Molecular testing to optimize therapeutic decision making in advanced colorectal cancer

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Abstract: Colorectal cancer (CRC) is a leading cause of cancer death in the United States. In recent years, therapeutic advances have prolonged the survival of patients with advanced disease. Along with the addition of new treatments, an increasing body of literature explores the potential benefit of using molecular testing to define tumor, circulating, or host biomarkers of benefit to specific treatment strategies. At present, testing for specific mutations in exons 2, 3, and 4 of KRAS and NRAS has become accepted practice to select patients for treatment with epidermal growth factor receptor (EGFR)-targeted agents. Additionally, testing for the BRAF V600E mutation is used to refine decisions based on patient prognosis. The presence of the uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) *28 polymorphism is associated with toxicity from irinotecan, although it has not been universally applied. Nonetheless, molecular markers to predict response and toxicity of cytotoxic therapy are evolving. While the development of selection biomarkers for anti-angiogenic treatments has not proved fruitful to date, improved development strategies and novel targeted agents are anticipated to revolutionize the approach to treatment of advanced CRC in the near future. This review summarizes currently available data to select treatment strategies in patients with advanced CRC.

Keywords: Colorectal cancer (CRC); biomarker; RAS; BRAF

Introduction

Despite the adoption of widespread screening efforts, a significant fraction of the 136,830 patients diagnosed with colon or rectal cancer will ultimately succumb to advanced disease (1). Indeed, colorectal cancer (CRC) is the second leading cause of cancer death in the United States, accounting for an estimated 50,310 deaths in 2014 (1). Over the past several decades, there have been a number of changes in the treatment options for patients with advanced CRC that include the incorporation of new drugs as well as the recognition of the benefit of surgical resection for selected patients with metastatic disease.

The first drug to demonstrate activity for the treatment of advanced CRC was 5-fluorouracil (5-FU). Initial efforts sought to optimize the dose and schedule of this agent while demonstrating the benefit of the addition of leucovorin. Over the past couple of decades, two additional cytotoxic agents have been added to the therapeutic arsenal for the treatment of advanced CRC. Irinotecan is an intravenous camptothecin analog that was initially developed for advanced CRC that was refractory to leucovorin-modulated 5-FU and is now incorporated into multiple lines of therapy (2,3). Oxaliplatin is a platinum derivative that has minimal single-agent activity in advanced CRC. However, the drug has substantial activity when combined with a fluoropyrimidine (4).

In recent years, two classes of biologic agents have been developed to advance outcomes in this disease by targeting vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) signaling respectively. Bevacizumab is a monoclonal antibody that binds to circulating VEGF isoform A with activity in multiple lines of treatment in advanced CRC (5). Ziv-aflibercept is a related agent that binds to multiple VEGF isoforms, and is now approved for use in the second-line in combination with irinotecan-based chemotherapy (6). Regorafenib is an oral multi-kinase inhibitor with anti-VEGF as well as potential anti-proliferative effect (7). While not solely an anti-VEGF agent, it perhaps can best be categorized as an extension of the anti-angiogenic paradigm in CRC. Cetuximab is a recombinant mouse/human monoclonal antibody that binds to the EGFR and was the first EGFR-targeting agent to demonstrate activity in irinotecan- and oxaliplatin-refractory CRC (8). Its use has now been expanded to multiple lines of therapy (9-11). Panitumumab is a very similar human monoclonal antibody targeting EGFR that has comparable activity to cetuximab as a single agent and in combination with chemotherapy (12,13).

While the application of each of these new treatments for advanced CRC has pushed the median survival to approximately 30 months or greater in recent clinical trials (14,15), each individual agent is not universally effective and is associated with nontrivial toxicity. Therefore, significant recent efforts have been made to understand both factors associated with survival regardless of therapy (prognostic biomarkers) and factors that identify patients more or less likely to benefit from a specific intervention (predictive biomarkers). In this review, we will discuss the current status of molecular biomarkers to select chemotherapy, anti-EGFR agents, and anti-VEGF agents in advanced CRC focusing on clinical studies and with a look forward to emerging and future developments.

