Review Article

Colorectal cancer genomics and designing rational trials

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Abstract: The widespread use of next generation sequencing (NGS) has led to a refined understanding of the genomics of colorectal cancer (CRC). However, progress in the use of molecular biomarkers in standard practice has been slow, and there is no approved targeted therapy for CRC based on a positive predictive marker yet. In this review, we will first summarize biomarkers with clinical utility in standard practice or targeted therapy trials and then consider how to rationally design clinical trials to more effectively target CRC. Specifically, we will discuss current clinical applications of genomic information consisting of the use of the MAPK (mitogen-activated protein kinase) pathway genes *KRAS*, *NRAS*, and *BRAF* as prognostic and predictive biomarkers for standard treatment, risk stratification by primary tumor site and consideration of tumor laterality in patient selection for epidermal growth factor receptor (EGFR) antibody treatment, and the evaluation for genomic biomarkers, including *BRAF* V600E, *HER2* amplification, and gene rearrangements, for targeted therapies in clinical trials. Applying lessons from targeted therapy trials in CRC, we now appreciate that both tumor genomics and tissue of origin affect targeted therapy response and that the development of resistance to targeted therapies is dynamic and often subclonal. Based on these understandings, we propose the design of adaptive clinical trials that evaluate real-time pharmacodynamic markers and monitor tumor subpopulations during the course of treatment to overcome challenges targeting genetic drivers in CRC.

Keywords: Colorectal cancer (CRC); genomics; targeted therapies

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Genomics of colorectal cancer (CRC)

The description of the adenoma-carcinoma sequence by Vogelstein and Fearon in 1990 first led to an understanding of CRC as a disease of successive genomic changes (1) and subsequent genomic studies have refined and expanded this understanding. Among the most important milestones, The Cancer Genome Atlas (TCGA) network performed a comprehensive molecular characterization of 224 resected colon and rectal tumors, demonstrating similar patterns of genomic alterations in these tumors, identifying recurrent genomic alterations, and characterizing tumors based on mutation frequency as ultramutated, hypermutated, and non-hypermutated (2) and the CRC Subtyping Consortium analyzed gene expression profiles to describe a consensus molecular subtype (CMS) classification consisting of four subgroups based on results from six independent transcriptomic-based subtyping systems (3). Despite the rapidly advancing understanding of genomic alterations in CRCs, until recently the clinical utility of molecular information in standard practice was limited to the use of *KRAS* as a negative predictive biomarker of response to EGFR (epidermal growth factor receptor) targeting antibodies. With decreasing costs and turnaround time, next generation multi-gene sequencing panels of tumor tissue and circulating free DNA (cfDNA) have entered the clinic and

the use of these methods has been rising exponentially (4). In this review, we will discuss the increasing incorporation of genomics in the treatment of patients with metastatic colorectal cancer (mCRC) both in the context of standard practice and clinical research.

Standard genomic markers in current clinical practice

Our knowledge of the genomic landscape of human cancers has rapidly accelerated with technological advances in sequencing, from capillary-based sequencing technologies to the modern massively parallel sequencing of today (5). Next generation sequencing (NGS) assays are highly sensitive, can analyze a large panel of genes, and detect novel mutations, small insertions and deletions (indels), copy number alterations, and select gene fusions and rearrangements from small amounts of DNA (6). A detailed description of technical aspects of NGS is beyond the aims of this review, but its fundamental principle is the spatial separation of individual DNA molecules and simultaneous analyses of millions of individual molecules. As each nucleotide in the sequences of each of the DNA strands is individually analyzed, the data are recorded and compiled computationally. The compiled data enable the bioinformatic analysis of multiple genes from multiple samples (7).

