Curr Gastroenterol Rep (2015) 17: 6
DOI 10.1007/s11894-015-0431-7

GI ONCOLOGY (R BRESALIER, SECTION EDITOR)

Molecular Markers Predictive of Chemotherapy Response in Colorectal Cancer

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Published online: 8 February 2015

Abstract Recognition of the molecular heterogeneity of colorectal cancer (CRC) has led to the classification of CRC based on a variety of clinical and molecular characteristics. Although the clinical significance of the majority of these molecular alterations is still being ascertained, it is widely anticipated that these characteristics will improve the accuracy of our ability to determine the prognosis and therapeutic response of CRC patients. A few of these markers, such as microsatellite instability and the CpG island methylator phenotype (CIMP), show promise as predictive markers for cytotoxic chemotherapy. *KRAS* is a validated biomarker for epidermal growth factor receptor (EGFR)-targeted therapy, while *NRAS* and *PI3KCA* are evolving markers for targeted therapies. Multiple new actionable drug targets and potential response biomarkers are being identified on a regular basis, but most are not ready for clinical use at this time. This review focuses on key molecular features of CRCs and the application of these molecular alterations as predictive biomarkers for CRC.

Keywords Biomarker · Colorectal cancer · Treatment · Molecular · MSI · Microsatellite instability · Methylation · CIMP · KRAS · NRAS · BRAF · TP53 · PIK3CA · MET · MEK · EGFR

This article is part of the Topical Collection on *GI Oncology*

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Introduction

Historically, the use of surgery, chemotherapy, and/or radiation therapy for colorectal cancer (CRC) has been guided by the TNM stage alone [1]. However, it is well known that the TNM stage is, at best, a modestly accurate predictor for response to treatment. It has been increasingly recognized that there are tumor molecular characteristics that more accurately guide the management of CRC patients by improving our ability to identify individuals at high risk of disease recurrence and to determine therapies that will be most effective in specific patients. This review focuses on recent advances in the discovery and validation of predictive markers that can be used to guide the treatment of CRC patients. We will discuss both validated and emerging biomarkers for conventional cytotoxic chemotherapy as well as for targeted therapies, such as anti-EGFR therapy. Mutant *KRAS* has served as the paradigm for predictive biomarkers for anti-EGFR therapy. However, the complexity of the molecular genetics and epigenetics of CRC and of the molecular biology of signaling pathways has revealed that the development and validation of other biomarkers for targeted therapy will be challenging. Many potential candidate genes for targeted therapy have been recently identified, creating opportunities for companion predictive marker assays. Predictive gene signature platforms are also under active investigation. In just the last few years, this area has greatly expanded as researchers and clinicians attempt to develop CRC treatments that will be optimally effective for specific patients.

Molecular Characterization

The identification of specific chromosomal abnormalities and gene mutations in CRC over 30 years ago provided the first glimpse into the potential for these molecular alterations to be

used to guide therapy for CRC. Since that time, tremendous advances have been made in our understanding of the molecular pathology of CRC. There are three commonly recognized molecular subclasses of CRC that are characterized by their forms of genomic and epigenomic instability: chromosome unstable (CIN; also referred to as microsatellite stable (MSS) CRC), microsatellite unstable (MSI), and the CpG island methylator phenotype (CIMP) [2]. Although this classification scheme is believed to oversimplify the molecular complexity of CRCs, it has been shown that CIN, MSI, and CIMP CRCs each have characteristic clinical and molecular phenotypes.

Recent Advances in the Molecular Characterization of CRC

The Cancer Genome Atlas (TCGA) Network performed a genome-scale analysis of 276 CRC samples, noting heterogeneity in the gene expression signatures and mutation profiles of the different individuals' tumors [3]. Of those that underwent whole genome sequencing, 16 % were found to be hypermutated, which meant that they had a substantially higher density of sequence mutations compared to the other CRCs. The vast majority of these hypermutated cases were also MSI and/or CIMP, although a previously unrecognized class of hypermutable CRCs was also observed. In addition to the common driver genes already known to occur in CRC (e.g., *APC*, *KRAS*, *TP53*), the non-hypermutated CRCs had a variety of gene mutations in other genes, including 24 that occurred with a reasonably high frequency. The findings from the TCGA and others have led multiple groups to develop new classification schemes that are more accurate and consistent than our current scheme based on CIN, MSI, and CIMP [4].