Molecular tests to individualize cytotoxic chemotherapy

Uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1)

UGT1A1 is the key enzyme for inactivation of the active metabolite of irinotecan, SN-38 through glucuronidation. The UGT1A1*28 polymorphism is characterized by an extra TA repeat in the gene's promoter region, and is associated with reduced protein expression and therefore reduced glucuronidation. The presence of two UGT1A1*28

alleles underlies Gilbert's syndrome. Initial studies demonstrated that toxicity, particularly hematologic toxicity, was significantly increased in patients with one or two alleles of UGT1A1*28 (16). This resulted in its notation in the prescribing information; however, management strategies for patients homozygous or heterozygous for this allele have not been standardized. In a study of 250 Caucasian patients with advanced CRC treated with the FOLFIRI (5-FU, leucovorin, irinotecan) regimen, including 22 (8.8%) homozygous and 114 (45.6%) heterozygous for UGT1A1*28, this marker was associated with an increased risk of hematologic toxicity in the first cycle but without statistically significant effect in subsequent cycles (17). A recent phase I study used flat doses of irinotecan to establish the maximum tolerated dose according to UGT1A1 genotype for an every 3-week regimen (18). In addition to the established association of UGT1A1*28 polymorphisms with irinotecan-associated toxicity, it has also been hypothesized that the reduced rate of elimination may actually be a predictive marker for response. Indeed, in the above-mentioned study of patients with CRC treated with FOLFIRI, response was increased in the UGT1A1*28 homozygous group (17). Nonetheless, this did not translate into a statistically significant increase in survival. While it is clear that genetic heterogeneity at UGT1A1 is associated with altered metabolism of SN-38, other drug-metabolizing enzymes influence the final toxicity and efficacy outcomes. The optimal management of irinotecan based on UGT1A1 status therefore remains incompletely defined (19).

Excision repair and cross-complementation group 1 (ERCC1)

The ERCC1 protein is a key component of the nucleotide excision repair complex, which is a primary method of repair of platinum-DNA adducts. Therefore, it has been hypothesized that low ERCC1 expression may be associated with sensitivity to oxaliplatin-based chemotherapy in cancer (20). An initial study of 50 patients treated with an oxaliplatin and 5-FU combination regimen suggested that a group of patients with higher RNA expression of ERCC1 had a 4.8-fold higher risk of death (95% CI, 2.09-15.88) compared to those with lower expression (21). In a 91-patient phase I study of escalating doses of capecitabine with oxaliplatin, ERCC1 RNA expression in a metastatic site was associated with time to treatment failure (22). Kim et al. studied expression of ERCC1 by immunohistochemistry (IHC) in 70 patients with advanced CRC treated with an oxaliplatin-containing

regimen (22). Survival was longer in those who did not have detectable ERCC1 expression according to this method (P=0.0474). However, this was not confirmed in a much larger analysis (n=1,197) of the MRC FOCUS trial, which failed to confirm any predictive benefit of ERCC1 IHC (23). Questions have been raised about the specificity of the antibody used to assess ERCC1 and about the concordance of ERCC1 expression in the primary and metastatic tumor tissue (24,25). Thus, the optimal method of assessment of assessment of ERCC1 status is not yet clearly defined, as different methods may give different results (26).

A large number of studies have evaluated the predictive utility of a polymorphism in ERCC1 (ERCC1-118 C>T) to predict outcomes of patients treated with oxaliplatin. While several studies have suggested that the T containing genotype is associated with better response and/or survival (27,28), several others have come to the exact opposite conclusion (29-31), and indeed further studies suggest no association (32,33). A potential explanation for this discrepant data is that the polymorphism may have different implications in a Caucasian versus Asian population (34,35). Given the limited and contradictory clinical data, measuring ERCC1 expression or gene polymorphisms by any method cannot be recommended for use to select oxaliplatin-based treatment at this time.

