Published in final edited form as:

Clin Gastroenterol Hepatol. 2016 May ; 14(5): 651–658. doi:10.1016/j.cgh.2016.02.008.

Molecular Biomarkers in the Personalized Treatment of Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is a disease in which pathogenesis is influenced by genetic and epigenetic events that occur with tumor initiation and progression. Precision oncology is becoming increasingly important in the management and therapy of CRC since large variation exists in individual patient prognosis and response to chemotherapy that is due to molecular heterogeneity. Certain biomarkers have been identified that can be utilized to predict clinical outcome beyond staging, and to inform treatment selection. Molecular testing is routinely performed in clinical practice for the selection of patients for targeted biologic agents or immunotherapy, and is advocated for prognostic stratification. Estimating prognosis can inform treatment decisions with avoidance of under or over treatment and also guide the intensity of patient follow-up. Classifiers of CRC have been developed that integrate genetic and/or epigenetic features which can provide prognostic and predictive information. The mutational status of *KRAS* and *BRAF*^{V600E} combined with analysis of the DNA mismatch repair system with/without CIMP has been shown to identify colon cancer subtypes with distinct clinical features and prognoses. Gene expression profiling has also been used to subtype CRCs and can overcome the limitations of single/limited gene testing. A recent effort identified four consensus molecular subtypes of biological relevance that were associated with different patient outcomes. Efforts to validate and refine these subtypes to include additional genomic features are ongoing. In addition to the potential for molecular subtypes to predict therapeutic efficacy, they can inform the development of new agents in a subtype-specific manner to accelerate drug-discovery efforts. The focus of this article is to highlight molecular markers that can inform clinical decision-making in patients with CRC. Molecular profiling-based stratification

Keywords

Predictive markers; Prognostic markers; Colorectal cancer; Molecular subtypes; RAS; BRAF; MSI; DNA Mismatch Repair; Immunotherapy; Targeted Therapy; Biologics; anti-EGFR; anti-VEGF

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Conflicts of Interest: None

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Technological primer

Molecular testing has become routine in patients with metastatic CRC to select patients for targeted therapy. While analysis of individual genes has been the norm, the use of next-generation sequencing (NGS) platforms allows for high throughput genomic analysis that has become commonplace and is commercially available. NGS can detect most genomic alterations with analysis of the entire coding sequence of multiple genes and can probe introns from genes that are often altered in solid tumors. This technology has the ability to identify somatic alterations including base substitutions, insertions/deletions, copy number alterations and rearrangements in a single assay from limited archival tissue. NGS of sporadic CRCs has confirmed prior identified genetic alterations and has classified new alterations. A newer technology for mutation detection is the use of circulating tumor (ct) DNA which is an alternative to tissue-based mutational analysis¹. In a recent study in metastatic CRC patients, ctDNA and patient-specific candidate tissue mutations was detectable in the cell-free DNA from the plasma in a high proportion of treatment naive patients². Furthermore, changes in ctDNA during first-line chemotherapy appeared to predict the later radiologic response. The applications of these 'liquid biopsies' has the potential to replace tumor tissue analysis in clinical practice, and can enable the monitoring of tumor burden and detect molecular resistance to therapy.

Several groups have reported molecular classifications of CRCs using gene expression data from NGS or expression arrays³⁻⁷, and in some cases, these subtypes provided predictive^{4,7} or prognostic information³⁻⁶. High throughput gene expression profiling reveals tumor subtypes that show overlap with previous classification systems utilizing microsatellite instability (MSI), CpG island methylator phenotype (CIMP), chromosomal instability (CIN) or *KRAS/BRAF* mutation status^{4,6}, which cannot be identified solely based on single mutations or epigenetic alterations. While subtyping efforts have been largely limited to gene expression profiling, advances in genomic technology enable tumor characterization using whole exome (entire human coding DNA) and whole genome (all coding and noncoding regions) sequencing (WGS) that are being increasingly utilized. WGS can provide additional data on chromosome or gene copy number and translocations. Advantages include the ability to achieve broad coverage of the genome and there is evolving ease of performance and declining costs. These technologies generate vast amounts of data for which bioinformatics approaches are being applied, yet important challenges remain regarding data interpretation and its clinical application. From a clinical practice standpoint, these technologies will need to produce relevant data in a relatively short time period for it have an impact on the management of individual CRC patients.

