Published in final edited form as:

J Oncopathol. 2013 September ; 1(3): 21–29. doi:10.13032/tjop.2052-5931.100056.

Using Molecular Markers to Guide Therapy of Metastatic Colorectal Cancer

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Abstract

Colorectal cancer remains one of the most commonly diagnosed cancers with almost one-fourth of patients presenting with metastatic disease at the time of diagnosis. As the repertoire of anticancer agents has expanded to treat colorectal patients with metastatic disease, life expectancies have increased and patients are remaining on therapy for longer periods of time. The exact way in which to combine chemotherapeutic and targeted agents remains a therapeutic challenge in an attempt to preserve efficacy while minimizing toxicity. A crucial need exists for reliable and reproducible biomarkers that can assist in personalizing the most advantageous therapy for patients based on the biology of their tumor that will prevent undue side effects and result in the longest duration of tumor stability. In this review, we discuss the completed studies for each agent currently approved for the treatment of metastatic colon cancer and emphasize a need for further prospective studies to solidify the use of biomarkers in this disease.

Keywords

metastatic colorectal cancer; biomarkers; 5-fluorouracil; irinotecan; oxaliplatin; bevacizumab; EGFR inhibitors

Colorectal cancer (CRC) remains the third most commonly diagnosed cancer in the USA with an estimated 75,000 new cases in 2012.¹ Approximately 20% of patients will present with incurable stage-IV disease. With current chemotherapy and targeted therapy options, average overall survival (OS) has increased to almost 2 years. There are 4 cytotoxic drugs and 5 targeted therapies approved in various combinations for the treatment of metastatic colorectal cancer (mCRC). A “Holy Grail” of oncologic therapy continues to be the ability to determine precisely what drugs are most effective in treating individual patient's tumors based on their molecular characteristics. This article aims to summarize the most up-to-date

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Conception and design: both authors

Collection and assembly of data: both authors

Data analysis and interpretation: both authors

Manuscript writing: both authors

Final approval of manuscript: both authors

Authors' Disclosures of Potential Conflicts of Interest: The authors indicated no potential conflicts of interest.

information on available biomarkers used to predict prognosis, risk of toxicity, and most importantly tumor response in patients with mCRC.

5-Fluorouracil

5-Fluorouracil (5-FU), first developed in the 1950s, remains the backbone of several cytotoxic combinations in the treatment of CRC and is arguably the single most effective agent for this disease. It primarily acts as a pyrimidine analog, inhibiting thymidylate synthase (TS), and preventing DNA replication.² By interfering with the action of TS, the rate-limiting enzyme in the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP), rapidly dividing cells are starved of dTMP and experience cell death (Fig. 1). Multiple studies have investigated the predictive and prognostic capabilities of either tumor TS expression or germline TS variants with regard to treatment with 5-FU; however, the vast majority are limited by small sample sizes and retrospective designs. A meta-analysis of 20 studies of CRC patients that correlated survival with TS expression found that those patients whose tumors expressed higher levels of TS seemed to have a shorter OS with a hazard ratio of 1.74 in the advanced setting.³ In terms of predicting response, the hypothesis that intratumoral levels of TS are related to response to 5-FU-based chemotherapy has been evaluated in multiple small studies, including a trial of 46 mCRC patients that reported an association between low TS levels and better 5-FU response.⁴ Similarly, a study of 36 advanced CRC patients with poor response to 5-FU-based chemotherapy found a correlation with high levels of tumor TS mRNA.⁵ Given that the majority of such studies are small and were performed retrospectively on archived tissue, using TS expression to guide use of fluoropyrimidines cannot be considered standard of care.

