Int. j. adv. multidisc. res. stud. 2024; 4(3):38-51

Received: 15-03-2024
Accepted: 25-04-2024

International Journal of Advanced Multidisciplinary Research and Studies

ISSN: 2583-049X

Molecular Biomarkers in Colorectal Cancer in the Era of Precision Medicine: A Review

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DOI: https://doi.org/10.62225/2583049X.2024.4.3.2738

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Abstract

Colorectal cancer (CRC) is a primary cause of death among cancer patients. This heterogeneous disease is characterized by alterations in many molecular pathways during its development. Mutations in RAS, combined with the mismatch repair gene defect are currently widely studied in clinics. Such biomarkers provide information for patient risk classification and for the selecting of the appropriate therapy alternatives. Nevertheless, likely, and robust prognostic markers that can identify "high-risk" CRC patients, who can benefit from adjuvant chemotherapy, in early stages, are currently absent. To solve this gap, genomic information has lately gained interest as a potential technique for estimating the likelihood of recurrence. However, due to several limitations of gene-based signatures, these have not yet been realistically used. In this review, we summarise the multiple molecular markers in clinical use for CRC, highlight potential indicators that might become indispensable over the next years, discuss recently discovered gene expression-based assays and emphasise the difficulties in biomarker development.

Keywords: Colorectal Cancer, Biomarkers, Molecular Markers, Early-Stage CRC, Gene Signature

Introduction

The global burden of colorectal cancer (CRC) is anticipated to increase by 60%, with 2.2 million new cases and 1.1 million deaths by 2030 [1]. This mortality can be largely due to the dissemination of the disease to other organs, with the liver being the most prevalent location of secondary metastases [2, 3]. Therefore, early therapy and surgical excision of the tumor is critical to enhance results in CRC patients. Surgical is the therapy of choice for early and locally advanced CRC. Current treatment regimens also advocate the systematic use of adjuvant therapy in CRC patients with lymph node involvement (stage III), although adjuvant treatment in stage II cancer is limited to clinically high-risk individuals and still topic of controversy. Since the MOSAIC study (Multicenter International Study of Oxaliplatin/Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer), oxaliplatin-based adjuvant chemotherapy has been the standard treatment for stage III CRC patients, and combinations of fluoropyrimidines with oxaliplatin led to improved overall survival and reduced risk of relapse in these patients [4, 5]. Fifty percent of stage III patients are cured by surgery, while 20% of patients will live due to the inclusion of adjuvant treatment and 30% will relapse within 2–3 years. Altogether, only 20% of stage III patients benefit from chemotherapy, exposing 80% of patients to useless damage [6]. In advanced disease, patients are treated with multimodality procedures including surgery and systemic treatments with chemotherapy and biologicals such as cetuximab, panitumumab and bevacizumab. Currently, CRC patient prognosis is based on clinicopathological criteria and largely focuses on the cancer stage at the time of diagnosis. The overall five-year survival rate is over 90% for stage I; it reduces to 70% for stage II, 58% for stage III, and less than 15% for stage IV [7]. Over the last couple of years, further diagnostic indicators, such as perineural invasion and poor histological differentiation, intestinal obstruction or perforation and advanced tumor stage (T4) at the time of surgery, became commonly accepted criteria to identify high-risk patients among early-stage CRCs.

Major technological developments in the field of molecular biology have led to the practical deployment of DNA testing on tumor tissue samples. The research for recognised oncogenes in colorectal tumors is a routine. The presence, or absence, of specific genes and mutations and the availability of targeted drugs have improved therapy selection and result, although this

is thus far limited to metastatic disease.

Prognostic/predictive gene panels are commercially accessible, but are not in general usage in Europe, due to their weak predictive value and lack of clear therapeutic guidelines, as well as their very expensive price. As such, the selection of the most advantageous treatment regimens for CRC patients, especially during early stages, remains a difficulty due to the paucity of appropriate markers. Prognostic markers provide treatment-independent prognostic information on patient outcomes, such as overall survival (OS) and relapse-free survival (RFS). Predictive biomarkers, on the other hand, help guide therapy decisions via treatment response information in biomarker-positive patients compared to biomarker-negative patients. In this review, we will explain the various prognostic and predictive molecular biomarkers already employed, as well as emphasising potential prospective biomarkers that might play a future role in the surveillance and treatment of CRC patients. Finally, we will highlight the key difficulties encountered in biomarker research and its translation into the clinical context.