Findings

CRC develops as a consequence of genetic and epigenetic alterations whose analysis in individual patients has the potential to indicate risk, inform prognosis, and predict treatment outcome. In patients with this malignancy, there is considerable stage-independent variability in patient outcomes that is due, in part, to molecular heterogeneity. This heterogeneity also makes it difficult to determine which patients will benefit most from

existing therapies including targeted agents. Analysis of somatic alterations in CRCs by the The Cancer Genome Atlas (TCGA) included whole-genome sequencing that identified 24 genes that were significantly mutated and included the expected *APC*, *TP53*, *SMAD4*, *PIK3CA* and *KRAS* mutations⁸. Commonly observed alterations enable a broad classification into 1) hypermutated tumors (~16%) of which three-quarters show high frequency MSI (MSI-H) and one-quarter have somatic MMR gene and polymerase ϵ (*POLE*) mutations⁸, 2) nonhypermutated tumors (~84%) with multiple somatic copy number alterations and aneuploidy that contain activating mutations in *KRAS* and *PIK3CA* and loss of heterozygosity of *APC* and *TP53* tumor suppressor genes, and 3) CIMP (~20%) that is commonly observed in MSI-H tumors and in some nonhypermutated CRCs. CIMP is characterized by a high frequency of genome-wide DNA methylation of CpG islands^{9, 10} that is believed to promote tumorigenesis by gene silencing due to methylation-mediated transcriptional repression. Sporadic MSI-H tumors are considered to be a subset of CIMP due to aberrant hypermethylation that inactivates *MLH1*¹⁰. Occurring more often in women and older individuals, CIMP-positive tumors are typically proximal, higher grade, and have deficient DNA mismatch repair (dMMR) resulting in MSI-H^{9, 11, 12}. *BRAF*^{V600E} mutations are strongly enriched in sporadic CRCs with dMMR and CIMP^{8, 13, 14}. CRCs with dMMR/MSI-H include both familial and sporadic types¹⁵. Approximately two-thirds are sporadic^{9, 10} and the other third of dMMR CRCs are due to germline mutations in MMR genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*) that cause Lynch Syndrome (LS). The presence of *MLH1* promoter hypermethylation and/or a *BRAF*^{V600E} mutation essentially excludes LS¹³. Components from these classifications of CRC outlined above are now used as diagnostic, prognostic, and predictive biomarkers. Additional common biomarkers may come from expression profiling and further genomic analyses.

Predictive and Prognostic Biomarkers

A prognostic factor is a clinical or biologic characteristic that is objectively measurable and that provides information on the likely outcome of the cancer in an untreated individual. Prognostic markers are helpful for identifying patients with cancer who are at high risk of metastatic relapse and therefore, potential candidates for adjuvant systemic therapy. In contrast, a predictive biomarker is defined as a marker which can be used to identify subgroups of patients who are most likely to respond (either in terms of tumor shrinkage or survival) to a given therapy. Importantly, prognostic factors define the effects of patient or tumor characteristics on patient outcome, whereas predictive factors define the effect of treatment on the tumor.

The personalized therapy of CRC has seen major advances in the setting of metastatic disease (stage IV) whereby monoclonal antibodies (mAbs) targeting tumor angiogenesis or the epidermal growth factor receptor (EGFR) signaling pathway have significantly improved patient survival when given in combination with cytotoxic chemotherapy^{16–19}. Antibodies targeting vascular endothelial growth factor (VEGF) [bevacizumab] or its receptor (VEGFR; aflibercept) or EGFR (panitumumab/cetuximab) are now standard of care when combined with FOLFOX [5-fluorouracil (5-FU), leucovorin (LV) and oxaliplatin] or FOLFIRI (5-FU/LV and irinotecan) in stage IV patients²⁰. To date, the identification of a predictive biomarker for anti-VEGF therapy remains elusive. In contrast to their benefit in the

metastatic setting, the addition of antibodies against VEGF or EGFR to standard adjuvant FOLFOX chemotherapy in patients with lymph node-positive, i.e., stage III, colon cancer has failed to improve outcome in randomized trials^{21–23}. Accordingly, there is no role for targeted therapy at this time in the treatment of non-metastatic CRC. In patients with stage III disease, micrometastases are responsible for disease relapse and the reasons for failure of targeted agents to improve their eradication awaits further study.

