

# Advances in Biomarkers: Going Beyond the Carcinoembryonic Antigen

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## Abstract

### Keywords

- ▶ carcinoembryonic antigen
- ▶ hereditary colon cancer
- ▶ polyposis
- ▶ Lynch syndrome

Using biologically available markers to guide treatment decisions in colorectal cancer care is becoming increasingly common, though our understanding of these biomarkers is in its infancy. In this article, we will discuss how this area is rapidly changing, review important biomarkers being used currently, and explain how the results influence clinical decision-making. We will also briefly discuss the possibility of a liquid biopsy and explore several exciting and new options.

Advances in biotechnology in the last decade have been tremendous. The technologies used in molecular biology, genetics, and biochemistry have catapulted our understanding of cellular processes and have led to several changes in how we practice medicine; and the coming years will be even more eventful. Not only can we now detect genetic mutations and achieve results in a fraction of the time, but we can do this using smaller tissue samples than ever before. Together, these capacities have enabled increasingly personalized therapeutic approaches for patients with colorectal cancer (CRC).

In the traditional paradigm of oncologic treatment strategies, three options were available for treating cancer: surgery, cytotoxic chemotherapy, and radiation. The choice of ideal treatment was made based upon the organ of origin, the histologic type of malignancy described, and the stage of the disease. In such a paradigm, all nonmetastatic colon cancers have surgery, and all stage III patients receive the same adjuvant chemotherapeutic agents, resulting in heterogeneous responses: some do well and some recur. We are rapidly closing in on using each patient's own unique genetic and tumor profile to help understand these discrepancies and inform medical decision-making, what is termed "personalized medicine." As we learn more about the genetic changes associated with CRC, we are also learning ways in which these mutations can be used to diagnose, prognosticate outcomes, and measure responses to treatments.

The term "biomarker" is somewhat difficult to define. At its essence, it is any biological substrate that can be detected, which is then used to help guide medical decision-making. Current biomarkers in routine clinical use today for CRC include measuring serum carcinoembryonic antigen (CEA) and detecting mismatch repair (MMR) gene and *KRAS* mutations in tumor tissues. Our goal is to review the current state of the science on biomarkers as it relates to CRC. We will discuss the use of CEA for monitoring of recurrence, the role of genetic testing in guiding surveillance, and treatment decisions and how genetic mutations may predict response to chemotherapy and outcomes. We will also briefly review how biomarkers can function as liquid biopsies and explore several new substrates that are currently under investigation. Given the pace of advancements in molecular biology, genetics, and amplification technologies, it is highly likely that in our careers we could do things only seen in the movies: from a single drop of blood we could diagnose the presence of CRC, understand precisely the genetic mutations present, how they impact outcomes and respond to treatment, and even follow that profile as it changes over time.

## Carcinoembryonic Antigen

The standard serum biomarker in use today for CRC is CEA. It is a superfamily of glycoproteins found on cell membranes that play an important role in cell recognition and adhesion. It

is thought to be intimately involved in the ability of CRC cells to metastasize; colorectal adenocarcinoma produces larger amounts of this protein as a result of alterations in posttranscriptional regulation.<sup>1,2</sup> Interestingly, most newly diagnosed patients present with normal levels as it is cleared in the liver before entering the systemic circulation, though in cases of distal cancers alternate circulation patterns through the internal iliac vessels may be associated with high levels.<sup>2</sup> Most modern assays for detecting CEA use a monoclonal technique that has a low false-positive rate; however, it can still be elevated in several disease states such as tobacco use, liver disease, and renal dysfunction.<sup>2</sup>

Unfortunately, CEA has little utility as a screening tool for diagnosing new CRCs due to poor sensitivity and specificity, particularly in early-stage cancer.<sup>2</sup> Fletcher calculated that if CEA were used to screen patients for CRC, at a sensitivity and specificity for early-stage disease of 40 and 90%, respectively, there would be 250 false-positive tests while missing 60% of cancers in asymptomatic patients.<sup>3</sup> Other biomarkers, such as CA 19-9 and CA 242, have fared no better and currently no serum test is recommended as a screening tool for diagnosing CRC.<sup>4,5</sup>