A detailed understanding of the metabolism of 5-FU has been beneficial for identifying patients at increased risk of toxicity, including varying degrees of myelosuppression, mucositis, and hand-foot syndrome. Dihydropyrimidine dehydrogenase (DPD) is the principal enzyme involved in the catabolism of 5-FU; patients with diminished activity of this enzyme can be exposed to severe or even fatal toxicity. While complete deficiency of DPD is rare, it is estimated that up to 3% of the general population has some degree of altered DPD activity due to polymorphisms within the *DPYD* gene.⁶ The prevalence of DPD deficiency is three-fold higher in African-Americans than in Caucasians, with African-American women having the highest prevalence of 12%.⁷ Unfortunately the *DPYD* gene is quite large, with over 30 single nucleotide polymorphisms (SNPs) identified, and a direct polymorphism–function relationship has not been established given that most variants have no bearing on the enzyme's utility. This has led to the use of functional assays to determine “true” DPD deficiency; however, these assays are somewhat cumbersome and have not made their way into the mainstream of patient care. While not routinely evaluated in all patients prior to initiating therapy, those with early and unusually severe signs of 5-FU toxicity should be evaluated for DPD deficiency with the caveat that no standardized guidelines on how to treat those patients with low DPD expression exist.

In terms of whether DPD expression can predict response to 5-FU, a few studies have reported an inverse correlation of enzyme expression with treatment outcomes in that

nonresponding tumors were found to have higher levels of intratumoral DPD expression.⁸ Conversely, higher response rates to 5-FU-based therapy have been reported in patients whose tumors had both low DPD and low TS expression. In addition, these patients had more favorable outcomes with increased median survival times of 16.3 months compared to 8.4 months for patients with high DPD or high TS expression. These findings are interesting in that those patients with low DPD activity due to genetic aberrations often have higher degrees of toxicity and subsequently receive lower doses of 5-FU; in theory the exposure to 5-FU is high in spite of dose reduction in such patients.

Irinotecan

Irinotecan is an inhibitor of topoisomerase I (Topo I), which serves to cleave double-stranded DNA during the replication process. Irinotecan was Food and Drug Administration (FDA) approved for the treatment of mCRC in 1996. The parent drug undergoes hydrolysis to produce the active metabolite SN-38, which then undergoes glucuronidation by uridine diphosphate glucuronosyl-transferase (UGT1A1) resulting in its inactivation. The ability to glucuronidate SN-38 in the liver is related to the genetic composition of TA repeats of the promoter region of the UGT1A1 alleles, with the wild-type allele (UGT1A1*1) having 6 TA repeats and the abnormal allele (UGT1A1*28) having 7 repeats.⁹ A meta-analysis of 9 studies that investigated the correlation between hematologic toxicity and UGT1A1 genotype in over 800 patients treated with irinotecan found that patients homozygous for the UGT1A1*28 allele had a higher risk of toxicity when compared to heterozygous or wild-type patients. This association, however, was only statistically significant for patients treated at doses of irinotecan higher than 150 mg/m².¹⁰ In 2004, the US FDA advised that the irinotecan package insert be updated to include the association between UGT1A1 genotype and risk of toxicity and the recommendation that patients with the UGT1A1*28/*28 genotype be treated with lower doses of irinotecan. Practically speaking, however, the data presented in the meta-analysis above indicated that even *28/*28 homozygotes are able to tolerate the doses of irinotecan used in common weekly or bimonthly regimens such as FOLFIRI (irinotecan in combination with infusional 5-FU/leucovorin).

Tumoral expression of Topo I, the target of irinotecan, has been evaluated preclinically and retrospectively in clinical trials as a marker of response to irinotecan-based therapy and as a means of predicting survival in irinotecan-treated patients. Over 1500 advanced colorectal patients enrolled in the FOCUS trial (fluorouracil, oxaliplatin, CPT-11: use and sequencing), which randomized patients to 5-FU alone versus 5-FU with irinotecan and 5-FU with oxaliplatin, had tumor available for analysis of Topo I in comparison to outcomes.¹¹ Patients with tumors that had moderate-to-high levels of protein expression (defined as staining >50% of tumor nuclei) were found to benefit from either the addition of irinotecan or oxaliplatin to 5-FU while those with low Topo I expression (defined as <10% staining) did not. This translated to an OS benefit for patients with high Topo I expressing tumors that received first-line combination chemotherapy versus those that received sequential single agents with a hazard ratio (HR) of 0.6 (95% CI 0.44–0.82). Further validation of these findings and further investigation of appropriate Topo I “cutpoints” in prospective studies are desirable before use of this marker to individualize irinotecan treatments can be considered standard of care.