RAS

EGFR targeting antibodies, the first molecular targeted therapy for CRC, brought genomic data to the clinical assessment of CRC patients. These drugs improve survival in metastatic disease, but response is seen in only about 10% of unselected CRC cases (8,9). EGFR expression, the logical marker for these agents, was found not to correlate with tumor response (10), and thus a search began for predictive markers to guide patient selection. The major breakthrough in this area was the identification of *KRAS* exon 2 mutations as predictors of lack of benefit (11,12). KRAS, a small GTP-binding protein, lies downstream of EGFR and acts as signal switch whose activation engages effectors that control proliferation, differentiation, and survival (13). Subsequently, activating *KRAS* hotspot mutations in exons 3 and 4 and in *NRAS* were found to also predict for lack of benefit from EGFR therapies, refining the population of patients for these agents (14). Clinical data suggest that the use of EGFR inhibitors in patients with *RAS* mutant tumors may be associated with harm and shorter survival (14) and it has been speculated that this may be due to the inhibition of wild-type RAS within these tumors (15). The prognostic value of *RAS* mutations is more controversial, but increasing data associate *RAS* activating mutations in CRC with shorter survival and increased risk of recurrence after resection of metastases (16-18).

BRAF

BRAF encodes a serine/threonine protein directly downstream from RAS in the canonical mitogen-activated protein kinase (MAPK) pathway and mutations in this gene occur in up to 12% of mCRC patients (19). The majority of these consist of a substitution of glutamic acid for valine at the V600 hotspot in exon 15. The resultant V600E BRAF mutant is constitutively activated and can signal independent of RAS activation (20). The clinical validation of this biomarker contrasts with that of *RAS*, as *BRAF* V600E was demonstrated early on to be a strong negative prognostic biomarker in mCRC and later was appreciated as a negative predictive marker for EGFR inhibitor treatment. *BRAF* V600E mutation is associated with shorter overall survival, estimated around 14 months (21,22). This survival interval is similar to what was seen before modern combination chemotherapies, suggesting limited activity of second line treatment in this subgroup. *BRAF* V600E is associated with the poor prognostic features of T4 primary tumors, poor tumor differentiation, and peritoneal carcinomatosis (23-25). Whether the presence of this mutation should alter first line treatment remains controversial, but molecular subgroup analysis of the TRIBE trial suggests that for fit patients the combination FOLFOXIRI-bevacizumab can achieve better overall survival compared to FOLFIRI-bevacizumab (16).

In contrast to the strong prognostic effect of *BRAF* V600E, the predictive effect of *BRAF* V600E on EGFR inhibitor response has been less clear, likely due to the poor prognosis of this subgroup overall and a more subtle effect compared to *RAS* mutation; EGFR inhibitor treatment does not appear to cause harm in *BRAF* V600E mCRC, presumably due to the low levels of activated wild-type RAS in these tumors where high extracellular signal-regulated kinase (ERK) activation feedback suppresses upstream signaling. Three systematic reviews have evaluated *BRAF* V600E as a predictive marker for response to anti-EGFR therapy and all have found no significant benefit for

EGFR inhibition in *BRAF* V600E mCRC (26-28). SWOG 1406 which tested irinotecan-cetuximab combination as a control arm in the second or third line setting in *BRAF* V600E mCRC found a response rate of 4% and median progression-free survival of 2 months to this combination, further supporting limited activity of cetuximab in this population (29). The National Comprehensive Cancer Network (NCCN) guidelines recognize that *BRAF* V600E mutation makes response to panitumumab or cetuximab highly unlikely (30).

It has recently become appreciated that not all BRAF mutants act the same (31). Two groups of non-V600 BRAF mutants have been described (32). The first group acts as constitutively activated proteins that signal as dimers, independent of RAS, and the second group consists of hypoactive BRAF mutants that bind more tightly than wild-type BRAF to RAS and exhibit enhanced binding and activation of wild-type CRAF to amplify upstream signaling. Hypoactive BRAF mutants have been associated with improved survival and increased sensitivity to EGFR inhibitors (32-34).