Methods

We searched PubMed (www.ncbi.nlm.nih.gov/pubmed) for full-text articles from 2017 to May 31, 2023, using the keywords: colorectal cancer; biomarkers; molecular markers; early-stage CRC; gene signature. The full-text articles found were carefully examined. In addition, all abstracts presented at international conferences between January 2020 and October 2023 were examined.

Current biomarkers in CRC

Most CRC cancers are sporadic (70%–80%), with roughly 20% being of family origin [8]. CRC is regarded a heterogeneous disease and is known to be formed from the accumulation of genetic and epigenetic alterations [9]. The genes most often mutated in CRC patients include APC (about 80%–82% of cases), TP53 (48%–59%), KRAS (40%–50%), and PIK3CA (14%–18%) [10]. Recently, new suggestions for the detection of molecular biomarkers in CRC tumor tissues were established, in order to assist in disease prognosis, surveillance, and treatment. Some biomarkers are based on the mutational status of genes known to be critical in CRC carcinogenesis (NRAS, KRAS, BRAF) or connected with defects in the DNA mismatch repair mechanism (MMR) (Table 1). These defects are the main method by which microsatellite instability (MSI) status is determined [11].

Table 1: Current clinical used molecular biomarkers in colorectal cancer (CRC)

Status	Prevalence	Prognostic Value	Predictive Value		Ref.
			Non-Metastatic	Metastatic	
Microsatellite instability (MSI) high	overall: 15% stage II/III: 15% stage IV: 4.5%	Increased overall survival in stage II	× 5-FU monotherapy (stage II) √ FOLFOX (stage III)	√ Immuno-therapy	[11–24]
KRAS/NRAS mutation	45%	Bad prognostic (debatable in stage II)	No impact on treatment	× Anti-EGFR therapy	[25–38]
BRAF mutation	7–10%	Bad prognostic especially in the metastatic setting	Currently no impact on treatment	× Anti-EGFR therapy (conflicting data) √ Combi therapy: Anti-BRAF/Anti-EGFR	[12–30,39–43]
Benefit: √; No benefit: ×.					

Microsatellite instability

Microsatellites are little tandem repeats of DNA sequences dispersed throughout the genome. MSI status derives from a damaged DNA mismatch repair (MMR) mechanism, often caused by the inactivation of the four MMR genes (MSH2, MLH1, MSH6 and PMS2). A faulty MMR system leads to a failure in the correction of the insertion or deletion of repeating units during DNA replication, leading to a hypermutable phenotype (MSI-high is characterized by instability at two or more loci). MSI status can be determined by two separate methods—immunohistochemistry analysis (IHC) or PCR. [12]. Reduced expression of the MLH1, MSH2, MSH6, and PMS2 genes, validated by immunohistochemical analysis, characterises malignancies as MSI (microsatellite instable, also referred to as deficient MMR, dMMR) in contrast to MSS (microsatellite stable, also referred to as proficient MMR, pMMR). Alternatively, traditional PCR can be conducted to analyse microsatellite length in malignancies to normal tissue to determine aberrant microsatellite lengths observed in the tumor.

