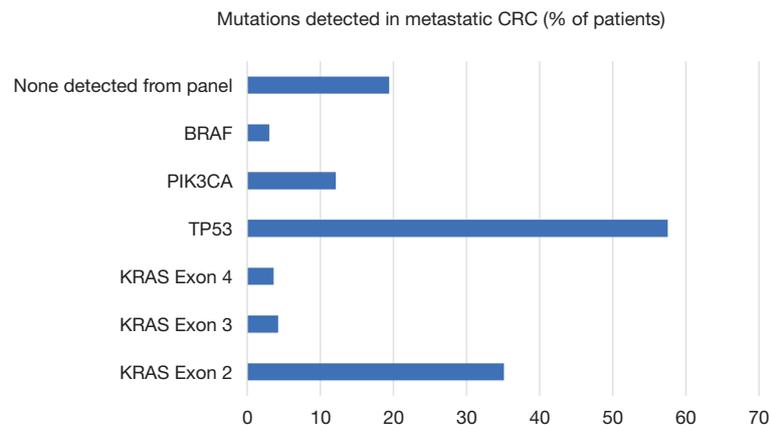
Figure 2 Representative proportions of detected mutations in metastatic colorectal cancer [Adapted with permission from (28)].

with the majority in *G12D*, *S* or *V* and *G13C* or *D*. Exon 3 (codon 61) accounted for 10% of mutations and exon 4 (codon 146) 8.5% (*Figure 2*). There were clear differences between exon 3 and 4 tumors. Exon 4 tumors were larger, more likely to be solitary, and associated with longer disease-free survival. Kwak *et al.* performed meta-analysis of codon 13 mutations on overall survival; they confirmed both codons 12 and 13 were associated with poorer overall survival compared with wild type tumors and there were no differences between the 2 groups (29). Interestingly, when patients were separated based on EGFR therapies, those that received EGFR therapy had similar outcomes if they harboured codon 13 mutations or were WT. Such meta-analyses do have limitations, as other mutation status and confounding factors are not controlled for in these trials (e.g., number of mutations samples, NGS platform used). Recently Margonis *et al.* have confirmed that *KRAS* mutations were associated with poorer response to pre-hepatectomy chemotherapy; however, codon 13 mutations correlated with the worst responses, poorer than codon 12. These data suggest differences in biology between sites of mutation and subsequent differences in resistance to chemotherapy (30).

Interestingly, in the Frankel study, pattern of recurrence was also influenced by genotype. *BRAF* mutant patients suffered multifocal recurrence and *KRAS* mutant patients recurred in liver and lung in a similar fashion to wild type patients. Vauthey *et al.* similarly captured all *KRAS* mutations and found *KRAS* mutation was associated with poorer outcome following resection of CRLM (31). Of note, their data were striking for the low level of *KRAS* mutations within their cohort but this could simply be due

to selection or referral bias. Interestingly, in a separate cohort of CRLM patients that had hepatic artery infusion chemotherapy, *KRAS* status did not influence outcome, suggesting this technique may overcome some of the aggressive features of *KRAS* mutant disease (32). The literature however is seeded with studies that have analysed only exon 2 mutations, some of these have shown poorer overall survival in *KRAS* mutant patients (33,34), while others (35) have observed no influence. Importantly, the patients in Frankel's cohort demonstrate that up to 20% of *KRAS* mutations are harboured in exons 3 and 4 and therefore must be considered when assessing *KRAS* status, as these mutations convey very different recurrence and survival profiles. Data from Janakiraman *et al.* confirm this finding that exon 4 mutant tumors are larger, have less malignant potential when compared with exon 2 and 3 mutations (36) and correlate with functional studies in mice performed by Poulin *et al.* (11) that show different phenotypes generated as a result of *G12D* (exon 2) or *A146T* (exon 4) mutations. Future studies should adopt a broader next generation sequencing platform to permit detection of all *KRAS* mutations and to consider them in the context of all genetic aberrations in individual patients to permit personalisation of therapies.

Increasing complexity: KRAS association with stage and molecular subtypes of disease

Following early sequencing genomic sequencing studies, investigators have begun to focus less on individual mutations and on characterisation of the pattern of mutations and gene expression to subclassify CRC.

Guinney *et al.* subclassified CRC into the consensus molecular subtypes (CMS) in 2015 in an effort to resolve inconsistencies in assessment of the disease and to promote clinical targeting and translation (37). *KRAS* mutant tumours were distributed throughout the 4 subtypes but were overrepresented in CMS 3 disease.

CALGB/SWOG 80405 assessed patients with metastatic colorectal cancer treated with first line therapy plus either bevacizumab or cetuximab. The study showed no significant difference in overall survival between groups. Innocenti *et al.* in their analysis of mutations in the trial showed that patients with tumours with high mutational burden survived longer, while patients with both *BRAF* and *KRAS* mutant tumours had poor overall survival (38). Williams *et al.* (39) looked at early colorectal tumours and again found that those with high mutational burden survived longest; similarly to findings by Van den Eynde and colleagues (40), they showed that tumour infiltrating lymphocyte response was the greatest predictor of outcome. Immune infiltration superseded dMMR, pMMR/*BRAF^wt*/*KRAS^wt*, pMMR/*BRAF^mut*/*KRAS^wt*, pMMR/*BRAF^wt*/*KRAS^mut* profiles and indeed transcriptional CMS subtypes in predicting outcome. Lal *et al.* (41) showed that *KRAS* mutation and CMS2 and 3 were independently associated with reduced immune cell infiltration, associated with poorer outcome. Pitroda *et al.* (42) found that liver metastases when considered in isolation by CMS RNA signatures >30% were unable to be classified accurately. They derived their own signature that showed once again that *KRAS* was strongly associated with the worst subtype of disease. Furthermore, they derived an immune subtype associated with the most favourable outcomes and a canonical subtype that predicted intermediate outcomes. These studies suggest that mutant *KRAS* is associated with the most aggressive disease and provides the stimulus for development of an aggressive, stromal/mesenchymal microenvironment that promotes metastatic progression as described in recent murine modelling studies.