Gene Expression Signature Assays and Mutation Signature Assays

With the advent of next-generation sequencing and improving high-throughput technologies, gene signature assays have been developed for CRC. Two assays have been validated and approved for clinical use: Oncotype DX (Genomic Health, Inc.) and ColoPrint (Agendia) [5, 6]. These expression signature assay platforms appear to be best used for prognosis, rather than prediction of chemotherapy benefit [7, 8]. Multiple other assays, such as UW-OncoPlex (University of Washington), attempt to more broadly assay somatic alterations to identify clinically actionable and emerging somatic mutations [9]. The uptake of such assays into the clinic varies widely, but is likely to become a standard-of-care instrument in the future.

Cytotoxic Chemotherapy

Currently, CRC is predominantly managed with surgical resection for early stage CRC and with a combination of surgery and chemotherapy for advanced disease. 5-Fluorouracil (5-FU) is the backbone cytotoxic agent used in most regimens. While originally given as a single agent, 5-FU is now most commonly administered in combination with oxaliplatin (FOLFOX) for adjuvant therapy for stage III (and high-risk stage II) CRC and with either oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) for metastatic CRC [10–13]. Consequently, predictive biomarkers for CRC have initially focused on these conventional cytotoxic chemotherapy agents.

Established Molecular Markers

There has been considerable interest in identifying predictive molecular markers for chemotherapy effect both in the adjuvant and metastatic settings. Candidate biomarkers that have been heavily scrutinized include mutant *TP53*, thymidylate synthase (TS) expression, and amplified ERCC1, among others [14–16]. Unfortunately, none of these markers has been established as a predictive marker that is ready to be used clinically. However, despite the lack of success so far, recent data has shown that MSI is a robust prognostic marker for survival and is also a promising marker for 5-FU responsiveness [17, 18].

Microsatellite Instability

MSI was discovered in the early 1990s during studies to identify potential tumor suppressor genes in CRC [17]. MSI is recognized by the presence of a high frequency of frameshift mutations in microsatellite DNA and results from inactivation of the DNA mismatch repair (MMR) system. A portion of MSI tumors are due to germline mutations in one of the MMR genes, *MLH1*, *MSH2*, *MSH6*, or *PMS2*, which cause Lynch syndrome. The majority (80 %) of MSI cases is sporadic and is due to hypermethylation of the *MLH1* gene promoter [17, 19]. This is often associated with mutant $BRAF^{V600E}$ and *CIMP* [20].

A large number of studies have demonstrated that individuals with proximal MSI CRCs have a better prognosis than stage-matched MSS CRCs, particularly when no adjuvant therapy is given [18]. More recently, MSI has also been shown to predict lack of benefit of adjuvant 5-FU in stage II–III colon cancer patients (and possible harm in stage II patients) [21–23]. However, its role as a predictive marker with modern combination chemotherapy regimens, such as FOLFOX and FOLFIRI, is uncertain [24, 25]. This uncertainty is a consequence of differences in the studies' chemotherapy regimens and assays for MSI, which make it difficult to compare studies and develop a robust and generalizable conclusion about the role of MSI as a predictive marker for conventional chemotherapy.

There is continued investigation of the use of MSI to predict response to chemotherapy for modern combination regimens. A recently published retrospective analysis of stage III

patients treated with adjuvant FOLFOX in the N0147 trial demonstrated a predictive value for MSI [22]. This marked difference in chemotherapy response prediction capability between single-agent 5-FU and combination regimens may be partly due to variability in multivariate models, as newer models often account for *KRAS* and *BRAF* status [26]. The ability of MSI to predict response to oxaliplatin may reflect effects unrelated to inactivation of the MMR system, as was concluded by investigators in the NSABP-07 trial. The benefit from oxaliplatin in individuals with MSS tumors may attenuate the beneficial effect of MSI status on survival [27]. Although MSI was retrospectively shown to predict improved disease-free survival (DFS) with adjuvant irinotecan and 5-FU (IFL regimen) in the CALGB (Alliance) 89803 trial, MSI has not consistently served as a predictor of benefit from combination chemotherapy with 5-FU and irinotecan [28–30].