While CEA may be a poor candidate to assist in screening or diagnosis, it has some utility in predicting prognosis and monitoring for recurrence; elevated levels have been shown to correlate with poor outcomes and might be a useful strategy for defining patients at higher risk of recurrence.<sup>1</sup> Preoperative CEA levels also serve as a benchmark to follow treatment outcomes—levels that do not fall within 6 weeks of resection are concerning for residual disease, either local or metastatic. Serial measurements after curative resection can also give an early warning of recurrence or metastatic disease; it is the most frequent indicator of recurrence in asymptomatic patients, is more cost-effective than radiology for detecting curable recurrent disease, is highly sensitive for liver metastases, and can improve survival when included as part of an intensive surveillance plan.<sup>1,6,7</sup> Using CEA to detect recurrence has been shown to provide 5 months lead time before developing other cancer-related symptoms, though this has not been shown to result in improved survival.<sup>8</sup> Current National Comprehensive Cancer Network (NCCN) recommendations include measuring CEA levels at diagnosis and every 3 to 6 months for the first 2 years, followed by every 6 months for the next 3 years in all patients with stage I to III CRC who would be candidates for further treatment if detected.<sup>9</sup> An increase of 30% above baseline is generally considered significant enough to warrant further evaluation, though no widely accepted definition is reported in any recommendation.<sup>1</sup>

For patients with metastatic disease, CEA changes during treatment can indicate response to therapy. While an elevated preoperative CEA is associated with poor outcomes in all patients, a fall in the CEA to normal levels after complete hepatic disease resection is highly predictive of improved survival.<sup>10</sup> Furthermore, CEA is a good biomarker when monitoring response to chemotherapy; the predictive value of a rising CEA during chemotherapy is so suggestive of progression that further imaging and testing may not be

needed before changing treatment regimens.<sup>4,11</sup> Serial CEA measurements every 2 to 3 months while on chemotherapy are beneficial as persistent rises can detect progression even before it may be apparent on imaging; thus, ineffective regimens can be changed quickly, reducing costs and improving quality of life.<sup>4</sup>

Despite the demonstrated benefits of using CEA as a biomarker for recurrence and response to treatment, there are some significant limitations. Notably, it has low sensitivity and specificity for early stage CRCs, so it is not only a poor screening tool as a noninvasive alternative to colonoscopy, it has limited ability to prognosticate in patients with early-stage disease who have normal levels—a significant proportion of those with the disease. It can also be falsely elevated in several common scenarios, such as patients who smoke and have either renal or liver dysfunction; therefore, a high level in an otherwise asymptomatic patient still demands further diagnostic testing. In addition, there is some concern that serial testing after curative treatment is not cost effective in terms of lives saved and improved survival.<sup>2</sup> While using CEA as a biomarker has some advantages—its easily measured via a serum test and can be repeated frequently with little impact on the patient—it also has some significant drawbacks; therefore, the search for better substrates continues.

## Indications for Genetic Testing

### Polyposis Syndromes

CRC is a heterogeneous disease. Most CRC occurs sporadically, but approximately 30% of cases demonstrate a familial predisposition, and a positive family history in unaffected persons doubles their risk of developing CRC.<sup>12</sup> However, only one-third of patients with a family history have identifiable germline mutations. Given their differential risk profiles, and the benefit of initiating early screening protocols in affected persons, the importance of identification of patients with hereditary syndromes is well-recognized. Accordingly, well-defined clinical parameters exist for selecting patients who should undergo genetic testing for suspected hereditary syndromes.<sup>13,14</sup>