MSI tumors can be found in around 15% of all CRC patients [13]. Of the 15%, 3% are associated with Lynch syndrome, an inherited cancer syndrome associated with a genetic predisposition to CRC, also known as hereditary non-polyposis CRC (HNPCC). MSI was initially introduced as a screening method for the discovery of the Lynch syndrome [14]. The other 12% of MSI malignancies are related to spontaneous hypermethylation of the promoter of the MLH1 gene. Of note, the prevalence of MSI is stage-dependent. In stage II/III CRC, up to 15% are dMMR, whereas only 4%–5% of stage IV CRCs are dMMR [15]. MSI tumors are distinct in terms of clinical and pathological characteristics; they are more frequent in the right colon, are more typically related with a younger age and show poor differentiation with a large lymphocyte infiltrate. Overall, MSI-high patients demonstrate a better prognosis compared to MSI-low (MSS) individuals. Recently, the addition of the DNA mismatch repair status to clinicopathological variables has enhanced prognostic projections in various cancer types and particularly in CRC patients, leading to its inclusion into the NCCN and ESMO guidelines [16]. It has been stated that MSI stage II patients do not require chemotherapy, as they seem to have a better prognosis and no favourable effect of 5-FU has been shown in this subgroup [17]. However, MSI status was only kept as a meaningful prognostic marker in localized CRC as its predictive relevance is not adequately shown yet. A meta-analysis comprising of 5998 patients from 19 different studies has thrown some questions on the employment of MSI status as a defining criterion for the postoperative therapy of stage II CRC patients, since they discovered no significant link between MSI status and overall or relapse-free survival [18]. However, a very recent comprehensive meta-analysis, combining 38 trials with 12,110 patients, further verifies the predictive importance of MSI status in stage II CRC [19] and underscores the need to implement MSI screening for all resected stage II CRC patients. The MSI status is less useful in stage III patients, as the risk differences are minor between MSI-high and MSS patients [20, 21]. Interestingly, patients with MSI tumors and substantial deletions in HSP110 T17 exhibit a superior response to 5-FU-based treatment [22].

With the introduction of a new era of onco-immunology and the success of checkpoint inhibitors in various tumor forms, such as melanoma and non-small-cell lung cancer, MSI status in CRC patients has gradually become a factor of vital interest for a lot of researchers. Emerging studies suggest that tumours with MMR deficits respond better to checkpoint inhibitors [23], likely due to their higher mutational load and immune cell infiltration [24]. In 2017 the US Food and Drug Administration (FDA) approved pembrolizumab, a monoclonal anti-PD1 antibody, for therapy in MSI-high patients, independent of cancer type [25]. Additionally, Nivolumab and Ipilimumab are approved for refractory stage IV MSI-high patients [26]. MSI status is the first biomarker-only based indication for therapy, independent of the original cancer. Importantly, MSI status might become a predictive marker for stage III MSI-high patients. Indeed, given the clear advantage of checkpoint inhibitors in MSI-high metastatic patients, new trials have started to evaluate immunotherapy, as a stand-alone or in conjunction with chemotherapy, in stage III MSI-high CRC (ATOMIC study, NCT02912559).

Nevertheless, not all mCRC patients react to immunotherapy within the MSI-high patients and given the high cost of these treatments, new predictive biomarkers are urgently needed to identify intrinsic and acquired resistance. PD-L1 expression in tumors did not suggest better survival outcomes in patients treated with immunotherapy, which concerns the efficacy of PD-L1 as a predictive marker for checkpoint-inhibition therapy in mCRC [27]. Studies to develop biomarkers in this growing area of clinical research are needed.

Ras mutation

KRAS is a downstream effector of the epidermal growth factor receptor (EGFR). In CRC patients, KRAS mutations are prevalent in 45% of metastatic tumors [28] and roughly 15%–37% of early-stage and is more often seen in pMMR compared to MSI tumors. Through epidemiological cohort studies, KRAS mutations were expected to predict outcome in CRC patients [29]. This prognostic value in stage III pMMR [30, 31], but not MSI, malignancies was supported by post hoc analysis of data gathered from adjuvant clinical investigations, including studies of trials PETACC-8 and N0147. Initially, only KRAS codon 12 mutations (in particular, c.35G > T, also known as G12V), but not codon 13 mutations, were associated with decreased survival in KRAS wild-type CRC [32, 33]. More recent data now verify the bad prognosis of both exon mutations [34]. In these latter trials, a 1.5 higher incidence of recurrence and death was reported in KRAS mutant patients compared to KRAS wild-type individuals. Assessing RAS mutational status in non-metastatic CRC might aid to understanding the lack of efficacy of anti-EGFR therapy in early-stage CRC. In addition, mutations in KRAS and BRAF (for BRAF see paragraph below) relate to worse progression-free survival (PFS) and overall survival (OS) of metastatic CRC (mCRC) patients compared to with non-mutated malignancies. Five randomized trials were employed to evaluate the prognostic importance of KRAS mutations and a total of 1239 CRC patients with metastases from five randomized trials (FIRE-1, FIRE-3, AIOKRK0207, AIOKRK0604, RO91) were included in the analysis. In this meta-analysis, more frequent KRAS exon 2 variations, i.e., G12V and G12D did not have a significant influence on OS, although the KRAS G12C-variant was connected with a poorer OS when compared to the non-mutated tumors (multivariate HR 2.26 (1.25–4.1), p = 0.001). A similar trend for OS was identified in the KRAS G13D-variant (multivariate HR 1.46 (0.96–2.22), p = 0.10).