Oxaliplatin

Discovered in the 1970s and approved by the FDA in 2002, oxaliplatin belongs to the platinum family of compounds that act to form cross-links in DNA, preventing DNA transcription, and resulting in cell death.¹² The body acts to repair these cross-links via nucleoside excision repair (NER), a multistep process integrating over 20 different genes.¹³ ERCC1 has been identified as a critical enzyme for the recognition of the damaged DNA area. With the help of endonucleases, ERCC1 removes the involved strand, allowing for the ensuing gap to be repopulated with undamaged base pairs. In general, tumors that harbor higher levels of the ERCC1 protein are thought to have more effective repair of cross-links induced by platinum agents, increasing the potential for resistance to these drugs. For example, in patients with stage-III colon cancer who received oxaliplatin-based chemotherapy, those with tumors that had expression of ERCC1 as determined by immunohistochemistry (IHC) had lower 5-year disease-free survival and OS when compared to tumors that had negative expression (54% and 60% vs. 72% and 78%, $p = 0.009$).¹⁴

Multiple studies have also been performed to determine potential predictive biomarkers of oxaliplatin toxicity. A retrospective analysis of polymorphisms in the *ERCC* genes of over 100 patients with advanced CRC who received oxaliplatin-based therapy reported an association with a variant of the *ERCC1* gene with an increased risk of neutropenia.¹⁵ In univariate analysis, patients with the rs11615 SNP of the *ERCC1* gene had a 43.8% incidence of Grade 3/4 neutropenia compared to 17.4% and 13.6% of patients with the CT and TT genotypes, respectively. The association was confirmed in multivariate analysis ($p = 0.010$). The various genotypes of the *ERCC1* gene are thought to result in alterations of the mRNA of the gene, with the CC variant resulting in decreased mRNA stability and hence lower ERCC1 protein levels. Therefore, with a reduced capacity to repair damage induced by oxaliplatin, patients with the CC genotype are thought to be at increased risk for oxaliplatin toxicity. Interestingly, however, there was no association with *ERCC1* variants and peripheral neuropathy, a common dose-limiting toxicity of oxaliplatin.

Other groups have found genetic variations in additional genes that predict an increased incidence of oxaliplatin-induced neuropathy. The glutathione S-transferases (GST) are a family of enzymes involved in detoxification of substances that could potentially interact with and damage DNA. GSTP1 is a particular isoenzyme that is highly expressed in CRC and works to detoxify platinum agents, with various polymorphisms responsible for resistance to chemotherapy as well as increased toxicity to treatment.¹⁶ One SNP in the *GSTP1* gene (I105V) results in decreased GSTP1 activity and has been reported in Asian populations to result in higher responses to FOLFOX (5-FU combined with oxaliplatin) in mCRC patients (56.1% vs. 37.6%, $p = 0.04$), as well as result in longer progression-free survival (PFS) and OS.¹⁷ In addition, patients who possessed the I105V variant had increased incidence of Grade 3/4 neuropathy (19.5% vs. 7.2%, $p = 0.02$) after 8 cycles of treatment.