Several polyposis syndromes have been linked to specific genetic mutations, thus any patient presenting with more than 10 polyps, hamartomatous polyps, or polyps at a young age (under 30 years), should prompt genetic evaluation. While the involved genes are known for many of these diseases, de novo mutations within a family can occur, so clinical suspicion outside of family history should prompt evaluation and can be enough to establish the diagnosis as the patient may have a mutation not identifiable by conventional methods.<sup>15,16</sup> The most commonly occurring hereditary syndrome is familial adenomatous polyposis. There are several mutations that occur in the *APC* gene, each with a known associated phenotype that gives rise to several presentations including an attenuated form.<sup>17,18</sup> Another similar polyposis syndrome has been described that involves an autosomal recessive mutation in the *MUTYH* gene, known as *MUTYH*-associated polyposis (MAP). Hamartomatous polyposis syndromes are much less common and de novo mutations occur as well, so clinical suspicion is paramount (see ►Table 1).

**Table 1** Heritable mutations in colorectal cancer

Syndrome	Mutated gene	Inheritance pattern
FAP, attenuated FAP	<i>APC</i>	Autosomal dominant
MAP	<i>MUTYH</i>	Autosomal recessive
Peutz–Jeghers	<i>STK11, LKB1</i>	Autosomal dominant
Juvenile polyposis	<i>SMAD4, BRMP1A</i>	Autosomal dominant
Cowden, Bannayan–Riley–Ruvalcaba	<i>PTEN</i>	Autosomal dominant

Abbreviations: FAP, familial adenomatous polyposis; MAP, MUTYH-associated polyposis.

Source: Table adapted from Syngal et al.<sup>14</sup>

### Lynch Syndrome

Lynch syndrome (LS), the most common heritable form of CRC, it is an autosomal dominant inherited disease with variable penetrance, and accounts for approximately 3% of all CRCs.<sup>19,20</sup> It generally results from inheritance of germline mutations in MMR genes, though rarely de novo mutations, which rise at a rate of about 2%, can also cause the syndrome.<sup>21</sup> Patients with LS can be identified using several strategies. Traditional clinical criteria for identifying persons at risk for LS, such as Amsterdam criteria and Bethesda guidelines, set forth parameters primarily based on family history and age at diagnosis. Both have undergone modifications in the efforts of improving sensitivity and specificity. Computational models, such as MMRpredict (Edinburgh, Scotland, UK), offer better sensitivity and specificity, but are less convenient to use in everyday practice.<sup>22</sup>

While these methods can be used to recognize patients at risk for LS, tumor testing confirms the diagnosis. The Center for Disease Control recommendations support the use of universal genetic screening for the presence of common mutations in MMR genes for all patients with newly diagnosed CRC.<sup>23</sup> However, the utilization of resources with universal genetic testing is significant, and this guideline is not collectively endorsed. Alternative recommendations include screening for patients with CRC under the age of 70 years and those over 70 years with a family history concerning for LS.<sup>24,25</sup>

Strategies to assess MMR deficiency use either immunohistochemistry (IHC) to identify impaired protein expression of the four MMR genes commonly mutated in LS (*MLH1*, *MSH2*, *MSH6*, *PMS2*), or polymerase chain reaction (PCR) to detect microsatellite instability (MSI), which suggests improperly functioning MMR proteins. Results of IHC and PCR generally agree, however, IHC is slightly less sensitive and specific. Even so, due to the increased availability of IHC, and its cost savings in comparison to PCR, it is often the first step in tumor analysis.<sup>26,27</sup> Once an MMR protein deficiency is identified, testing with PCR can confirm MSI. This involves testing five markers, known as the Bethesda panel (BAT 25, BAT 26, D5S346, D2S123, and D17S250), though other commercially available panels are widely used.<sup>28,29</sup> MSI tumors