Differences in genomics between right and left primary tumors

The different ontogenesis of right- and left-sided colorectal tumors suggests biological differences within CRC, but it was not until recently that this variable started to be analyzed thoroughly in the metastatic setting. The two major clinical implications of primary tumor laterality are that right-sided tumors have a worse prognosis (35,36) and are insensitive to anti-EGFR therapy (37-39). The difference in prognosis appears to be driven by a higher rate of mitogenic mutations in right-sided tumors, with an increased frequency of RAS, PI3K, and TGFβ pathway alterations. Among microsatellite stable mCRC, 80% of tumors with a right-sided primary harbor a hotspot *RAS* mutation or *BRAF* V600E compared to 46% of left-sided primary tumors. Left-sided tumors often have no discernable mitogenic genomic driver, and growth factor ligand expression is higher in left colon primaries (40). Specifically, left-sided tumors express higher levels of amphiregulin and epiregulin, which are associated with increased sensitivity to EGFR antibodies (41). Microsatellite instability-high CRC, while rare in metastatic disease, occurs much more commonly in the right colon. All mCRC cases should be evaluated for microsatellite instability as immune checkpoint inhibitors are associated with high response rates in this population (42,43).

Genomic markers for targeted therapies in clinical trials

BRAF V600E

BRAF V600E is the most prevalent potentially targetable alteration in mCRC. RAF inhibitors are FDA approved for the treatment of *BRAF* V600 mutant melanoma, non-small cell lung cancer (NSCLC), and Erdheim-Chester disease (44,45). While RAF inhibitors have a high response rate in these diseases, these drugs have limited efficacy in *BRAF* V600E CRC as single agents (46). It is thought that high levels of basal receptor tyrosine kinase (RTK) signaling, particularly EGFR, in CRC underlie rapid adaptive resistance because ERK inhibition releases these receptors from negative feedback suppression (47,48). Receptor reactivation causes a rebound in ERK phosphorylation both by reactivating ERK signaling and by recruiting RAS which forms RAF dimers, which are insensitive to current RAF inhibitors (as these drugs selectively inhibit RAF monomers). Based on this understanding, clinical trials have tested combination therapies of RAF and EGFR inhibitors with modest response rates of 10–25% (29,44,49-52) (*Table 1*). Adding a mitogen-activated protein kinase (MEK) inhibitor to RAF and EGFR inhibitors leads to more profound inhibition of ERK signaling and improved response rates (53). The ongoing phase 3 BEACON trial will evaluate whether doublet or triplet targeted therapy extends survival compared to standard therapy containing irinotecan and cetuximab for patients with *BRAF* V600E mCRC. Early results from the safety lead-in (n=29) for the triplet combination of the RAF inhibitor encorafenib, MEK inhibitor binimetinib, and cetuximab showed an overall response rate of 48% in patients with *BRAF* V600E mCRC (54).

While combination therapy has increased response rates in mCRC, most patients progress within a few months of starting targeted therapy. Reactivation of ERK signaling, rather than alterations in parallel signaling pathways, appears to underlie resistance in patient tumors (59,60). ERK inhibitors and novel RAF inhibitors that inhibit mutant monomers and dimers have been proposed as new agents that may be able to overcome resistance (59,60).

Table 1 Clinical trial efficacy data for targetable genomic alterations in mCRC

Alteration	Therapy	Number of patients	ORR (%)	Median PFS (months)	Reference
BRAF V600E	V	21	5	2.1	(46)
	V + P	15	13	3.2	(49)
	V + I + C	54	16	4.4	(29)
	D + T	43	12	3.5	(50)
	D + T + P	83	18	NR	(53)
	E + C	50	22	4.2	(52)
	E + C + A	52	27	5.4	(52)
	E + B + C	29	48	8.0	(54)
HER2 amplification	Tras + Lap	27	30	5	(55)
	Tras + Pert	34	38	NR	(56)
NTRK fusion	Laro	4	50	NA	(57)
ALK fusion	Ceritinib	1	NA	NA	(58)

V, vemurafenib; I, irinotecan; C, cetuximab; D, dabrafenib; T, trametinib; P, panitumumab; E, encorafenib; A, alpelisib; B, binimetinib; Tras, trastuzumab; Lap, lapatinib; Pert, pertuzumab; Laro, larotrectinib; NR, not reached; NA, not applicable; ORR, objective response rate; PFS, progression-free survival; mCRC, metastatic colorectal cancer.