KRAS and BRAF oncogenes

KRAS and *NRAS* mutation status predict the efficacy of anti-EGFR antibodies with clinical benefit restricted to patient tumors with non-mutated *KRAS* or *NRAS* genes^{16–18}. More than one-third of CRCs carry mutations in exon 2 of *KRAS*, and an additional 15% of tumors were found to carry mutations at exons 3 and 4 of *KRAS* and exons 2, 3 and 4 at *NRAS* that predict resistance to anti-EGFR antibody therapy^{16, 17, 24, 25}. It is now recommended that expanded *RAS* mutation testing be performed for all patients being considered for anti-EGFR mAb treatment. In addition to *RAS*, mutations in the phosphoinositide 3-kinase catalytic subunit alpha (PIK3CA; exon 20 vs exon 9), which is part of the EGFR signaling pathway, may confer resistance to anti-EGFR therapies although further study is needed²⁵. The association of mutations in *KRAS* with prognosis in patients with CRC has produced conflicting data^{26–28}, yet recent data from adjuvant studies in node-positive, i.e., stage III, colon cancer have shown that *KRAS* exon 2 mutations are associated with poor clinical outcome^{29, 30}. Furthermore, the adverse impact of *KRAS* mutations on prognosis appears to be stronger in the distal compared to the proximal colon cancers³¹.

A subset (~8%) of CRCs carry a point mutation (V600E) in the *BRAF* oncogene that is mutually exclusive with mutation in *KRAS*³². Patients whose tumors carry *BRAF*^{V600E} mutations have been consistently shown to have a poor prognosis in the metastatic setting^{33–36}. In addition, data from clinical trials of adjuvant therapy in stage III disease also reveal an association between mutant *BRAF*^{V600E} and adverse outcome^{14, 26, 29}. The adverse prognostic impact of mutant *BRAF*^{V600E} is particularly evident for overall survival and this finding may be explained, in part, by poor survival after recurrence that was observed for *BRAF*^{V600E} mutant colon cancers^{37, 38}. In contrast to patients with *BRAF*^{V600E} mutant melanoma³⁹, CRCs that harbor *BRAF*^{V600E} mutations were found to be resistant to inhibition of the BRAF/MEK/ERK signaling pathway by vemurafenib⁴⁰. Resistance to vemurafenib was later found to be due to feedback activation of EGFR when BRAF is inhibited⁴¹. This finding has led to ongoing clinical trials that investigate combinations of inhibitors of BRAF, EGFR, and MEK with or without chemotherapy⁴². In a recent report, the combination of a BRAF inhibitor plus a MEK/ERK inhibitor demonstrated modest activity in a subset of patients with *BRAF*^{V600E} mutant metastatic CRC⁴³.

DNA Mismatch Repair/Microsatellite Instability (MMR/MSI)

BRAF^{V600E} mutated vs non mutated tumors are associated with poorer clinical outcome in dMMR and proficient (p) MMR tumors, with its effect being somewhat attenuated in dMMR cancers³⁷.

Studies have consistently shown that CRCs with dMMR/MSI-H have a more favorable prognosis compared to those tumors with pMMR/MSS^{15, 25, 37, 44, 45}. These studies include clinical trials comparing surgery alone with 5-FU-based chemotherapy^{25, 44}, and trials comparing 5-FU-based regimens^{15, 37, 46}. Of note, the survival benefit of dMMR/MSI-H status appears to be stronger among colon cancers with earlier stage *vs* later stage disease⁴⁶. Patients with dMMR/MSI-H stage II colon cancers have an excellent prognosis and do not receive benefit from 5-FU-based adjuvant therapy^{47, 48}. In stage III patients, a pooled analysis of two adjuvant chemotherapy trials that evaluated FOLFOX alone or combined with cetuximab showed that patients with dMMR *vs* pMMR tumors had significantly better patient DFS and OS that was limited to the FOLFOX arm⁴⁹. Given that dMMR/MSI-H testing evaluates patients for LS and provides prognostic information, NCCN guidelines recommend such testing for all newly diagnosed CRC cases. While conflicting reports exist for the association of CIMP-positive *vs* negative tumors with clinical outcome, a meta-analysis concluded that CIMP was associated with a poor prognosis in both MSI-H and MSS tumors^{50, 51}. In a recent study using only CIMP-positive tumors, overall survival was decreased in those lacking *MLH1* methylation compared to those with *MLH1* hypermethylation that is consistent with the close association between *MLH1* hypermethylation and MSI-H and the reduction in significance after controlling for MSI status⁵².