Microsatellite instability (MSI)

Approximately 15% of CRCs have evidence of defective DNA mismatch repair manifested as MSI. While some of these tumors are a result of hereditary defects in DNA mismatch repair enzymes (hereditary non-polyposis CRC), others are sporadic and the result of promoter silencing of the DNA mismatch repair enzyme MLH1 (36). Current data suggest that defective DNA mismatch repair confers an improved prognosis in most stages of CRC, but there is also evidence that these tumors are relatively resistant to 5-FU monotherapy in the adjuvant setting (37). The utility of MSI testing to direct treatment in advanced CRC is not established. A summary of candidate markers is provided in *Table 1*.

Molecular tests to individualize anti-EGFR therapy

KRAS and NRAS

The EGFR signaling pathway has been targeted in a number of different malignancies. Upon binding to ligands such as EGF, amphiregulin, or epiregulin, EGFR results in an intracellular signal that is transduced through several intracellular signaling pathways. A dominant pathway involves the activation of the G-protein intermediate RAS, and subsequent signaling through BRAF, MEK, and ERK (the MAP kinase pathway). Mutations in the RAS family of proto-oncogenes (KRAS, NRAS, HRAS) result in constitutive activation of MAP kinase pathway signaling that is independent of activation of receptor tyrosine kinases such as EGFR. Since the currently available EGFR antibodies cetuximab and panitumumab bind to the extracellular domain of EGFR to result in receptor internalization and block signaling, it has been hypothesized that mutations in RAS render the activity of EGFR antibodies irrelevant.

The most common RAS mutations in colon cancer occur in codons 12 and 13 of KRAS, and are present in 35-45% of cases. An initial study of 30 patients confirmed preclinical data that tumors harboring this mutation would be resistant to EGFR antibody therapy (38). These findings were subsequently confirmed in prospective retrospective analyses of large clinical trials that randomized patients to treatment with or without EGFR antibodies. For example, in the CRYSTAL trial that compared initial treatment with the combination of leucovorin, 5-FU, and irinotecan (FOLFIRI) to the same treatment with cetuximab, there was a significant interaction between KRAS mutation status and survival outcome (9). Many other similar studies and a meta-analysis came to similar conclusions (12,39-41). Therefore, KRAS mutation testing has been integrated into guideline-based management of advanced CRC (42).

Multiple questions have been raised by these data, perhaps most prominently is whether all mutations in RAS behave similarly. Initially, it was questioned whether codon 13 mutations were equivalent to codon 12 mutations in predicting the lack of efficacy of EGFR antibodies. Several retrospective studies suggested that the predictive effect of KRAS codon 13 mutation was incomplete (43,44); nonetheless, targeted studies have not suggested that benefit in this population is worthy of changing the paradigm (45).

Subsequently, other mutations in KRAS and similar mutations in NRAS have been evaluated as potential predictors of lack of response to EGFR antibodies in CRC (46). The other mutations frequently encountered include mutations in codons 59, 61, 117, and 146. They are generally activating in cell line models (47). Recent secondary analysis of several key studies of EGFR antibodies in the treatment of advanced CRC have demonstrated

Table 1 Summary of reviewed candidate molecular markers in colorectal cancer

Marker	Putative mechanism	Status
Cytotoxic agents		
UGT1A1*28 polymorphism	Reduced glucuronidation of SN-38, resulting in reduced clearance and increased exposure	Associated with toxicity but management strategies not standardized
ERCC1 expression	Reduced repair of DNA-platinum adducts	Contradictory data, methodology has been questioned
ERCC1 polymorphisms	Altered expression of ERCC1 resulting in differential repair of DNA-platinum adducts	Contradictory data which may be result of interaction with race/ethnicity
MSI	Reduced sensitivity to 5-FU	Insufficient data in advanced disease
EGFR antibody therapy		
RAS mutation	Constitutive pathway activation independent of receptor signaling	Strongly predictive for absent activity
BRAF V600E mutation	Constitutive pathway activation independent of receptor signaling	Strongly prognostic, does not consistently predict lack of efficacy
EGFR expression/amplification	Increased target availability	Evidence of response in EGFR expression negative patients
Amphiregulin/epiregulin	Autocrine signaling loop	Methodological issues and prognostic implications need to be clarified
PI3KCA mutation	Constitutive pathway activation independent of receptor signaling	Contradictory data from small studies
PTEN loss	Loss of negative regulator of EGFR mediated PI3K pathway signaling	Contradictory data from small studies
Antiangiogenic treatments		
VEGF or VEGFR expression	Activated angiogenesis through VEGF pathway	Not predictive
Circulating angiogenic factors	Activated angiogenesis through angiogenic pathways.	Not predictive although IL-8 prognostic