Interestingly, the underlying cause of MSI may affect whether MSI is predictive for chemotherapy responsiveness. In a retrospective analysis of stage II and III colon cancer patients that received adjuvant 5-FU or placebo, Sinicrope et al. compared individuals with MSI CRCs secondary to germline mutations (i.e., Lynch syndrome) to those with sporadic MSI tumors [31]. Individuals with germline mutations had improved DFS after 5-FU adjuvant chemotherapy, while patients with sporadic MSI CRCs did not receive benefit (p= 0.006). These findings suggest that recognizing both molecular features and their etiology is important for determining the utility of predictive biomarkers. Additional studies are needed to clarify whether the disparate MSI effect in single-agent 5-FU vs. in modern multi-agent therapies is also related to the etiology of MSI. Thus, MSI is largely accepted now as a prognostic marker, but its role as a predictive biomarker is more controversial, which has led to its continued scrutiny (Table 1).

Emerging Markers

CIMP

CIMP is found in 5–40 % of CRCs and is defined by an exceptionally high frequency of aberrantly methylated CpG dinucleotides in the genome [45]. In normal cells, the majority of DNA is methylated at CpG dinucleotides except in CG-rich regions ("CpG islands"). These CpG islands are found in approximately half of gene promoters and are usually unmethylated in normal cells. However, in the majority of CRCs, many of the CpG islands become aberrantly methylated. CIMP CRCs are distinct from other CRCs because they have an exceptionally high frequency of methylated CpG islands. CIMP is thought to promote carcinogenesis by silencing tumor suppressor genes via methylation-mediated transcriptional repression [2].

Since the initial discovery of CIMP in 1999, a number of assay panels have been developed to determine the CIMP status of CRCs. Unfortunately, there has been no consensus regarding which panel to use, which has, in turn, slowed the investigation and development of CIMP as a predictive biomarker [45]. Despite the lack of a "gold-standard" CIMP assay, there does appear to be an association between CIMP and poor prognosis and, albeit less consistently, between CIMP and a favorable response to 5-FU [45] (Table 2). Recently, in an exploratory retrospective analysis of a randomized trial of 5-FU/leucovorin (5-FU/LV) alone vs. with irinotecan (IFL regimen) in stage III colon cancer patients (CALGB/Alliance 89803), Shiovitz, et al. found that CIMP-positive individuals had worse overall survival [46•, 59]. These authors also found on sub-group analysis that patients with CIMP-positive colon cancers that were also MMR-intact (MMR-I) did better with IFL than with 5-FU/LV alone with a 5-year overall survival (OS) of 66 vs. 46 %, respectively. Furthermore, in this updated data analysis from this trial, CIMP was a stronger prognostic feature than MSI [28, 46•]. Other studies assessing the use of CIMP to predict response to FOLFOX therapy did not observe CIMP to be a significant predictor [60]. At this time, CIMP appears to have a strong potential to be a prognostic marker for CRC, but its use as a predictive marker for conventional chemotherapy will require further investigation.

BRAF

There is substantial evidence from the retrospective analysis of cohort and randomized clinical trials that mutant *BRAF* is a marker of poor prognosis [26, 36]. There is also some data to suggest that individuals with *BRAF*-mutant metastatic CRC may benefit from aggressive first-line therapy (Table 1). When the chemotherapy triplet regimen of 5-FU, oxaliplatin, and irinotecan (FOLFOXIRI) was compared to FOLFIRI, significantly improved response rate (RR; 60 vs. 34 %, respectively), progression-free survival (PFS; 9.8 vs. 6.9 months), and OS (22.6 vs. 16.7 months) were noted in unselected stage IV patients [32]. The clinical importance of these results is not clear as this regimen is not standard-of-care. Also, of note are results from the analysis of a small phase 2 trial suggesting improved PFS with the use of FOLFOXIRI + bevacizumab in patients with metastatic *BRAF*-mutant CRCs [42•]. In these patients, median PFS was 9.2 months and OS was 24.1 months. For approximate comparison, median PFS for *BRAF*-mutant CRCs was 5.6 months with FOLFIRI and 8.0 months with FOLFIRI + cetuximab [37]. However, in the MRC FOCUS trial of advanced CRC, mutant *BRAF* was not shown to be a significant predictive marker for any 5-FU-based chemotherapy regimen (5-FU/LV, 5-FU + oxaliplatin, or 5-FU + irinotecan) [61]. Thus, *BRAF* is best considered as an emerging marker for predicting response to conventional chemotherapy, which is still in need of further assessment.