Tumors with dMMR/MSI-H are hypermutated and express abundant frameshift peptides that serve as neoantigens to elicit a brisk immune response characterized by abundant tumor infiltrating lymphocytes (TILs)⁵³. CRCs with dMMR/MSI show increased expression of checkpoint proteins (PD-1, PD-L1, CTLA-4, LAG-3, IDO) compared to pMMR/MSS tumors that help enable them to evade immune destruction⁵⁴. In a recent phase II study, metastatic CRCs with MSI-H were treated with an anti-programmed death ligand-1 (PD-1) antibody (pembrolizumab) which achieved a 62% objective response rate compared to 0% for pMMR/MSS CRCs that did not respond to this therapy⁵⁵. Progression-free survival has not yet been reached for the treatment arm of this small patient cohort that included sporadic dMMR/MSI-H and Lynch Syndrome cases as well as some extracolonic malignancies associated with this syndrome. While only ~4% of metastatic CRCs are dMMR/MSI-H indicating that the target population is relatively small⁵⁶, the potential clinical benefit appears to be quite substantial and may potentially apply to earlier stage patients.

Classification by Molecular Subtype

While single gene analysis has been shown to provide valuable predictive and prognostic information, analyses from multiple studies support the feasibility of classifying CRCs by combining molecular markers or using gene expression profiling. The identification of CRC subtypes that link molecular features to clinical outcome can guide patient stratification and inform the rational design of drugs targeting specific pathways. In participants in an adjuvant chemotherapy trial, the combination of *KRAS* and *BRAF*^{V600E} mutations with the status of the DNA MMR system was shown to identify colon cancer subtypes with distinct clinicopathological features and prognoses. These data were then validated in an independent patient cohort (Fig. 1)¹⁴. Evaluation of a similar molecular classifier that included CIMP status yielded similar results in a large population-based cohort of

individuals with varying stages of CRC⁵¹, suggesting the clinical relevance of this classifier. Several studies have utilized gene expression profiling to develop expression signatures that can identify distinct tumor subgroups³⁻⁷. Using an unsupervised classification strategy involving over 1,100 individuals with colon cancer, three main molecular subtypes were identified based upon genetic, epigenetic and clinical features⁴. These colon cancer subtypes (CCS) included CCS1 tumors that were characterized by mutations in *KRAS* and *TP53* genes, displayed increased Wnt signaling, and showed evidence of marked CIN. CCS2 cancers were strongly enriched for CIMP-positive tumors, showed enhanced immune cell infiltration, and were frequently located in the right colon. CCS3 tumors consisted of both MSI-H and CIN tumors, but were enriched for *BRAF*^{V600E} and *PIK3CA* mutations and displayed a mesenchymal phenotype. A similar molecular classification developed by other investigators included at least three major subtypes that were largely based on three biological hallmarks of the tumor: epithelial-to-mesenchymal transition, deficiency in MMR genes and MSI-H, and cellular proliferation⁶. In these classifications, tumors with dMMR/MSI-H had the best prognosis whereas tumors with a mesenchymal expression phenotype had the worst outcome. These molecular subtypes show overlap with previous classification systems that include CIN, MSI, CIMP, or *KRAS*/*BRAF*^{V600E} mutation status, but cannot be identified solely based on single mutations or epigenetic features.

Current classifiers show superficial similarities, yet important differences exist that are likely related to differences in data processing and algorithms applied to diverse patient cohorts, sample preparation methods, and gene expression platforms. To address this issue, a CRC Subtyping Consortium was established to perform a cross-comparison of subtype assignments obtained by the various approaches on a common set of tumor samples⁵⁷. This effort identified four molecular subtypes (CMS) with distinguishing features: CMS1 [MSI immune, 14%] showing hypermutation; CMS2 (canonical, 37%) that is epithelial with marked WNT and MYC signaling activation; CMS3 (metabolic, 13%) that is epithelial with metabolic dysregulation; and CMS4 (mesenchymal, 23%) showing prominent transforming growth factor (TGF)- β activation, stromal invasion and angiogenesis. While validation in independent cohorts preferably from clinical trials is needed, the CMS groups can be considered to be the most robust classification system currently available for CRC with a clear biological basis that may inform future clinical stratification and subtype-based interventions.