HER2 amplification

Amplification of *ERBB2* (also called *HER2*) occurs in 2% to 6% of mCRC and is associated with insensitivity to EGFR antibody treatment (61-63). Targeting this RTK with the anti-HER2 antibody trastuzumab is one of the major successes in the treatment of breast cancer and since then further anti-HER2 drugs have demonstrated benefit in this disease. Treatment of colon cancer xenografts with *HER2* amplification indicated that trastuzumab and lapatinib, a small molecular inhibitor of EGFR/HER2 did not cause tumor regression as monotherapy, but the combination demonstrated tumor shrinkage (64). This combination was studied in the phase 2 HERACLES study in 27 patients with HER2-positive mCRC and 30% of patients achieved an objective response (55). Cohort B of the HERACLES trial, which is currently ongoing, will evaluate the activity of the antibody-drug conjugate TDM-1 and TDM-1 together with the anti-HER2 antibody pertuzumab in the second line setting. The multi-basket MyPathway study included a *HER2* amplified mCRC cohort treated with trastuzumab plus pertuzumab of 37 patients and found a response rate of 38% (56). The clinical significance of *HER2* mutations in mCRC has been less studied, and *HER2* mutations are more commonly concurrent with *RAS* mutations in mCRC than is *HER2* amplification. In two CRC patient-derived xenografts (PDXs) with *HER2* mutations, treatment with trastuzumab plus the pan-HER inhibitor neratinib or lapatinib produced tumor regression (65). There is initial evidence of efficacy in *HER2*-mutated NSCLC and breast cancer patients treated with targeted therapy (66,67), but if these results will be replicated in mCRC remains to be seen.

NTRK fusions

The NTRK family of kinases includes three genes, *NTRK1*, *NTRK2*, and *NTRK3*, which encode three transmembrane receptors—TRKA, TRKB, and TRKC, respectively. Fusions involving the *NTRK* genes have been identified, at low frequencies, across various tumor types including CRC and involve numerous partners (68). Selective NTRK inhibitors, such as larotrectinib and entrectinib, have been associated with high response rates in tumors with these fusions (57), including responses seen in mCRC patients (69).

ALK, ROS, and RET fusions

Recurrent gene fusions involving *ALK* and *ROS* have been described primarily in NSCLC, but infrequently are present in other tumors types, including CRC (70,71).

Targeted therapies against ALK or ROS kinase, including crizotinib, ceritinib, and alectinib, are FDA-approved for NSCLC with these alterations. In CRC, these fusions are present in 0.2% to 2.4% of patients (72,73). An analysis by Foundation Medicine of 3,117 advanced CRC cases genotyped with their NGS panel identified 6 cases (0.2%) with *ALK* fusions. One of these patients was treated with ceritinib and achieved a partial response lasting 9 months, until the tumor acquired a *KRAS* mutation (58).

RET kinase fusions are frequent in papillary thyroid cancer and have been described rarely also in lung adenocarcinoma and chronic myelomonocytic leukemia. In the Foundation Medicine series of 3,117 advanced CRC cases, *RET* fusions were present in 6 cases (0.2%). None of these patients had another driver mutation, and one of them was treated with regorafenib, which has activity against RET kinase. This patient had clinical and CEA tumor marker responses, but unfortunately no further follow-up was available as the patient died of unrelated causes shortly thereafter (74). In *RET*-rearranged lung adenocarcinoma drugs such as cabozantinib and lenvatinib have demonstrated initial activity (75,76), but in *RET*-rearranged mCRC these multikinase inhibitors have not been studied.

Designing rational trials

As noted above, currently targetable genomic alterations in mCRC are found in only a minority of cases and it has been difficult to bring targeted therapy for matched genomic alterations in mCRC to the clinic. Here we discuss some of the challenges and lessons learned in the delivery of precision medicine for mCRC.