Table 1 Studies of candidate biomarkers with conventional cytotoxic treatment of colorectal cancer (+/- targeted therapy)

Biomarker	Mutation prevalence	Stage	Biomarker Features	Treatment[b]	Median DFS/PFS (months)[e]	Median OS (months) [e]
Unselected[a] [32–35]	100 %	IV		FOLFOX	7.2	
				FOLFOX + cetux	7.2	
				FOLFIRI	6.9–8.0	16.7–18.6
				FOLFIRI + cetux	8.9	
				FOLFOXIRI	9.8	19.9
				FOLFOXIRI + bev	13.1	22.6
MSI [22, 23, 31]	12–16 %	II	All	5-FU[c]	HR 2.3*	HR 2.95
			Germline MSI		HR 0.31	
			Somatic MSI		HR 1.5*	
		III	All		HR 1.01*	
			Germline MSI		HR 0.26	
			Somatic MSI		HR 0.77*	
MSS [22, 23, 31]	88 %	II		5-FU[c]	HR 0.84*	
		III			HR 0.64	
KRAS [34, 36, 37, 38•, 39]	37–40 %	IV	Exon 2 mutation	FOLFOX	8.6–9.2	19.2
				FOLFOX + cetux	5.5	
				FOLFOX + pan	7.4	15.5
				FOLFIRI	8.1	17.7
				FOLFIRI + cetux	7.6	17.5
	3–4 %		Exon 3 or 4 mutation	FOLFIRI		16.7
				FOLFIRI + cetux		16.2
NRAS [39, 40••, 41]	2–3 %	IV	Exon 2–4 mutation	FOLFOX	8.0	17.8
				FOLFOX + pan	7.3	17.1
BRAF [36, 37, 39, 42•]	5–18 %	IV	V600E mutation	FOLFIRI	5.6	10.3
				FOLFIRI + cetux	8.0	14.1
				FOLFOXIRI		
				FOLFOXIRI + bev	9.2	24.1
RAS wild type [33, 34, 37, 38•, 39, 40••, 43••, 44]	53 %	IV	*KRAS* exon 2 wild type	FOLFOX	7.2–8.6	19.4–19.7
				FOLFOX + cetux	7.7	
				FOLFOX + pan	9.6–10.0	
				FOLFIRI	8.7	23.9
				FOLFIRI + cetux	9.9–10.0	21.0
				FOLFIRI + bev	10.3	24.9–28.7
						25.0
			Wild type for expanded *KRAS* and *NRAS*	FOLFOX	7.9	20.2
				FOLFOX + pan	10.1	25.8
				FOLFIRI[d]	8.8	21.6
				FOLFIRI + cetux[d]	10.4–10.9	25.1–33.1
				FOLFIRI + bev	10.2	25.6
				FOLFOXIRI+pan[d]	11.3	

MSI microsatellite unstable, *MSS* microsatellite stable, *5-FU* 5-fluorouracil, *FOLFOX* 5-FU, leucovorin, oxaliplatin, *FOLFIRI* 5-FU, leucovorin, irinotecan, *FOLFOXIRI* 5-FU, leucovorin, oxaliplatin, irinotecan, *cetux* cetuximab, *pan* panitumumab, *bev* bevacizumab, *DFS* disease-free survival, *PFS* progression-free survival, *OS* overall survival

[a] These data reflect trials where biomarkers were not used to guide the therapy choice, often predating recognition of predictive biomarker. These data are meant to serve as a reference for the biomarker-guided therapy outcomes

[b] For stage IV cases, only first-line therapy data are included

[c] Reported hazard ratios are for adjuvant 5-FU/LV vs. surgery alone. Median survival data were not reported

[d] These data include individuals who are wild type for *KRAS*, *NRAS*, and *BRAF*

[e] These data are reported in phase 2–3 trials. The appropriate comparisons are not noted, but can be found in the associated references. Unavailable data values were left blank