are regarded as MSI-H (high) if > 30% of the markers are mutated, MSI-L (low) if at least one and < 30% of the markers are mutated, and microsatellite stable (MSS) if no markers are mutated.<sup>30</sup> To determine the etiology of MSI in patients without a clear family history of LS, further analysis of the pattern of protein loss can be useful. In patients with a MLH1 deficiency, testing for BRAF mutations and MLH1 hypermethylation should follow, as these are often seen in sporadic CRC with MSI but rarely seen in LS, which is associated with direct mutations in *MLH1* or *MSH2*.<sup>31–33</sup> Alternatively, if loss of MLH2 and/or MSH6 protein occurs, genetic testing should evaluate *MSH2*, *EpCAM*, and *MSH6*.<sup>24,34</sup>

Until recently, there has been little clinical utility for understanding the genetics of sporadic CRC. Over the last decade, advances in molecular techniques and genomic profiling have improved our understanding of cellular processes, allowing for the development and approval of several targeted therapies for use in the CRC. This has resulted in an explosion of scientific interest in establishing genetic biomarkers with prognostic and predictive value that can be used to help guide treatment decisions.

## Mutation Analysis to Guide Treatment

### Targeted Therapy in Metastatic Colorectal Cancer

The development of several “targeted therapies” for use in metastatic CRC has led to an increasing drive to use each patient’s unique tumor mutational profile to help inform treatment choices (see ►Table 2). This is the beginning of personalized medicine—neither two persons, nor do two tumor cells harbor the same profile. Tumor mutation profiles can even change over time as new mutations accumulate or respond to treatment. The beginning of this was the understanding of the impact of *KRAS* and *NRAS* mutations. The

**Table 2** Clinical relevance of colorectal cancer genetic mutations

Mutated gene	Clinical impact of mutation
<i>KRAS</i>	Lack of response to anti-EGFR targeted therapy
<i>NRAS</i>	Possible limited benefit of anti-EGFR targeted therapy
<i>BRAF</i>	Poor prognostic indicator
MMR with MSI	Limited benefit of adjuvant chemotherapy in Stage II Good prognostic indicator
LOH 18q/CIN	Possible resistance to fluorouracil
<i>Top 1</i>	Possible increased response to irinotecan
<i>ERCC1</i>	Possible increased response to oxaliplatin or fluorouracil
<i>UGT1A</i>	Increased risk of toxicity from irinotecan
<i>DPYD</i>	Increased toxicity with fluorouracil

Abbreviation: EGFR, epidermal growth factor receptor; LOH, loss of heterozygosity.

protein products of these genes are involved in intracellular signaling pathways that promote cell growth and development via MAP kinase activation. Mutations in these genes result in constitutive activation of the MAPK pathway and are present in ~40% of CRCs at *KRAS* exon 2 (codons 12 and 13).<sup>35,36</sup> Patients with *KRAS* mutations do not respond to the targeted anti-epidermal growth factor receptor (anti-EGFR) therapy, including cetuximab and panitumumab, which block activation of the pathway in patients with wild type (WT) *KRAS* genes. Additionally, and perhaps more importantly, patients with *KRAS* mutations have been shown to have worse outcomes when given anti-EGFR-targeted chemotherapy, highlighting the importance of assessing *KRAS* status before the initiation of these therapies.<sup>37,38</sup> For those patients with metastatic CRC and no *KRAS* mutations, anti-EGFR therapy can confer a significant survival benefit.<sup>39</sup>