Both genomic alteration and tissue histology influence response to targeted therapy

Where the same genomic alteration has been identified in mCRC and other tumor types, differing responses have been seen in mCRC compared to other tumor types. This is most dramatic for V600E *BRAF* where the BRAF inhibitors vemurafenib or dabrafenib are associated with a response rate of over 50% in melanoma and less than 10% in mCRC and combined RAF and MEK inhibitors, for example dabrafenib plus trametinib, achieves responses of about 70% in melanoma and 12% in mCRC (50,77). Also *HER2* amplified mCRC appears relatively insensitive to single agent trastuzumab and dual HER2 inhibitor therapy is needed for responses, while trastuzumab alone is effective in treatment of *HER2* amplified breast and gastric cancers (78,79). These differences have been attributed to the high RTK environment of the colorectum, where the activity of the targeted therapy is attenuated by release of negative feedback loops, reactivation of receptors, and a rebound in pathway signaling. Thus combination therapy is required to effectively inhibit pathway signaling and achieve clinical benefit. For example, in *BRAF* V600E mCRC, analysis of phosphorylated ERK expression levels before treatment exposure and after 14 days of treatment in paired tumor biopsies showed greater ERK inhibition with the triplet of RAF, MEK, and EGFR inhibition (dabrafenib, trametinib, and panitumumab) compared to baseline or to RAF and EGFR inhibition alone (dabrafenib and panitumumab), mirroring the improved response rate for the triplet, but the degree of ERK inhibition achieved with the triplet (60%) remained less than that seen for RAF inhibition (dabrafenib) in melanoma (84%) (53), providing a mechanistic explanation for the difference in activity between these tumor types. These data suggest that the activated oncogene (e.g., V600E *BRAF* or amplified *HER2*) remains a driver in CRC, but combination strategies that effectively inhibit the signaling network in mCRC must be devised to achieve greater clinical benefit.

Resistance is dynamic and often involves subclonal populations

The most common mechanism of acquired resistance to EGFR inhibitors in mCRC patients is the emergence of *KRAS* hotspot mutations (80-82). These mutants are thought to emerge from selection of a minor, pre-existing clone (83). Analysis of cfDNA in patients with progression through EGFR inhibitors indicates that the prevalence of these *KRAS* mutations in cfDNA is lower than that seen in patients with baseline *KRAS* mutant mCRC and the percent mutant reads decreases with time from last EGFR inhibitor exposure (82). These data suggest that resistance results from selection of subclonal populations and is dynamic based on exposure to the selective pressure. In patients with *BRAF* V600E mCRC progressing through targeted therapies, serial studies of cfDNA identified the emergence of more than one *RAS* mutation in 25% of patients (53), consistent with multiple, subclonal, resistant populations. In a patient at Memorial Sloan Kettering Cancer Center with *BRAF* V600E mCRC treated with vemurafenib/panitumumab who developed resistance