*Result does not meet statistical significance at $p<0.05$

Table 2 Emerging predictive biomarkers in colorectal cancer

Biomarker	Biomarker prevalence	Stage	Application(s)
CIMP [45, 46•]	24 %	II–III	• Controversial whether CIMP-positive individuals derive greater benefit from adjuvant 5-FU as a single agent • CIMP-positive, MMR-I individuals had longer 5-year OS with IFL vs. 5-FU/LV. CIMP-negative, MMR-D patients had a trend toward benefit from IFL. Other sub-groups did not appear to benefit from the addition of irinotecan to 5-FU/LV
TP53 [47]	52 %	II–III	• Wild-type *TP53* was predictive of benefit from adjuvant CAPOX + cetuximab vs. CAPOX alone in the EXPERT-C trial, whereas there was no difference among *TP53*-mutant cases. *TP53* is not yet routinely used for chemotherapy response prediction
PIK3CA [39, 48••, 49–52]	10 % Exon 9 3 % Exon 20	IV	• Exon 20 mutations are predictive of worse outcomes with anti-EGFR therapy • PI3K inhibitors are being evaluated • *PIK3CA* mutations may predict benefit from aspirin for secondary prevention
MET [53•, 54]	Increased after exposure to anti-EGFR therapy	IV	• Over-amplification or increased expression of MET is associated with resistance to anti-EGFR therapy
MEK [55, 56]	Increased after exposure to anti-EGFR therapy	IV	• Selective MEK-1/2 inhibitors are currently being evaluated in clinical trials, alone and in combination with other targeted therapies
HER2 [57]	2–3 %	IV	• Dual blockade of HER2 and EGFR has increased efficacy compared to either agent alone in preclinical models
BRAF [36, 58]	5–18 %	IV	• BRAF inhibitors have been poorly effective as single agents, but continue to be evaluated in combination therapies

CIMP CpG island methylator phenotype, *MMR-I* mismatch-repair-intact, *MMR-D* mismatch repair-deficient, *5-FU* 5-fluorouracil, *LV* leucovorin, *IFL* irinotecan, 5-FU, LV, *CAPOX* capecitabine, oxaliplatin

Anti-angiogenic Therapy

In the decade since the FDA approval of the vascular endothelial growth factor (VEGF)-targeted agent bevacizumab for CRC, multiple studies have demonstrated superiority of bevacizumab-based chemotherapy to cytotoxic chemotherapy alone for the treatment of metastatic CRC [62, 63]. Notably, bevacizumab-related hypertension occurs more often in individuals who respond to treatment [64, 65]. Consequently, hypertension is considered a pharmacodynamic biomarker, but lacks the predictive capability needed to guide the selection of optimized individual therapeutic regimens. Overall, to date, no reliable biomarkers for prediction of response to anti-angiogenic therapy have been found, although there are some promising potential biomarkers, which will be discussed below.

Multiple retrospective studies have attempted to identify predictive markers of bevacizumab response in CRC [66]. Recently, retrospective studies have suggested an association between carcinoembryonic antigen (CEA) levels and response to bevacizumab-based chemotherapy. Lower baseline CEA levels suggest a higher likelihood of treatment response and longer median PFS and OS [67•, 68]. Prager et al. assessed baseline CEA levels in a cohort of patients receiving first-line therapy with chemotherapy (FOLFOX, XELOX, FOLFIRI, or XELIRI) and bevacizumab and compared them to patients who received a cetuximab-based regimen. The CEA classification of high vs. low was somewhat arbitrarily derived as above or below the study median value, questioning the use of this threshold in other populations, but the results were validated in an independent cohort. Arguing against the use of CEA, a similar unrelated retrospective analysis did not observe that CEA predicted response to either bevacizumab or cediranib [69•]. Additional suggested predictive biomarkers for bevacizumab treatment response and/or efficacy include: level of tumor VEGF-A expression, germline genetic variations in pericyte maturation genes, phosphatase and tensin homolog (PTEN) loss, and levels of phosphorylated AMP-activated protein kinase (pAMPK) [47, 70–73]. However, despite the identification of a number of plausible predictive markers for responsiveness to bevacizumab, none of these markers has been validated as a clinically useful biomarker. Baseline serum VEGF and CEA levels appear to have the highest potential to be used as predictive markers to date, but will require additional studies using stringent thresholds and validated assays before they are ready to be used clinically [68, 74].

Similar to the situation for bevacizumab the newer anti-angiogenic agents regorafenib and aflibercept lack predictive markers [75]. Thus, with a deficiency of predictive biomarkers, the selection of anti-angiogenic therapy should be guided by other factors at this time, including potential side effects and tumor molecular features that predict benefit or harm from other agents.

EGFR-Targeted Therapy

Two targeted anti-epidermal growth factor receptor (EGFR) monoclonal antibodies have been approved in combination with chemotherapy for the treatment of metastatic CRC.