Bevacizumab

Angiogenesis is a critical component of the process of tumor growth and metastasis.¹⁸ Vascular endothelial growth factor (VEGF) helps regulate the complex process of angiogenesis via its effects on cell migration and proliferation. Bevacizumab is a humanized monoclonal antibody that binds soluble VEGF, thus preventing the activation of the VEGF receptor and slowing tumor growth via disruption of angiogenesis (Fig. 2). Bevacizumab was first approved for use in mCRC in 2004 based on the results of a randomized study in previously untreated mCRC patients.¹⁹ The median survival for patients receiving chemotherapy and bevacizumab was longer when compared to chemotherapy alone (20.3 months vs. 15.6 months, $p < 0.001$). Since then, multiple studies have been conducted in an attempt to better understand the predictive properties of VEGF expression (both within tumors and in the circulation). A recent report of the predictive and prognostic capabilities of circulating VEGF-A levels in over 1800 patients with a variety of cancers, including colorectal, lung, and renal cell carcinoma, found that higher pretreatment levels of VEGF-A in the plasma demonstrated trends toward poorer outcomes but had no predictive power for outcomes of patients treatment with bevacizumab-containing regimens.²⁰ The hazard ratios for both PFS and OS were similar in groups who demonstrated both high and low VEGF-A levels, and there was no correlation found between plasma levels of VEGF-A and expression of VEGF-A in the tumor. In general, the data from studies of VEGF and related markers have produced conflicting results that have failed to generate a reproducible angiogenic marker.²¹ This search has been complicated by, most importantly, the complexities surrounding the biology of angiogenesis as well as the diverse effects on tumors seen with inhibition of VEGF. Recently, 2 additional drugs that target the VEGF pathway, regorafenib and aflibercept, were approved for the treatment of mCRC, providing additional mechanisms for inhibiting angiogenesis in mCRC.^{22,23} While aflibercept has high affinity for soluble VEGF ligands and placental growth factor, regorafenib is a multitargeted tyrosine kinase inhibitor that inhibits signaling via VEGF receptors, mutant BRAF, PDGF, TIE, KIT among other receptors.²⁴ It has already been reported that in patients treated with regorafenib, neither *KRAS* mutations nor *PIK3CA* mutations correlate with treatment outcomes.²⁵ A search for biomarkers indicative of regorafenib response is ongoing, and given its single-agent activity, may prove more productive than the search for biomarkers of bevacizumab activity has been.

Cetuximab and Panitumumab

Kras Mutations

The epidermal growth factor receptor (EGFR) belongs to the HER family of transmembrane receptors whose aberrant expression has been implicated in the pathogenesis of many tumor types.²⁶ The overexpression of EGFR in CRC tumors is well documented and is associated with worsened OS and an increased risk of developing distant metastases.²⁷ Cetuximab and panitumumab are monoclonal antibodies targeting EGFR that are indicated for the treatment of mCRC patients in multiple lines of therapy (Fig. 2).^{27,28} The RAS family of signal transduction proteins is activated upon phosphorylation of EGFR, and carries out many of the effects of EGFR activation. Activation of *KRAS* via mutations in codons 12, 13, or 61 of

exon 2 triggers a number of pathways, including the RAF/MEK/ERK and PI3K/AKT/MTOR pathways, both of which are implicated in cell growth and proliferation. The EGFR antibodies were initially studied in patients with IHC-determined “overexpression” of the receptor as a presumptive biomarker.²⁹ This biomarker was subsequently found to be a poor predictor of outcome. Subsequent analysis of several key clinical trials established the mutational status of the KRAS protein as the first robust and clinically applicable biomarker of response in the treatment of CRC. Tumor samples from patients enrolled on clinical trials comparing treatment with cetuximab or panitumumab to best supportive care have been analyzed for KRAS mutations. Analysis of trials using both cetuximab and panitumumab has demonstrated a significant association between KRAS mutational status and the efficacy of EGFR inhibition in terms of OS and PFS.^{30,31} For patients with mutated KRAS tumors, no difference in terms of survival was observed between those treated with an anti-EGFR antibody and those receiving best supportive care. In the case of panitumumab, KRAS wild-type patients exhibited a markedly higher response rate of 17% compared to 0% for the mutant group.³⁰ Subsequently, several studies of combinations of the EGFR inhibitors with chemotherapy backbones confirmed improved outcomes in patients with KRAS wild-type tumors. In a pooled analysis of 845 mCRC patients enrolled on both the cetuximab combined with irinotecan in first-line therapy for metastatic colon cancer (CRYSTAL) and oxaliplatin and cetuximab in first-line treatment of metastatic colorectal cancer (OPUS) trials, in which cetuximab was combined with FOLFIRI and oxaliplatin plus infusional 5-FU/leucovorin (FOLFOX) respectively, patients with KRAS wild-type tumors had statistically and clinically significant improvements in median OS with the addition of cetuximab compared to chemotherapy alone (23.5 months vs. 19.5 months, respectively).³² In randomized studies of the addition of panitumumab to FOLFOX in first line and FOLFIRI in second line, significant improvement in PFS was documented for patients with KRAS wild-type tumors receiving EGFR therapy with a nonsignificant, albeit strong, trend toward improved OS.^{33,34} In a recent analysis of the panitumumab randomized trial in combination with chemotherapy for metastatic colorectal cancer to determine efficacy (PRIME) trial, which randomized patients to receive FOLFOX with or without panitumumab, a statistically significant OS advantage for the addition of panitumumab was noted when patients harboring any KRAS (including codons 12, 13, and 61) or NRAS mutation were excluded.³⁵ Interestingly, in this same study, patients with KRAS-mutated tumors who were treated with panitumumab plus FOLFOX had worse outcomes, with a reduction in OS from 19.3 months with chemotherapy alone to 15.5 months with the addition of panitumumab.³³ While it is still unclear in exactly what combinations of chemotherapy EGFR inhibitors are best utilized, these studies do underscore the importance of preemptive KRAS testing for all patients being considered for EGFR inhibitor therapy.