At present and as evidenced by the MRC COIN trial, there is a large gap in our understanding as to the mechanism of resistance that underlies EGFR-directed therapies, and optimum combination therapies for patients with advanced CRC (43). This large randomised controlled trial showed no difference in progression free and overall survival in patients receiving first line palliative oxaliplatin plus or minus cetuximab in both *KRAS* wild type and mutant patients. Indeed, a recent phase II trial (44) has demonstrated that patients carrying polymorphisms in non-functional receptor KIR2DS4 respond positively to cetuximab therapy. The only group that seemed to benefit in COIN were those wild type patients on infusion fluorouracil and cetuximab, with limited disease. One positive factor from COIN was the stratification in response seen between *KRAS* mutant and wild type patients, with *BRAF* mutant patients faring worst of all. Future trials will look to stratify patient subsets based on molecular subtypes, while it is clear that novel strategies and targets within the microenvironment of metastases need to be trialled to improve understanding of the disease and resistance mechanisms. One hundred eighty-eight trials have recently recruited, or are ongoing, using KRAS as a biomarker in metastatic colorectal cancer. These trials, though too numerous to list, give an appreciation of the importance of KRAS to the future understanding of metastatic CRC and in developing appropriate therapies for patients (clinicaltrials.gov).

Mechanism of KRAS-driven metastasis

Recent murine modelling studies have provided an insight into the role of mutant KRAS in generating and maintaining CRC metastases. Using an inducible *Kras* allele (exon 2, G12D) in combination with conditional null *Apc* and *Tp53* alleles, the 'iKAP' model, Boutin *et al.* showed that the model faithfully progressed from adenoma to metastasis when *Kras* was mutated, but not in the presence of wild type *Kras* (45). Exome sequencing revealed that mutant *Kras* cell populations were homogenous in metastases while heterogeneous in primary tumours suggesting the dependency of metastases on this subpopulation of cells. Genetic elimination of mutant *Kras* led to absence of this population at primary and secondary sites and subsequent apoptotic elimination of cells, suggesting that mutant *Kras* is important for maintenance of CRC metastases. High levels of TGF-β expression were seen, in keeping with a mesenchymal phenotype and consistent with strong overlap with the most invasive CMS4 CRC seen in humans. Incredibly when mutant *Kras* was switched off in advanced tumors, these tumors reverted to a benign adenomatous morphology.

De Pinho's group have also recently demonstrated that *Kras* mutant cells engage myeloid derived suppressor cells via CXCR2 signalling which then promote immunosuppression and evasion of checkpoint blockade (46). This mechanism is believed to be reliant on interferon regulatory factor 2. Similarly, our group have observed an important role of CXCR2-positive MDSCs in driving metastases in mutant *Kras* CRC. Our findings suggest Notch signalling, downstream of tumour CXCL5 expression, plays

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a critical role in engagement of these cells and subsequent immunosuppression (Jackstadt *et al.*, In Press, Cancer Cell). The importance of these observations lies in resistance to immunotherapy seen in CRC (47). Elimination of T cell suppressive cells from these tumours in the future may render them amenable to targeting by immunotherapy (48).

Additionally, Chu *et al.* have demonstrated that activation of IGF-1R downstream of mutant Kras leads to reversible induction of metastasis in murine models, raising the possibility of a role for MEK inhibition in a subset of patients with metastatic CRC (49). Batlle's group has shown on an *Apc*, *Kras* mutant background that TGF-β produced by tumor stroma regulates immunogenicity by influencing T cell responses. In experimental models TGF-β inhibition released this block on T cell responses and sensitised metastases to PD1 checkpoint inhibition (50), indicating a potential therapeutic strategy for future trials. In this setting it will be interesting to observe the development of parallel tumor biopsies and serial ctDNA monitoring to assess response to therapy and assessment of reasons for resistance to specifically targeted therapies (51). Indeed, Dr. Corcoran and his group have already shown that tumor heterogeneity can drive molecular resistance to therapy and influence lesion-specific responses to targeted therapies (52).

Conclusions

KRAS mutant CRC patients have poorer disease free and overall survival. Patients tend to develop aggressive tumours that metastasize with high concordance in mutation status between primary and secondary sites. Insights from modelling studies suggest mutant *KRAS* works at multiple levels to sustain an aggressive phenotype of colorectal cancer. Recent work in human cohorts suggests, however, that the site of mutations may influence tumor behaviour and outcome. KRAS remains an important biomarker of aggressive disease, and trials have shown it can stratify response to therapy and recurrence after resection. Work into KRAS mutations in the context of the transcriptional profile of metastatic CRC is ongoing. It is likely these aggressive tumors will require multiple individually targeted therapies, in combination with real-time assessment of resistance mechanisms, to prevent recurrence in adequately resected CRLMs in the future.

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Footnote

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Ethical Statement: The authors are 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.

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