Translation

Prognostic biomarkers can explain some of the variability in outcomes seen among CRC patients, inform patient management and follow-up strategies, and serve as stratification factors. Predictive biomarkers can enable the selection of patients for targeted therapy and are individualizing oncologic practice. The prime example is expanded *RAS* testing to select metastatic CRC patients for anti-EGFR therapy. There remains, however, a critical need to identify additional predictive biomarkers to enhance therapeutic outcomes. Several biomarkers provide both prognostic and predictive information. One such example is dMMR/MSI-H which has been consistently associated with favorable patient survival rates^{25, 37, 44, 45}, and evidence suggests that it can predict for lack of benefit from adjuvant 5-fluorouracil^{47, 48}. While data are limited, patients with dMMR/MSI-H CRC appear to

receive benefit from standard FOLFOX as adjuvant chemotherapy as do pMMR/MSS cases^{49, 58}. A potentially practice-changing development is the therapeutic benefit from immune checkpoint inhibitors observed in patients with metastatic CRC⁵⁵. To advance the personalized care of patients with CRC, guidelines from the NCCN and a forthcoming consensus guideline authored by the several professional organizations recommend that dMMR/MSI testing be performed in all newly diagnosed CRC patients for the identification of Lynch Syndrome. In addition, it is recommended that *BRAF*^{V600} mutation analysis be performed in conjunction with dMMR/MSI testing for prognostic stratification, and that expanded *RAS* testing be performed to identify candidates for anti-EGFR therapy.

Roadblocks and limitations

Despite advances in the molecular profiling of CRCs, progress in identifying predictive markers beyond *RAS* status remains limited, with a prime example being lack of predictive biomarkers for anti-angiogenesis drugs. This issue is complicated by whether the target is the tumor *vs* the tumor microenvironment or both. CRCs are molecularly heterogeneous tumors and differences can also be found between the primary tumor and its metastases. For biomarker studies, important issues include standardization, reproducibility, and interpretation that must be ensured before use in the clinical setting. Given the limitations of single gene analysis, gene expression profiling has identified intrinsic tumor subtypes that are associated with clinical outcome^{3–7}, yet further study is needed to establish the consistency of these signatures and to perform external validation. In addition to analysis of pretreatment tumors, expression profiling at the time of treatment resistance and progression may further inform therapeutic decision-making. Recent advances in genomic technology enable comprehensive tumor characterization using whole genome or whole exome analyses which are being increasingly utilized due to evolving ease of performance and declining costs. Obstacles exist including the need for sophisticated bioinformatic analysis and the need to perform testing/analysis in a time frame that is realistic for use in the clinic.

Conclusions

Advances in the molecular characterization of CRC have led to the personalized treatment of this malignancy which is evolving at a rapid pace. In patients with metastatic CRC, expanded *RAS* testing for patients who are being considered for anti-EGFR therapy is routine and current guidelines recommend *BRAF*^{V600E} mutational analysis in conjunction with MMR testing for prognostic stratification. Universal testing of all newly diagnosed CRCs for *KRAS/BRAF* and MSI/MMR will have the added benefit of increasing the detection of patients with Lynch Syndrome. Biomarker combinations of *KRAS*, *BRAF* and MMR status with/without CIMP can identify colon cancer subtypes with distinct prognoses. Moreover, gene expression profiling can identify colon cancer subtypes that show some overlap with biomarker-based classifiers, and can enable prognostic stratification with the potential for subtype-based targeted interventions. Advances in genomic technology enable more comprehensive tumor characterization using whole genome or whole exome sequencing, yet pose new challenges for data interpretation and clinical application.

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Figure 1. Categorization of stage III colon cancers into 5 subtypes based on mutations in *BRAF^{V600E}* and *KRAS*(exon 2) and MMR status (*top*) identifies subtypes with distinct clinicopathological features and prognoses¹⁴. DFS is shown for individual molecular subtypes in an independent cohort of stage III colon cancer patients used for external validation of the subtype classifier (*bottom*). (Reprinted with permission from Sinicrope FA, et al. Molecular markers identify subtypes of stage III colon cancer associated with patient outcomes. *Gastroenterology* 2015;148:88–99.)

Fig. 2.

Proposed taxonomy of colorectal cancer reflecting significant biological differences in the gene expression-based consensus molecular subtypes (CMS). Prognostic value of CMS1 (yellow), CMS2 (blue), CMS3 (pink) and CMS4 (green) with Kaplan-Meier survival analysis is shown in the aggregated cohort for overall survival (n = 2,129) [left] and for relapse-free survival (n = 1,785) [right]. (Reprinted with permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: Nature Medicine, doi: 10.1038/nm.3967. Epub 2015 Oct 12. Guinney J, et al. The consensus molecular subtypes of colorectal cancer. Nat Med 2015;21:1350–6.)