Braf Mutations

One downstream effector of the KRAS pathway is the protein kinase BRAF, which when mutated results in constitutive activation of the RAS/RAF/MEK pathway³⁶ and uncontrolled cell growth. A study aimed at defining the clinical characteristics and outcomes of CRC patients whose tumors contain a BRAF mutation found significant differences in terms of age, sex, and frequency of microsatellite instability.³⁷ Out of 475 patients with tumors

analyzed, 12% were found to have harbor BRAF mutations with these patients being older, more likely to be female with tumors that were more commonly in the proximal colon. Almost 80% of BRAF mutant tumors were noted to demonstrate microsatellite instability, a molecular characteristic of patients with tumoral loss of mismatch repair proteins. Prognostically speaking, this study also found that patients with BRAF mutated tumors had a statistically significant decrease in OS when compared to their BRAF wild-type counterparts. While microsatellite instable tumors are typically associated with improved outcomes, the presence of a BRAF mutation has been reported to be an independent prognostic factor for survival, regardless of the potential positive effects of the mismatch repair proteins.³⁸ Retrospective analyses of clinical trials combining cetuximab with chemotherapy have also demonstrated the prognostic capabilities of a BRAF mutation; however, no differences in outcome were noted between treatment arms, indicating that BRAF does not appear to share the same predictive properties as KRAS in determine response to EGFR inhibitors.^{32,39}

Egfr Ligands

The molecules responsible for binding to and activating the EGF receptor are initially constructed as transmembrane proteins that, via proteolysis, become soluble growth factors that can bind via endocrine, paracrine or autocrine mechanisms.⁴⁰ In humans, seven such peptides have been described, including transforming growth factor- α (TGF- α), amphiregulin, and epiregulin. This was first described by Khambata-Ford et al.⁴¹ in a trial of mCRC patients receiving cetuximab as monotherapy who underwent gene profiling analysis performed on pretreatment biopsy specimens. Their study reported that patients with tumors that highly express epiregulin and amphiregulin were more likely to have disease control from cetuximab with significantly longer PFS of 157 days versus 57 days for those with low-expressing tumors. In a smaller study of 36 mCRC patients treated with either cetuximab or panitumumab, the pattern of IHC expression of the seven EGFR ligands was evaluated as a potential predictive model of response to EGFR inhibition.⁴² In patients with KRAS wild-type tumors, disease control rates (DCR) were significantly higher for those patients with tumoral staining of four EGFR ligands, namely amphiregulin, TGF- α , epiregulin, and epidermal growth factor. The authors then separated the patients into a positive group (comprised of cases with expression of at least two of the four ligands) and a negative group with immunoreactivity to none. PFS was significantly longer in the patients with positive ligand staining when compared to the negative group (231 days vs. 79 days, $p = 0.000008$) with the positive group having a response rate of 53% compared to 0% in the negative group. Although this study was limited by its small sample size, the ease with which IHC can be performed on tumor samples makes this a feasible approach that deserves further study.