While rare reports note *EGFR* mutations that preferentially affect cetuximab vs. panitumumab responsiveness [76•], generally, these agents are thought to have equivalent efficacy [77]. The addition of panitumumab in the first line in unselected stage IV patients showed a 1.4-month increase in PFS compared to FOLFOX alone (PRIME trial [38•]), while cetuximab combined with FOLFIRI in the CRYSTAL trial added a 0.9-month PFS benefit [33]. The OPUS study, which combined cetuximab and FOLFOX in unselected metastatic patients, failed to demonstrate a survival benefit [34].

Established predictive markers

KRAS exon 2

Shortly after the clinical benefit of cetuximab and panitumumab was shown in patients with metastatic CRC, it was noted that anti-EGFR therapy efficacy was confined to individuals with *KRAS* wild-type tumors, with no response or even harm to patients with *KRAS*-mutant tumors [78, 79]. For example, stratified analysis in the OPUS study noted improved survival in individuals with KRAS wild-type CRCs (7.7 vs. 7.2 months in cetuximab + FOLFOX vs. FOLFOX alone, $p=0.02$), but worse survival in individuals with *KRAS*-mutant tumors (5.5 vs. 8.6 months, $p=0.02$) [34]. Importantly, this original definition of mutation status was restricted to *KRAS* exon 2, and codons 12 and 13. Interestingly, EGFR expression does not appear to correlate with response to either cetuximab or panitumumab [80, 81]. *KRAS* mutation status is now clearly established as the first clinically useful biomarker to predict response to anti-EGFR therapy.

Expanded RAS testing

Multiple retrospective studies have now shown that *KRAS* testing restricted to exon 2 misses cases resistant to anti-EGFR therapy. "Expanded *RAS*" testing is now advocated, which also tests exons 3 (codon 61) and 4 (codons 117, 146) of *KRAS* and exons 2–4 of *NRAS* [1]. In the PRIME trial, 17 % of patients with CRCs that were wild type for *KRAS* exon 2 were found to have a mutation in another *RAS* exon: 4 % *KRAS* exon 3, 6 % *KRAS* exon 4, 3 % *NRAS* exon 2, and 4 % *NRAS* exon 3 (and 0 % *NRAS* exon 4) [40••]. While the numbers were small, any *RAS* mutation was associated with a statistically significant worse PFS (hazard ratio (HR) 1.31, 95 % confidence interval (CI)=1.07–1.60). Those patients with no RAS mutations detected using the expanded RAS protocol ("expanded wild type") were most likely to receive benefit (HR 0.72, 95 % CI=1.04–1.62). Similarly, in the FIRE-3 study, 16 % of individuals who were wild type at *KRAS* exon 2 were found to have a *RAS* mutation by expanded *RAS* testing [43••]. Comparing FOLFIRI + cetuximab to FOLFIRI + bevacizumab, PFS in the expanded wild-type population was 10.4 vs. 10.2 months ($p=0.54$) and OS was 33.1 vs. 25.6 months ($p=0.011$), respectively. These absolute differences were greater than when evaluating the entire intent-to-treat population, which included a more limited assessment of *KRAS* mutation status.

Anti-EGFR therapy has been evaluated beyond its use with doublet chemotherapy in metastatic CRC. Expansion of anti-EGFR therapy to adjuvant therapy in non-metastatic CRC has failed to show a benefit, even within *KRAS* wild-type CRC [82, 83•]. This suggests a difference in tumor biology in stage III as compared to stage IV patients. The application of anti-EGFR therapy is also being explored with triplet therapy for metastatic CRC. There is phase 2 clinical trial data that first-line panitumumab + FOLFOXIRI is efficacious in patients with wild type *KRAS*, *HRAS*, *NRAS*, and *BRAF* with a median PFS of 11.3 months [44].

Based on the above information, the National Comprehensive Cancer Network (NCCN) currently advocates for full testing of *KRAS* and *NRAS* in all cases of metastatic CRC to guide therapy selection [1] (Table 1). *KRAS*, *NRAS*, and *BRAF* mutations appear to be mutually exclusive [39]. Retrospective studies suggest that the clinical outcomes predicted by *KRAS* mutations are irrespective of the mutated codon [84•, 85]. It remains controversial whether patients with *KRAS* G13D mutations might gain benefit from anti-EGFR therapy; the apparent responsiveness of CRCs with *KRAS* G13D mutations may be an effect of codon 13 mutations reflecting a worst prognosis in early stage and metastatic CRC [86, 87]. Currently, the role of EGFR pathway testing in non-metastatic CRC is not established since anti-EGFR therapy has failed to show efficacy in stage II or III CRC patients. As *BRAF* lacks the predictive value for anti-EGFR therapy, *BRAF* testing is currently considered optional for *KRAS*/*NRAS* wild-type tumors [1, 37]. Given the evolving knowledge about the *RAS* pathway, clinical trials that include anti-EGFR therapy should be critically evaluated in the context of their inclusion/exclusion criteria and *RAS* testing strategies.