Though eliminating patients with known mutations in *KRAS* results in improved response rates to EGFR-targeted therapies in combination with chemotherapy, still only approximately 10% of *KRAS* WT patients respond to cetuximab as monotherapy, suggesting the need for further efforts to improve patient selection by better identification of those who will respond to this therapy.<sup>38,40–42</sup> Accordingly, investigators have become increasingly interested in studies revealing a lack of response among tumors with other RAS family mutations, specifically on the *NRAS* gene, designated “extended RAS” or “expanded RAS” mutations.<sup>43–45</sup> Nearly 20% of *KRAS* WT tumors bear one of these mutations and patients with these mutations may also have limited benefit from anti-EGFR therapies, suggesting an opportunity to further improve patient selection by excluding a significant portion of *KRAS* WT, who would be unlikely to respond to anti-EGFR therapy.<sup>43,45</sup> While testing for *KRAS* mutations in codons 12 and 13 is widely recommended to help guide selection of chemotherapeutic agents, testing for other *KRAS* and *NRAS* mutations is more controversial.<sup>25,46</sup> Still, current NCCN recommendations state that patients with any *KRAS* or *NRAS* mutation should not be treated with cetuximab or panitumumab.<sup>25</sup>

*BRAF* is another important protein in the MAP kinase pathway. Mutations in *BRAF* occur in approximately 15% of CRC, and in contrast to *KRAS*, evidence concerning the efficacy of anti-EGFR therapy in *BRAF* mutated CRC is conflicting. Several studies show no benefit of anti-EGFR therapy in patients with *BRAF* mutations.<sup>35,47,48</sup> However, pooled data from the CRYSTAL and OPUS trials suggest that *BRAF* status does not predict a response to anti-EGFR therapy.<sup>49</sup> Some of the difficulty in arriving at definitive conclusions stems from the relatively low incidence of *BRAF* mutations in patients with CRC.<sup>35,36</sup> The prognostic significance of *BRAF* mutations is more certain, as patients with this mutation have consistently been shown to have poor outcomes in relation to those with WT *BRAF*, with reduced overall survival and progression-free survival.<sup>44,50,51</sup> Current NCCN recommendations include testing of tumors for *BRAF* mutations in stage IV disease, though this is of prognostic value only.<sup>25</sup> Even so, testing in metastatic patients, particularly those who have failed other chemotherapy regimens, may yield additional benefit to patients in terms of identifying those who might be

eligible to participate in clinical trials with *BRAF* inhibitors, which are likely to be of more clinical utility.<sup>43</sup>

### Microsatellite Instability in Stage II Colon Cancer

MSI occurs in about 15% of sporadic *KRAS* WT CRCs. It is associated with right-sided lesions and poorly differentiated, mucinous histology.<sup>52,53</sup> MSI testing for the purposes of identifying patients with LS should be performed according to previously described guidelines. Additionally, the NCCN recommends MSI testing of all tumors in patients with Stage II disease. MSI indicates improved prognosis in comparison to MSS tumors and MSI status can also be used to aid in assessing the likelihood that patients will benefit from adjuvant chemotherapy.<sup>25</sup>

Despite the predominance of poorly differentiated histology among patients with MSI tumors, these patients tend to have improved outcomes and a decreased likelihood of metastases in comparison to patients with MSS tumors.<sup>54</sup> In this respect, MSI is regarded as a strong positive prognostic indicator.<sup>55,56</sup> There is evidence to support that patients with MSI tumors and Stage II/III CRC do not have the same improvement in disease-free survival and overall survival with adjuvant fluorouracil (5-FU)-based therapy as compared with their MSS counterparts.<sup>52,53</sup> Furthermore, the use of 5-FU in MSI patients with Stage II CRC has been associated with adverse outcomes.<sup>53</sup> However, these results were found in the setting of regimens lacking oxaliplatin, an agent almost universally used in the adjuvant treatment of CRC today. More recent data from randomized trials (PETACC 3 and QUASAR) have not confirmed these findings.<sup>57,58</sup> Taken together, it is currently recommended that tumors from patients with stage II CRC be tested for MSI. Decisions about whether patients with Stage II CRC and MSI receive 5-FU-based chemotherapy should be individually tailored and based on risk assessment.<sup>25</sup> The same trend is not seen in stage III CRC, where a clear survival benefit is seen with adjuvant chemotherapy in all patients.<sup>59</sup>