At present, most advanced patients are treated using multi-modality approaches, including surgery and systemic treatments [35]. The inclusion of an anti-EGFR drugs (cetuximab and panitumumab) to the normal chemotherapy regimen has been demonstrated to improve survival, as well as lessen the risk of cancer progression, when compared to solo chemotherapy treatment [36]. However, this benefit is confined to those who do not have mutations in downstream effectors of EGFR, such as KRAS and NRAS, due to the constitutive activation of the downstream MAPK pathway [37, 38, 39]. As activating mutations in KRAS and NRAS occur in around 40% and 7% of CRC patients, respectively [40], mutational analysis is essential prior to treatment with anti-EGFR antibodies. The mutational investigation should include KRAS and NRAS codons 12 and 13 of exon two, 59 and 61 of exon three, and 117 and 146 of exon four.

Of note, not all wild-type KRAS patients react to anti-EGFR treatment, and the likely emergence of drug resistance is a severe issue. Anti-EGFR therapy leads to the creation of KRAS, NRAS, BRAF and EGFR ectodomain mutations, which drive the MAP kinase pathway activation despite EGFR suppression. Studies in which patients were re-challenged with anti-EGFR indicated a greater overall response rate, primarily likely due to the fact that resistant clones deteriorate exponentially after drug removal [41]. Additional biomarkers are needed in order to determine the individuals within the wild-type KRAS population who are likely to respond to anti-EGFR therapy, as well as to identify those that have developed resistant, as this form of customised treatment is often rather expensive.

BRAF mutation

The BRAF gene has mutated in 10% of CRC [42]. BRAF-activating mutations most often occur in codon 600 (BRAF V600E), which accounts almost 90% of all BRAF mutations [43, 44]. This mutation is often mutually exclusive with other RAS mutations [45]. BRAF p.V600 mutational analysis is indicated in patients with pMMR-positive that demonstrate a loss of MLH1. In these patients, the BRAF p.v600 mutation excludes Lynch syndrome.

Kalady and colleagues integrated 21 trials encompassing 9885 CRC patients. They determined that BRAF-p.V600-mutated tumors are usually related with four or more positive lymph nodes, high-grade histology, MSI status, higher prevalence in females, and are often located in the right side of the colon, while wild-type tumors can be detected in any section of the colon [46]. Several retrospective investigations demonstrated that microsatellite stable (MSS) patients with BRAF mutations have more than a two times greater risk of relapse and mortality than those with wild-type BRAF [47, 48, 49, 50, 51, 52, 53, 54]. In recent investigations, the presence of BRAF mutations was observed to impair patient survival in stage III and IV (objective response rate (ORRs) <10%, with a PFS of around two months, and OS of four to six months) but not stage II CRC [55]. Although larger trials are needed, these current results do not support the assessment of BRAF status in stage I and II CRC. Additionally, there is currently no evidence that patients with BRAF-mutated tumors are less likely to benefit from standard chemotherapy drugs (irinotecan and oxaliplatin in

the MRC FOCUS Trial). Altogether, measuring the mutational status of BRAF p.V600 has until very recently (BEACON trial, see below) been exclusively a predictive marker for stage III-IV CRC, with minimal impact on therapeutic decision.

Interestingly, in most of the early BRAF investigations, MSI status was not clearly included in the analysis. It is currently known that MSI BRAF-CRC and MSS BRAF-CRC present different prognoses and outcomes, with a shorter OS and RFS in BRAF-MSS patients but no difference is documented in MSI BRAF-CRC compared to wild-type. These data strongly show that the BRAF-CRC subtype should not be classed as one entity. Along this line, the response of BRAF mutant cancers to specific anti-BRAF therapy remains constrained and varies greatly within BRAF V600E cohorts. This variation in treatment resistance might be explained by physiologically distinct subpopulations within BRAF-mutated tumors. Accordingly, Barras and colleagues have established two subgroups based on gene expression data, BM1 and BM2, which are independent of MSI status, PI3K mutation, gender and sidedness. Whereas BM1 subtype is characterized by KRAS/AKT pathway activation, mTOR/4EBP deregulation and EMT, BM2 demonstrates considerable deregulation of the cell cycle. Further dissection of the different themes of BRAF-mutated malignancies might be exploited for biomarker generation, as well as for therapeutic targeting [56]. In the future, the discovery of different subgroups of BRAF-CRC can help clinicians choose more suitable drugs, as standard treatment regimens are not sufficient for BRAF-MSS patients.