Phosphatidylinositide 3-Kinase (PI3K) Pathway Aberrations

Another well-described downstream target of KRAS is the PI3K pathway which, through activation of AKT, results in cell-growth regulation via mTOR⁴³ as well as suppression of apoptosis among other cellular effects. PI3K, which can be directly stimulated by KRAS via binding of its p110 α catalytic subunit, phosphorylates PIP₂ to PIP₃ which results in

activation of AKT. The tumor suppressor phosphatase and tensin homolog (PTEN) acts to undo PIP₃ phosphorylation, halting intracellular activation of AKT and preventing AKT's actions on cell growth and survival. Preclinical data have suggested that PTEN expression status as well as the presence of PI3K mutations in colon cancer cell lines correlate with sensitivity to cetuximab.⁴⁴ A study of mCRC patients treated with irinotecan and cetuximab evaluated the association between IHC staining of PTEN in both primary and metastatic tumors and treatment outcomes.⁴⁵ While PTEN status of primary tumors had no bearing on response or survival, a higher percentage of patients with PTEN-positive metastases had response to treatment compared to those whose tumors lacked PTEN expression. There was a statistically significant improvement in the PFS of patients with PTEN-positive metastases of 4.7 months compared to 3.3 months for those with PTEN-negative tumors ($p = 0.05$). A meta-analysis of 8 studies that evaluated PTEN status in CRC patients receiving cetuximab-based therapy also reported an association between improved PFS with PTEN positivity as well as higher objective response rates to EGFR inhibitors.⁴⁶ Because of accuracy issues related to IHC evaluation, we do not consider PTEN expression to be a marker that is currently useable in the clinic.

Mutations that result in constitutive activation of *PIK3CA*, the p110 α subunit of PI3K, have been reported in up to 15% of CRCs.⁴⁷ A study of over 1000 CRC tumors evaluated the relationship between the presence of *PIK3CA* mutations with various clinicopathologic parameters as well as associations with other intratumoral molecular aberrations.⁴⁸ The authors reported the presence of a *PIK3CA* mutation in approximately 12% of tumors and classified them as being more proximal tumors with mucinous histology occurring in older individuals. There was also a statistically significant association with the concurrent finding of a *KRAS* mutation ($p < 0.04$). The presence of such mutations has been reported to have a prognostic role as well with one study of 1170 colon and rectal cancers showing that compared with *PIK3CA* wild-type tumors, those that have concomitant mutations in both exons 9 and 20 have significantly worse OS (HR: 2.68, $p = 0.0008$).⁴⁹ Tumors with a mutation in either exon 9 or 20 had no change in cancer-specific outcomes. In terms of predicting treatment response, multiple studies have reported an association with mutations in the *PIK3CA* gene and poor outcomes when treated with EGFR inhibitors.^{50–52} One particular study of over 1000 tumor samples collected from patients treated with cetuximab found that of the 14% that harbored a *PIK3CA* mutation, those with exon 20 mutations (but not exon 9 mutations), had lower response rates, shortened PFS and worse OS when compared to their wild-type counterparts.⁵⁰

Conclusions

The repertoire of approved therapies for patients with mCRC has recently expanded, however, our knowledge of how to best sequence these drugs, the best combinations in which to use them, and in what populations of patients to target them remains less well understood. It is, however, certain that the era of testing agents in unselected populations is rapidly coming to a close. As the molecular biology of CRCs is better elucidated, studies of novel agents should include early evaluation of specific biomarkers, either within tumor specimens or in the peripheral blood, which will serve to predict response and prevent undue toxicity from treatment.

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Figure 1. Mechanism of Action of 5-FU

Acting as a pyrimidine analog, 5-FU is converted to its active metabolite fluorodeoxyuridine monophosphate, which then inhibits TS and prevents further DNA synthesis. 5-FU catabolism is mediated by DPD, 80% of which occurs in the liver.

Figure 2. The VEGF and EGF Receptor Signaling Pathways with Approved Targeted Therapies in mCRC