Emerging Predictive Markers

PI3K/AKT/PTEN

Distinct from the RAS/RAF pathway, members of the phosphoinositide-3-kinase (PI3K), pathway, PIK3CA, AKT, and PTEN also are downstream effectors of EGFR signaling. Mutations in these genes may also affect anti-EGFR therapy responsiveness. In retrospective studies, patients with CRCs that have *PIK3CA* exon 20 (kinase domain) mutations have much worse outcomes with cetuximab compared to patients with *PIK3CA* wild-type CRCs [39]. *PIK3CA* exon 9 (helical domain) mutations do not seem to serve as a predictive marker for anti-EGFR therapy, which highlights the complexity of the effects of the specific mutations on the functions of the altered

kinases. Of interest, *PIK3CA* exon 9 mutations are often seen with *KRAS* mutations and *KRAS* remains the stronger predictive biomarker. Collectively, approximately 70–80 % of cases unresponsive to EGFR-targeted therapy appear to be secondary to mutations in *KRAS*, *NRAS*, *PI3KCA,* and other proteins relevant to EGFR signaling [39, 53•, 57].

PIK3CA mutations may also be beneficial in guiding secondary prevention options. Large studies have demonstrated that aspirin reduces adenoma and CRC formation [88]. More recent observational data suggests that the aspirin benefit is limited to individuals with *PIK3CA*-mutant tumors [48••]. The use of aspirin in secondary prevention remains a complex issue because of the risks of aspirin use; further study is required.

TP53

TP53 is not in the EGFR pathway or traditionally thought of as a predictive biomarker for EGFR-targeted therapy. However, in the phase 2 EXPERT-C trial, which added cetuximab in the adjuvant setting for high-risk stage II rectal cancer patients, exploratory retrospective analysis suggested *TP53* mutation status did predict benefit from cetuximab [89•, 90]. Patients with *TP53* wild-type tumors who received cetuximab in addition to adjuvant capecitabine and oxaliplatin (CAPOX) had improved PFS and OS compared to CAPOX alone (Table 2). However, among individuals with *TP53*-mutant tumors, there was no difference in survival with the addition of cetuximab. Of note, only 60 % of patients were wild type for *KRAS* (exons 2 and 3) and *BRAF*, but the interaction between *TP53* and treatment arm remained significant when adjusting for *KRAS* mutation status. At this time, *TP53* mutation status is not routinely assessed in rectal cancer patients.

Emerging Mechanism-Driven Therapy

Several hallmark cellular changes necessary for carcinogenesis are now recognized, including the induction of angiogenesis, evading growth suppressors, and activation of invasion and metastasis [91] The development of newer agents for CRC has focused on key pathways in CRC that mediate these hallmark behaviors of cancer cells [92], with the aim of counteracting the cellular signaling changes that result from driver gene mutations in these central signaling pathways. Beyond the EGFR pathway, none of these agents are currently ready for clinical use in patients with CRC. However, several agents are on the horizon and show promise for future implementation. Ideally, these agents will have companion diagnostic assays that will be used to select individuals who have the highest likelihood of response.

PI3K Inhibitors

PI3K pathway inhibitors have been developed, but their role in the treatment of CRC is currently unclear. Newer investigational agents have shown some promise in the preclinical setting for *PI3KCA*-mutant tumors [49, 50]. However, once applied to the clinical setting, PI3K inhibitors have largely failed to show significant clinical benefit in CRC, despite the hypothesis that they would be effective given the high frequency of mutations in *PIK3CA*, *PTEN*, and other PI3K signaling pathway members in CRC [51, 52]. Further study of these agents is likely to focus on the efficacy of this class of agents in combination with targeted therapies, as guided by tumor molecular characteristics, such as concurrent *KRAS* and *PIK3CA* mutations [93].