Several studies have indicated that BRAF mutations (which are RAS wild-type and may therefore benefit from anti-EGFR therapy) predict the lack of reactivity to anti-EGFR treatments in CRC [57, 58, 59, 60]. Two additional meta-analyses validated this conclusion in KRAS-wild type mCRC patients [61]: the addition of anti-EGFR antibodies in BRAF mutant mCRC patients did not lead to an improved outcome compared to the standard therapy or optimum supportive care. This concept was recently challenged by a meta-analysis performed by Rowland and colleagues. The authors concluded that there is presently inadequate information to clearly state that KRAS wild-type/BRAF-mutated metastatic malignancies respond differently to anti-EGFR therapy compared with KRAS wild-type/BRAF wild-type tumors [62]. Thus, information addressing the response of EGFR-targeting medicines in BRAF-mutant CRCs remain inconsistent.

As noted previously, BRAF inhibition approaches in metastatic BRAF-mutated patients have displayed a bad prognosis. Indeed, pre-clinical research have demonstrated that BRAF inhibition in CRC leads to the robust adaptive feedback of signaling networks, including the activation of EGFR, resulting to the restoration of MAPK signaling and supporting tumor growth [63, 64]. As a follow-up, pre-clinical investigations combining anti-EGFR and/or MEK or HER therapy with BRAF inhibitors were done and revealed promising benefits [65, 66, 67], which led to the launch of clinical trials. Recently, the phase-three-study BEACON CRC showed that patients with BRAF V600E mutated mCRC benefit from the doublet or triplet targeted combination therapy of encorafenib (a BRAF inhibitor), and cetuximab (an anti-EGFR antibody) or the latter ones combined with binimetinib (a MEK inhibitor) in a second or third line setting [68] (http://clinicaltrials.gov/show/NCT02928224). This has led to FDA approval of both the doublet and triplet therapy as a treatment for patients with advanced BRAF-V600E-mutated mCRC following up to two prior lines of therapy. This approval is a milestone, providing a chemotherapy-free targeted combination in a challenging subset of CRC patients. The extent of clinical benefit may, however, be overestimated, due to a faulty control arm of the BEACON study, which has been the focus of criticism. Further experiments are underway to test this new combinatorial approach in the first line.

Importantly, given the overlap of BRAF V600E mutations with a large frequency of MSI, checkpoint inhibitors may play a critical role in this CRC population. For all of the above-mentioned reasons, and to further shed light on the efficacy of BRAF as a biomarker, NCCN guidelines have advocated routine testing of BRAF mutational status in advanced mCRC patients.

Future biomarkers; ctDNA and Tumor Mutation Burden

In this chapter, we will give biomarkers, which show promising results and might therefore translate into the clinical scenario (please refer to Table 2). Promising approaches, such as liquid biopsy, have the ability to deliver clinically significant information [69]. Liquid biopsy refers most of all to the collection and evaluation of circulating tumor cells, cell-free nucleic acids and tumor-derived exosomal vesicles, which are released by the primary or metastatic location of the tumor into the circulation or other fluids. The identification of ctDNA can be challenging whilst ctDNA can contain up to 50% of total cell-free DNA in later metastatic stages, it can represent less than 1% or even be undetected in earlier tumor stages. The promise for liquid biopsy in guiding treatment and monitoring the condition is significant and may soon be used to clinical practice. In recent years, ctDNA has been demonstrated to be a strong tool in assessing the adequacy of surgical tumor clearance and thus the risk of recurrence, in selecting the most successful targeted therapy and in following responses to systemic treatments [70, 71]. The reappearance or increase in ctDNA, along with the introduction of new mutations, is related with recurrence, progression and resistance to therapy. Therefore, ctDNA analysis looks as a more sensitive tool for monitoring disease progression compared to standard clinical procedures. Once the mutational profile of a given patient has been established by tumor biopsy, this unique profile can be employed to follow disease development with ctDNA measurements in a personalised method.