### Chromosomal Instability, Topoisomerase I, and Excision Repair Cross-Complementing Gene 1 Polymorphisms

Chromosomal instability (CIN) describes a state of high rates of gains and losses of chromosomes within tumors. The cause of CIN is unknown, but loss of heterozygosity (LOH) is a hallmark feature and LOH at 18q has generated attention due to its possible association with outcomes.<sup>60,61</sup> Additionally, there is some limited evidence to suggest that CIN, in general, may be indicative of resistance to both taxols and 5-FU.<sup>62,63</sup>

Topoisomerases allow for DNA unwinding, a process that is imperative for DNA replication and upregulated in the CRC.<sup>64</sup> The cytotoxic chemotherapeutic agent irinotecan binds and stabilizes the topoisomerase I (Top I)–DNA complex, preventing further DNA replication.<sup>65</sup> Accordingly, the UK MRC FOCUS trial showed that Top I overexpression predicts a response to irinotecan and possibly oxaliplatin, though this could not be verified in another study.<sup>66,67</sup> A similar story is seen for another gene, excision repair cross-complementing (*ERCC1*), which encodes the ERCC-1 protein and is responsible for repairing double strand breaks and cross-linking errors in DNA.<sup>64</sup> ERCC-1 deficiency is common in a CRC and can result



from either genetic mutation or promoter methylation.<sup>67</sup> ERCC-1 deficiency has been noted to predict response to oxaliplatin or 5-FU-based chemotherapy, but this could not be confirmed in other retrospective analyses.<sup>68,69</sup> A deal of additional research evaluating the utility of the above biomarkers is needed before widespread routine clinical use can be recommended.

### Predicting Chemotherapy Toxicity

The uridine diphosphate glucuronosyltransferase 1A (*UGT1A*) gene is located on chromosome 2q37. The gene transcribes several enzymes, among them, *UGT1A1* is commonly known for its role in the glucuronidation of bilirubin and a mutation here can result in increased concentrations of the active metabolite of irinotecan, thus increasing tumor responsiveness to this regimen in the presence of the mutation. However, this increased cytotoxicity also increases the risk of adverse reactions, such as neutropenia and diarrhea. Despite the known risk of toxicity in patients with the mutation, the recommendations do not currently endorse routine testing. Rather, they advise dose reductions in the setting of adverse reactions.<sup>70</sup> Dihydropyrimidine dehydrogenase (*DPD*) deficiency can also lead to chemotherapy-related toxicity as it is the enzyme responsible for metabolism of 5-FU. Deficiency of this enzyme is present in 3 to 5% of the population and is associated with severe adverse effects in response to 5-FU-based chemotherapy.<sup>69,71</sup> Deficiency generally rises through mutations in the dihydropyrimidine dehydrogenase gene (*DPYD*, mapped to chromosome 1p22) and patients who are homozygous for the mutant *DPYD* allele can have lethal reactions to 5-FU.<sup>72</sup> Despite the propensity for patients with *DPD* deficiency to develop toxicity in response to 5-FU, its association with response to therapy is uncertain. Given decreased adverse effects with newer dosing regimens and the lack of solid evidence to support its ability to predict response to therapy, testing for *DPD* deficiency is not currently standard.<sup>73,74</sup>

In addition to *DPD*, mutations in other enzymes participating in pyrimidine metabolism, such as thymidylate synthase and methylenetetrahydrofolate reductase, may predict clinical outcome, response, and toxicity to 5-FU. With regard to biological therapies, investigators have been interested in the utility of *PI3K* and *PTEN* to predict response to anti-EGFR therapy.<sup>75</sup> Similarly, others are exploring the capacity for soluble vascular endothelial growth factor (*VEGF*), plasma *VEGF*, and tumor *VEGF* to predict responses to treatment with anti-*VEGF*.<sup>74</sup> The clinical validity of these markers and more are yet to be defined.