ERCC1, excision repair and cross-complementation group 1; MSI, microsatellite instability; 5-FU, 5-fluorouracil; EGFR, epidermal growth factor receptor; PI3K, phosphoinositide-3-kinase; VEGF, vascular endothelial growth factor.

no trend for benefit for the addition of EGFR antibodies of patients to patients whose tumors these further RAS mutations (which we and others refer to as expanded RAS testing, see *Table 2*) (10,48-52). While no single study is statistically powered to evaluate the treatment by biomarker interaction for each of these additional mutations individually, this extended analysis has generally been accepted as standard prior to the prescription of an anti-EGFR agent (53). Moreover, with the application of next generation sequencing technology to simultaneous assessment of multiple candidate genetic markers, further refinement of this algorithm can be expected (54).

BRAF

Using analogous reasoning to that described for RAS mutations, the BRAF V600E mutation, immediately downstream of RAS, has been evaluated as a negative predictive marker for the efficacy of EGFR antibodies. However, establishing the BRAF V600E mutation as a negative predictive marker has been challenging due to its lower prevalence (approximately 5-10%) (39-41,55). Additionally, an activating mutation in BRAF conveys a strong prognostic significance, with mutated tumors conferring a poor prognosis (10). Most studies do not clearly demonstrate a negative predictive benefit for BRAF for the selection of EGFR antibodies (48). Nonetheless, BRAF mutation testing has been recommended due to its strong implications on prognosis and due to the availability of BRAF mutation targeted clinical trials (56). Indeed, we use this information to guide the intensity of therapy and surveillance for progression of disease on treatment.

Table 2 Benefit from anti-EGFR antibody treatment plus chemotherapy versus chemotherapy alone in exon 2 wild type and expanded RAS wild type patients

Trial	Line	Cytotoxic backbone	Antibody	N	Exon 2 RAS WT		N	Expanded RAS WT		Reference
					PFS HR (95% CI)	OS HR (95% CI)		PFS HR (95% CI)	OS HR (95% CI)	
COIN*	1	FOLFOX/ CAPOX	Cetuximab	729	NR	1.04 (0.87-1.23)	581	NR	1.02 (0.83-1.24)	(10)
PRIME	1	FOLFOX	Panitumumab	656	0.80 (0.67-0.95)	0.83 (0.70-0.98)	512	0.73 (0.60-0.88)	0.77 (0.64-0.94)	(48)
CRYSTAL	1	FOLFIRI	Cetuximab	666	0.70 (0.56-0.87)	0.80 (0.67-0.95)	367	0.56 (0.41-0.76)	0.69 (0.54-0.88)	(49)
OPUS	1	FOLFOX	Cetuximab	179	0.57 (0.38-0.86)	0.85 (0.60-1.22)	87	0.53 (0.27-1.04)	0.94 (0.56-1.96)	(50)
20050181	2	FOLFIRI	Panitumumab	597	0.73 (0.59-0.90)	0.85 (0.70-1.04)	421	0.70 (0.54-0.91)	0.81 (0.63-1.03)	(51)
PICCOLO*,#	2	Irinotecan	Panitumumab	460	0.78 (0.64-0.95)	1.01 (0.83-1.23)	323	0.68 (0.53-0.86)	0.92 (0.73-1.16)	(52)

*, the COIN and PICCOLO studies included KRAS codon 61 mutations in their primary analyses and the expanded RAS WT populations also exclude patients with BRAF mutations; #, the expanded RAS analysis of PICCOLO excludes a small number of PIK3CA mutations. EGFR, epidermal growth factor receptor; WT, wild type; PFS, progression free survival; OS, overall survival; HR, hazard ration; NR, not reported.