Table 2: List of promising future biomarkers in CRC

Biomarkers	Value	Therapy Involved	Target Group	Ref.
Currently Translated into Daily Routine				
ctDNA	Predictive/prognostic	√ Targeted therapies √ Chemotherapy duration	All CRC	[72–79]
Tumor sidedness	Prognostic/predictive	× Anti-EGFR therapy (RCC) √ Intensive Chemotherapy (RCC) √ Immunotherapy (RCC) (most likely)	All CRC/mCRC	[80–83]
ALK, ROS1, NTRK1-3 fusions	Predictive	√ ALK, ROS and TRK inhibitors	All CRC ALK, ROS and NTRK fusions	[84–88]
HER2 amplification	Predictive	√ Anti-HER2 strategies × Anti-EGFR strategies	All CRC with HER2 amplification	[89,90]
Need for Further Investigation				
CMS subtyping	Prognostic	Predictive value currently studied	All CRC	[12,91–94]
Immunoscore	Prognostic	Predictive value not yet determined	All CRC	[95–100]
Markers based on the stromal compartment	Prognostic/predictive	× Adjuvant chemotherapy in rectal cancer	All stages with a focus on stage II for the prognostic value	[101–105]
Tumor mutation burden	Predictive	√ Immunotherapy	mCRC	[77–79]
CIMP	Prognostic	Predictive value not yet determined	All CRC	[106–108]
PI3K	Predictive	× Anti-EGFR therapy × First-line chemotherapy	mCRC All CRC	[40,109–111]
miR-31-3p (low expression)	Predictive	√ Anti-EGFR therapy	KRAS wt mCRC	[112–116]
Promising Biomarker Specifically Focusing on Stage II CRC				
CDX2 (low expression)	Prognostic	Predictive value not yet confirmed	Stage II CRC	[117]
MYO5B (low expression)	Prognostic	Predictive value currently studied	Stage II CRC	[118]

Benefit: √; No benefit: ×.

Several studies have demonstrated the viability of employing ctDNA to closely track patients after surgery and identify those with a high risk of recurrence. The independent prognostic value of ctDNA was established in a recent phase III experiment (IDEA-France). In this trial, patients with positive ctDNA four weeks after main surgery were associated with a poor result compared to negative ctDNA patients were given a three months adjuvant therapy [72]. In addition to different mutations, methylation markers such as SEPT9 may be utilised as surrogate indicators for the detection of residual tumor burden following surgical resection [73].

The tumor mutation load, which may be assessed by ctDNA, was recently recommended to detect responders to checkpoint blockade therapy [74, 75]. It is not yet clearly proven whether TMB is an independent prognostic factor. Additionally, the definition of TMB, as well as the method utilised to assess it, could range widely between laboratories. Recently, it has been hypothesised that combining MSI and ctDNA to TMB can increase the prediction efficiency of checkpoint inhibitors [76]. Further upcoming investigations employing larger cohorts are needed to validate these findings.

Tumor Sidedness

One of the most fascinating areas in mCRC is the impact of the primary tumor location (PTL). It has been known for decades that the colon has two different embryological origins, namely the midgut for the proximal colon (also referred to as right-sided colon) and the hindgut endoderm for the distal colon (also referred to as left-sided colon). Additionally, the two areas of the colon have varied blood supply, distinct microbiome populations and are related with various biological characteristics [77]. Although the dogma is not totally acknowledged yet among the scientific community, some studies support the concept that right-sided colon cancer (RCC) has a poorer prognosis than left-sided colon cancer (LCC) [78, 79]. Importantly, post-hoc analysis of the CRYSTAL and FIRE-3 trials, suggests a relationship between PTL and responsiveness to anti-EGFR therapy [80], since right-sided KRAS wild-type CRC did not seem to benefit from cetuximab treatment. Additional research necessary to explore PTL in order to firmly recognise it as an independent predictive biomarker for anti-EGFR therapy, especially as RCC and LCC are similarly characterized by distinct mutational landscapes. Nevertheless, based on the studies cited above and according to current guidelines, anti-EGFR therapy should be limited to left-sided KRAS wild-type CRC. LCC appears to benefit better from adjuvant chemotherapies such as 5-FU based regimes [80], whereas RCC might support more strict chemotherapy treatments in the metastatic scenario. Additionally, RCC predicts more favourable results with immunotherapies, as these tumors have a high antigenic load.