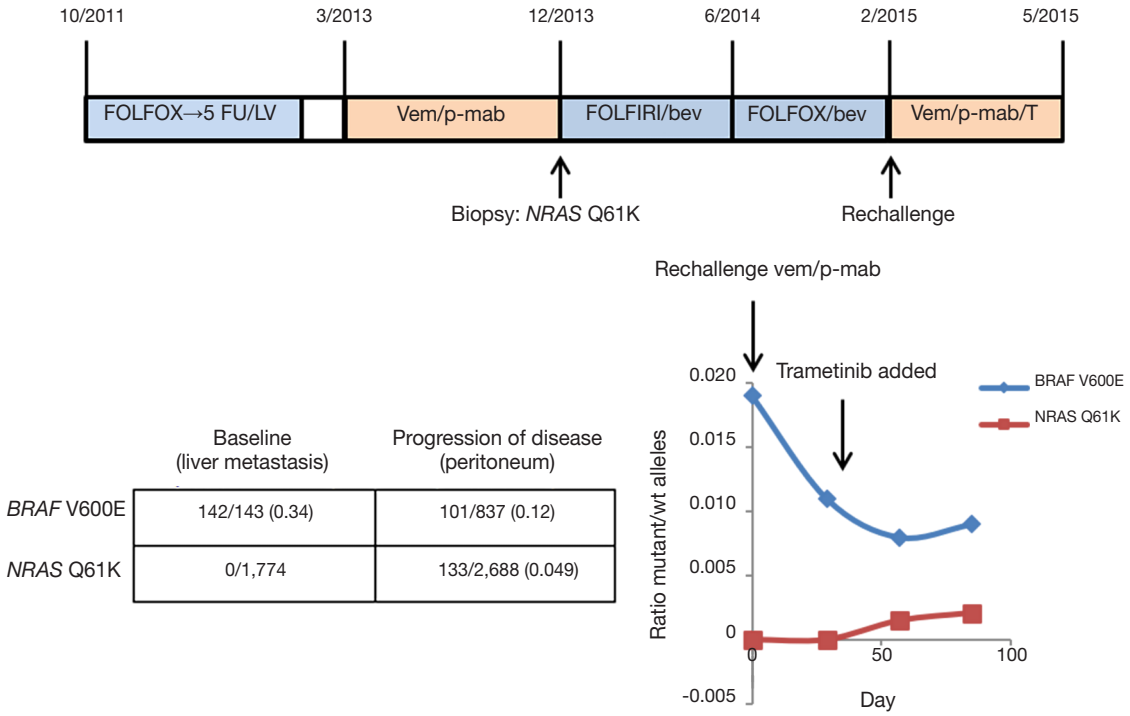

Figure 1 Rechallenge with RAF and EGFR inhibitors in a patient with V600E *BRAF* colon cancer. (Upper panel) Treatment history showing timing and duration of targeted therapy. (Bottom panel) *BRAF* and *NRAS* variant allele fractions in tissue samples (left) collected prior to all treatment and at the time of progression on vemurafenib/panitumumab and in serial plasma samples (right) collected every 4 weeks during a rechallenge with vemurafenib and panitumumab. In the absence of the selective pressure of vemurafenib/panitumumab treatment, the resistant *NRAS* Q61K becomes undetected in plasma, but with restarting treatment, the resistance alteration rapidly reemerges. The detection of the resistance *NRAS* Q61K mutation in plasma coincides with a plateau in suppression of *BRAF* V600E ctDNA. vem, vemurafenib; p-mab, panitumumab; bev, bevacizumab; T, trametinib.

with a newly detected *NRAS* Q61K mutation (60), the resistance alteration became undetectable off treatment, but reemerged after 8 weeks of rechallenge with vemurafenib and panitumumab (*Figure 1*). These data suggest clinical trials with intermittent schedules may be able to forestall resistance (84).

Adaptive trial design with real-time monitoring of tumor subpopulations

The initial experience with targeted therapy in mCRC recommends a future of drug development with real-time pharmacodynamic markers and with tracking of sensitive and resistant tumor populations, for example with circulating tumor DNA (ctDNA) analysis. The best treatment regimens will need to profoundly inhibit the target while minimizing toxicity, perhaps by taking advantage of real-time cfDNA analysis guiding intermittent dosing schedules. As RTK signaling plays a key role in relative resistance in the colorectum, future adaptive designs may further refine patient subpopulations by testing personalized combination regimens aimed at inhibiting the activated oncogene and the dominant reactivated receptor.

Conclusions

CRC results from successive genomic and epigenetic alterations, but it has not been straightforward to realize the promise of targeted therapies in mCRC. Often it has not been sufficient to directly inhibit the target, but rather addressing the signaling network that feeds into the target is required. At this time, negative predictive markers for response to EGFR targeted therapies and the positive predictive marker of microsatellite instability for response to immune checkpoint blockade are part of standard care. Emerging data support a developing role for targeted therapies in mCRC with *HER2*

amplification, *NTRK* rearrangements, or *BRAF* V600E mutations. Real-time pharmacodynamic and cfDNA analysis will allow further refining and optimizing targeted therapy approaches for mCRC.

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Footnote

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