MEK inhibitors

MEK inhibitors have been evaluated as MEK is downstream of EGFR in the RAS/RAF pathway. While responses were seen in melanoma and lung cancer, the pure MEK inhibitor RO4987655 failed to show benefit in *KRAS*-mutant CRC [94]. Despite the lack of efficacy of RO4987655, selumetinib (AZD6244) is a selective MEK-1/2 inhibitor that has shown in vitro and in vivo activity in CRC. It is currently being evaluated for the treatment of metastatic CRC in early phase clinical trials [55]. Selective MEK-1/2 inhibitors have shown in vitro efficacy in modulating sensitivity to cetuximab [56]. It remains to be seen whether MEK inhibitors will make it through multi-phase clinical trials and into clinical practice and whether specific biomarkers will be useful in selecting patients eligible for treatment.

Dual Pathway Blockade

Overcoming resistance to anti-EGFR therapies has been one focus of dual-targeted therapy trials. Amplification of the *MET* gene and increased expression of MET has been associated with both primary and secondary (acquired) resistance to cetuximab/panitumumab, distinct from the effect of mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* [53•]. MJ-56 has shown in vitro efficacy in human CRC cell lines, attenuating signaling of EGFR and c-MET [54]. HER2 amplification as a potential resistance mechanism also has been identified in *KRAS*/*NRAS*/*BRAF*/*PIK3CA* wild-type ("quadruple negative") tumors [57]. In preclinical studies, dual blockade of EGFR and HER2 in *HER2*-amplified colorectal xenografts was more effective than either agent alone suggesting that this regimen may be effective in patients with CRCs that have amplified *HER2*.

Some studies have used more than one targeted therapy or have used a multiple pathway agent in an attempt to block multiple key growth pathways in the tumor cell. Following

the recognition of the frequency of *BRAF* V600E mutations in melanoma, *BRAF* inhibitors were developed and implemented in clinical practice [95]. However, the success seen in the treatment of melanoma with these agents was not paralleled in CRC [58]. This has led to the use of *BRAF* inhibitors in combination with other agents in early phase dual agent trials. Dual blockade of the MEK and PI3K/mTOR pathways in mouse CRC xenografts improved results over monotherapy, but did not induce tumor regression [96]. Although based on solid conceptual grounds as a way to overcome resistance to single targeted therapy regimens, the use of multiple pathway inhibitors has had suboptimal results when applied to the clinical setting, often increasing toxicity but not improving treatment outcomes [97••, 98]. This observation has led to the consideration of a strategy involving targeted therapy and cytotoxic chemotherapy, which may cause better tumor response. Along this vein, there is early preclinical data that the proteasome inhibitor carfilzomib may be synergistic with irinotecan in CRC cell lines through effects mediated on the MEK/ERK and PI3K/AKT pathways [99]. Thus, new therapeutic targets for CRC continue to be explored and optimized (Table 2), but are not ready for clinical practice.

Conclusions

The next era of CRC treatment trials involves focusing on tumor genetic signatures, rather than single-gene alterations. This includes identification of new targets, combination targeted therapies, and possibly immune modulation. There is clear lack of benefit from EGFR-targeted therapies in individuals with metastatic CRC harboring *KRAS* exon 2 mutations. In addition, mutations in *KRAS* exons 3 and 4, *NRAS* exons 2–4, *PIK3CA* exon 20, and possibly *TP53* are predictive of lack of benefit. No clinically useful predictive biomarkers for anti-angiogenic therapy have been identified. Inhibitors of PI3K, MEK, MET, HER2, and other newer targets are under active investigation but are not yet ready for application to clinical practice. The increasing complexity of mutation profiling for therapies targeting EGFR and other endpoints suggests the need for greater molecular characterization of tumors before, and possibly after, starting treatment. Future trials will need to better determine eligibility based on who is predicted to respond to targeted therapy. In addition, combination targeted therapies and/or dual downstream inhibition of resistance mechanisms appear to be promising future approaches to CRC treatment.

Compliance with Ethics Guidelines

Conflict of Interest Stacey Shiovitz has received financial support from the National Institutes of Health (NIH T32-CA009515) and a 2014 Young Investigator Award from the Conquer Cancer Foundation of the American Society for Clinical Oncology. William M. Grady has received financial support from the National Institutes of Health (NIH RO1CA115513, P30CA15704, UO1CA152756, U54CA143862, P01CA077852) and a Burroughs Wellcome Fund Translational Research Award for Clinician Scientist.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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Papers of particular interest, published recently, have been highlighted as:
- • Of importance
- •• Of major importance

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