EGFR expression and amplification

During the initial development of EGFR antibodies in metastatic CRC, it was predicted that EGFR protein expression would be required for any activity. Therefore, only EGFR-expressing CRC was allowed on the initial clinical trials (57). However, subsequent analyses did not demonstrate a correlation with EGFR expression and response (58). Response to an EGFR antibody was observed in tumors that did not have immunohistochemically detectable expression of EGFR (59). Therefore, expression of EGFR has been abandoned as a predictive marker for the benefit of EGFR antibody therapy CRC. Similarly, EGFR copy number has been described as a poor candidate for development as a predictive marker of the efficacy of EGFR antibody therapy (60), despite at least one small study suggesting a provocative interaction between copy number and outcome (61).

EGFR ligand expression

Intratumoral expression of the EGFR ligands amphiregulin and epiregulin may reflect activation of the EGFR pathway through an autocrine signaling loop. An initial study using tissue obtained from pretreatment tumor biopsy mRNA on a single agent cetuximab study suggested that intratumor expression was strongly associated with disease control and progression free survival (62). A follow up analysis of tissue obtained on a study combining cetuximab and irinotecan demonstrated concordant results, and further demonstrated that the effect was only relevant in tumors with absent mutation in KRAS (63).

However, ligand expression may have prognostic implications that dilute the predictive utility and the optimal method of measurement is not yet standardized as concordance between tumor sites is modest (64,65). Thus, while the measurement of amphiregulin or epiregulin expression cannot be recommended to select EGFR antibody use in RAS wild type patients, they remain strong candidates for further development in ongoing studies of EGFR antibody therapy in CRC.

PI3K pathway alterations

In addition to the RAS pathway, EGFR signaling is mediated through a distinct pathway that involves phosphoinositide-3-kinase (PI3K), AKT and PTEN. Similar to the rationale for how RAS pathway mutations impact upstream EGFR-directed therapy, mutations in the PI3K pathway have been evaluated as a predictive marker for the efficacy of EGFR antibody therapy.

The α-catalytic domain of PI3K is encoded by the *PIK3CA* gene and is mutated in 10-20% of CRCs. Mutation in PIK3CA is not mutually exclusive with RAS mutations. The majority of mutations in PIK3CA occur in either exon 9 (the helical domain) or exon 20 (the kinase domain). Initial studies came to conflicting conclusions regarding the potential utility of PIK3CA mutations in predicting resistance to EGFR-targeted therapy (66,67). In a large study of 773 patients with evaluable specimens treated with EGFR antibody therapy, PIK3CA mutations were associated with a lower response rate in KRAS wild-type patients (17.7% *vs.* 37.7%; OR 0.35; 95% CI,

0.13-0.83; P=0.015) but not with reduced PFS or OS (40). Interestingly, the effect seemed to be confined to exon 20 mutations where response, PFS and OS were all statistically reduced compare to wild type patients. Nonetheless, exon 20 mutations are relatively rare (only 9 without KRAS mutation were analyzed in the study) and further studies are needed to confirm these findings and establish the magnitude of the effect. Indeed, a subsequent study on a subset of patients from the original cetuximab monotherapy phase III study did not find any effect of PIK3CA mutations as a predictive marker for benefit (41).

Phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a tumor suppressor gene which functions as a negative regulator of PI3K activation by EGFR. Loss of PTEN function results in sustained activation of PI3K effector proteins and has been reported to occur in the range of 20-40% of CRC (68,69). In small retrospective series of 27 patients with CRC who received cetuximab as part of their therapy, 10 of 16 patients with intact PTEN had objective response in contrast to none of the 11 patients with loss of PTEN (68). Interpretation of this study is limited by both the small numbers but also presence of KRAS mutations in 37% of the cases. A larger but also retrospective study found loss of PTEN in 20% of 111 cases of wild type KRAS CRC (69). In contrast to the prior study, loss of PTEN was not associated with response or progression-free survival although overall survival was shorter.