### Multigene Assays

Several multigene assays are available for the purposes of risk stratification. Oncotype DX (Genomics Health Inc., Redwood City, CA), ColoPrint (Agendia NV, Amsterdam, Netherlands), and ColDx (Almac, Craigavon, Ireland) each offer genetic panels to aid in predicting recurrence of CRC.<sup>25</sup> The 12 gene assay, Oncotype DX, was developed using data from NSABP studies and the Cleveland Clinic. Its predictive value, in terms of establishing a recurrence score for patients with Stage II and Stage III CRC, was validated in studies using tissue and data gathered in both the QUASAR and NSABP-07 trials.<sup>76-78</sup> The

ColoPrint is a similar panel, with 18 genes. In a retrospective cohort study comparing ColoPrint to clinical risk factors outlined by NCCN guidelines, ColoPrint was more accurate at predicting recurrence of stage II MSS CRC. The study was unable to show a benefit of chemotherapy according to risk stratification, however, this would best be appreciated in a randomized trial that is adequately powered to do so.<sup>79</sup> Finally, ColDx is a microarray-based assay was validated in its ability to assess recurrence risk in stage II CRC using specimens and data gathered from CALGB 9581.<sup>80</sup> Despite the capacity of these assays to determine risk of recurrence, no test has displayed the ability to predict a response to chemotherapy, therefore, the true impact of these diagnostics on oncologic outcomes is uncertain, and the utility of these assays in assistance with clinical decision-making has yet to be determined.<sup>25</sup>

### Limitations of Current Biomarkers and Future Possibilities

As of today, all analyses of tumor characteristics are dependent upon invasive biopsy. This includes evaluation of the specific tumor type and histologic features as well as genetic profile. Whether tissue is collected via endoscopic or percutaneous biopsy, or from a surgical specimen, a reasonably large volume of tissue is needed for interrogation. In most cases, these specimens are collected at the time of diagnosis, but performing the tests evaluating some biomarkers, such as *KRAS*, may be delayed until the patient recurs with metastatic disease. This sequencing may pose several problems. First, CRC is a heterogeneous disease with several distinct pathways to progression, thus each person and even each cell has the potential to harbor a different profile of mutations.<sup>81,82</sup> Second, tumor molecular profiles change over time, resulting in different behaviors, such as the ability to metastasize.<sup>83</sup> Third, tumor profiles can change with treatment as those cells with targeted features are destroyed by chemotherapy and others without are left behind to develop more genetic alterations.<sup>84</sup> A static sample of tissue from a single point in time is not able to reflect these ongoing and dynamic changes. Furthermore, if repeat tissue is needed for analysis, it involves an invasive procedure with inherent complications that can impact patients' care and treatment.

Development of a "liquid biopsy" is an ongoing research topic that is making considerable progress in recent years as amplification technologies are rapidly improving. The goal is to discover a substrate that is sensitive enough to detect the presence of early CRC, but also specific enough to discriminate from other adenocarcinomas, provide molecular profiling of the tumor to direct targeted therapies and assess for responses to treatment, offer prognostic information about the risk of recurrence and response to therapy, and can be drawn repeatedly over the course of treatment with little to no risk. While we have not yet identified the ideal candidate, several targets are showing promise.

Proteins circulating in the serum have long been used as biomarkers, including CEA and CA 19-9. These tests have the benefit being easily reproducible, but are not very sensitive or specific for diagnosing CRC and provide only rough