Molecular tests to individualize anti-VEGF therapy

A number of markers have been explored as potential predictors for the benefit of anti-VEGF strategies in advanced CRC. Initial studies hypothesized that altered intratumoral expression of VEGF would predict responsiveness to bevacizumab. However, examination of VEGF expression by in situ hybridization and immunohistochemistry did not correlate with outcome in a subset analysis of the pivotal AVF2107g study (70). Similarly, immunohistochemical analysis from the NO166966 study of XELOX and FOLFOX with or without bevacizumab did not demonstrate an association of VEGF, VEGFR1, or VEGFR2 on tumor cells or stroma with progression free survival (71).

Alternatively, it was proposed that baseline or pharmacodynamic changes in circulating angiogenic factors such as VEGF-A could predict efficacy of bevacizumab.

This was initially piloted in a phase II study of bevacizumab in rectal cancer that demonstrated no evidence for a predictive benefit of baseline plasma VEGF (72). Nonetheless, provocative data were generated that associated baseline soluble VEGFR1 and increases (>2-fold) in plasma PlGF with improved treatment effect. In the HORIZON studies testing the anti-VEGF kinase inhibitor cediranib, high circulating VEGF was associated with worse outcome. However, neither clear predictive effect of baseline VEGF nor soluble VEGFR2 was observed for the efficacy of cediranib versus placebo (HORIZON II) or cediranib versus bevacizumab (HORIZON III) (73). In a phase II study of FOLFIRI plus bevacizumab for patients with previously untreated advanced CRC, Kopetz *et al.* analyzed a panel of 37 baseline and on-treatment plasma angiogenic factors for association with PFS (74). Notably, both baseline VEGF and VEGFR2 were not associated with outcome. However, high baseline interleukin-8 levels were associated with a shorter PFS (11 *vs.* 15.1 months; P=0.03). Moreover, elevations in bFGF, PlGF, and HGF were observed in subsets of patients before radiographic evidence of disease progression. Despite these interesting data, there is no marker available to select for benefit of anti-angiogenic therapies at this time in advanced CRC or elsewhere (75,76).

Expression profiling

Recognizing that single molecular events only partly define the molecular architecture of CRC, a number of investigators have explored gene expression profiling as a mechanism to characterize subsets of CRCs that are likely to respond to a given intervention. For example, a relatively small study used an institutional training set to identify 7 genes from a Affymetrix U133 Plus 2.0 chip that were differentially expressed in patients who responded to 5-FU-based chemotherapy (77). After converting the signature to a RT-qPCR assay, the authors demonstrated that patients with a favorable predictive signature had a significantly greater response rate (58% *vs.* 13%, P=0.024), improved PFS (61% *vs.* 13% at 1 year, HR =0.32, P=0.009), and improved OS (32 *vs.* 16 months, HR =0.21, P=0.003). Such data are provocative and clearly larger studies are warranted.

Conclusions

It is an exciting time to be treating advanced CRC as the development of multiple new treatment strategies has

pushed the median survival to the range of 30 months. Nonetheless, the availability of selection markers to optimize therapy on an individual patient basis has not kept up with the pace of drug development. It has been established that UGT1A1*28 polymorphism is associated with irinotecan toxicity, although the optimal management strategy for this marker has not been identified. Performance of a test for mutations in exon 2 of KRAS has now been expanded to other sites in exons 3 and 4 of KRAS and analogous sites in NRAS, and is essential for any patient being considered for an EGFR targeted antibody. A mutation in BRAF (V600E) is associated with a poor prognosis, which may drive alternative treatment strategies for individual patients. Unfortunately, no established predictive biomarker for anti-VEGF therapy yet exists. Nonetheless, the application of new technologies and robust study designs to biomarker discovery and validation efforts is likely to expand the library of available molecular tests to optimize care for patients with advanced CRC in the near future.

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Footnote

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