prognostication.<sup>85</sup> They also do not offer any guides to treatment, but do tend to correlate with disease progression or response.<sup>86</sup> The potential pool of proteins from which to draw a biomarker is endless, but one candidate, Rab27b, has shown promise. In a recent publication, this GTPase involved in regulating secretory pathways and oncogenesis was shown to have elevated expression in tumor when compared with adjacent normal tissues, and high levels correlated with serum CEA elevations, lymph node metastases, and TNM stage, as well as overall survival rate in a cohort of 116 CRC patients.<sup>87</sup> Further work will need to be done to determine if serum expression of Rab27b correlates or offers improved sensitivity and specificity beyond current biomarkers. Rather than investigate known oncogenic proteins *de novo* in each cancer type, another strategy could be a more powerful way to identify candidate proteins. Surinova et al (2015) used a screening method to identify candidate glycoprotein biomarkers from tumor epithelia, then screened selected proteins in the plasma of CRC patients to identify those that reached the circulation. Overall, 88 proteins were identified and 70 fulfilled validation criteria with an independent cohort; further consensus testing identified 5 proteins that consistently predicted disease and performed better than CEA in diagnosing CRC, even when used together with the CEA.<sup>88</sup>

MicroRNA (miRNA) is another area of active investigation as a source of biomarkers for CRC. miRNA is small, noncoding single strands of RNA that are thought to play a role in posttranscriptional regulation of gene expression and are dysregulated in CRC.<sup>89</sup> Several candidates have been identified, including miRNA-375, miRNA-183, and miRNA-211 that correlate with detection, recurrence, or prognosis.<sup>89–91</sup> However, the understanding of these biomarkers and their native functions is poorly understood. In addition, testing for these substances is highly variable in commercially available kits, calling into question the reproducibility of using these small molecules in the clinical setting.<sup>92</sup> Furthermore, proteins and miRNA will never have the potential to provide meaningful genetic information about the tumor and its response to treatment, limiting the applicability of these biomarkers.

Perhaps the area showing most potential is in the detection and analysis of circulating DNA and circulating tumor cells (CTCs). Circulating cell-free DNA (cfDNA) is thought to arise from both normal and tumor cells as a result of normal processes as well as apoptosis and necrosis.<sup>93</sup> Amplification of cfDNA has been increasingly used to diagnose fetal genetic abnormalities in high-risk women, with positive tests warranting more invasive diagnostic procedures, and these cfDNA screening tests have also identified new incidental malignancies in the mother due to their high sensitivity.<sup>94</sup> Levels of serum cfDNA been shown to correlate with prognosis in metastatic CRC, and it also has the potential to be a source for detection of various genetic mutations, with a high concordance of *KRAS* mutations when compared with standard techniques.<sup>93,95</sup> Evaluating the level and pattern of methylation in cfDNA samples has also been shown to be sensitive for detecting CRC, especially for stage IV disease.<sup>96,97</sup> CTC can also be a highly sensitive and specific biomarker and provide specific genetic information about both the tumor and its

response to treatment. Using enrichment and depletion techniques, CTC can be isolated and detected by targeting their cell-surface markers, such as CK20, so that further analysis can be performed.<sup>98</sup> The discordance between CTC and primary tumor was low for both *KRAS* and *KRAS* mutations.<sup>99</sup> Furthermore, patients with known *KRAS* mutations in CTC were shown to have poor outcomes when treated with anti-EGFR therapy, the same as if primary tumor testing occurred.<sup>100</sup>

## Conclusion

As we approach an era of personalized medicine, the impact of each individual tumor cell and each person's unique genetic profile will become increasingly important. Our understanding of the utility of these mutations is only in its infancy. We currently use only CEA as a routine serum biomarker, and it has significant limitations. While we are fairly good at identifying heritable mutations, several persons with a strong family history do not have any mutation we currently test for, suggesting that there are still many more genes with influence over the development of CRC. Our understanding of how tumor genetic mutations can predict response to therapy and oncologic outcomes is poor and improvements in this arena could have a huge impact on our everyday practice and advances here will become progressively more valuable. Furthermore, the discovery of a "liquid biopsy" to diagnose, predict response, and prognosticate outcomes would be monumental, not only from a treatment standpoint, but from a quality of life and comfort standpoint of our patients. The future in biomarker understanding is wide open and there remains much to be learned.

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