NTRK, ALK and ROS

Overall, the occurrence of gene fusion in CRC is in the range of 0.5%–2% in CRC patients; yet, their prognostic and predictive significance is far from being unravelled [81]. Promising results from the STARTRK study indicated that entrectinib, a modest drug which selectively inhibits ALK, ROS1 and TrkA-B-C, was able to elicit outstanding responses in highly pre-treated mCRC patients carrying LMNA-NTRK1 [82], CAD-ALK [83] and STRN-ALK fusions [84]. As ALK fusions have recently been revealed to be implicated in resistance to BRAF inhibitors in melanoma [85], combinatorial therapy combining ALK inhibitors with other targeted drugs might yield to some therapeutic advantage in a segment of CRC patients. Similar to the approval of pembrolizumab in MSI tumors, the FDA approved the NTRK inhibitor entrectinib in NTRK-fusion-mutated malignancies of all organ types, including CRC, provided they do not have a known acquired resistance mutation, in 2019.

HER2 overexpression

HER2 (Errb2) is a transmembrane receptor of the EGFR family, and its activation leads to cell proliferation and apoptosis suppression. HER2 overexpression is associated to ERBB2 amplification or the activation of somatic mutations and is defined in clinical practice as IHC 3+ or IHC 2+ and ISH-positive disease. Among CRC patients, the prevalence of HER2 overexpression is reported to be around 5%, with ERBB2 amplifications recorded in 5.5% [86]. Recently, HER2 garnered a lot of interest in CRC, since two recent clinical trials, MyPathway (trastuzumab and pertuzumab) and HERACLES (trastuzumab and lapatinib), suggested promise therapeutic benefit for dual HER2 blockade in patients with HER2-amplified mCRC (reviewed in [86, 87, 88]). The prognostic utility of HER2, which is more usually identified in sigmoid tumors, is still under discussion and has recently been studied in Auclin *et al*.. There is currently growing emphasis in HER2 as a prognostic marker for anti-EGFR therapy. Data from many studies suggest that acquired amplifications of ERBB2 negatively predict efficiency and are connected with the development of resistance to EGFR-targeted therapies (reviewed in [89, 90]). The implementation of HER2 screening in normal practice can provide important information for guiding therapy options.

Consensus Molecular Subtypes

Genomic information has increasingly attracted attention as a viable alternative to clinicopathological criteria for predicting the patient's chance of relapse. In contrast to breast cancer, the identification of multiple genetic subgroups of CRCs have thus far proven unsuccessful when applied as prognostic indicators. In late 2016, a major consortium of diverse groups working on CRC united their efforts and revealed four molecular subtypes based on multi gene arrays and conserved across all studied studies. These subtypes are referred to as CMS1 (MSI-immune subgroup representing 14% of CRC cases), CMS2 (canonical subgroup accounting for 37% of cases), CMS3 (metabolic subgroup representing 13% of CRC patients) and CMS4 (mesenchymal representing 23% of CRC cases). CMS subtyping usually demonstrates a link with clinical outcomes. Besides CMS categorization, Isella and colleagues have proposed five CRC intrinsic subtypes (CRIS) which are recognised by different genetic, functional and clinical features. Even when some recent pre-clinical research have underlined the clinical value of CMS and CRIS subtypes by revealing varied drug efficacy between tested subtypes [91], the therapeutic impact of the designation of these subtypes remains rather constrained.

Immune cell infiltration

It is becoming increasingly evident that the tumor microenvironment has an essential role in disease development and tumor resistance. Along this line, the infiltration of tumors by lymphocytes has been suggested as a prognostic marker [92, 93, 94]. Based on this result, Galon and associates introduced the Immunoscore classifier, which evaluates the presence of CD3+ and CD8+ cells within the tumor and invasive boundaries [95, 96]. Indeed, patients with malignancies in which these lymphocytes may be detected (which are also called "hot" tumors) demonstrate greater relapse-free survival periods than patients with cancers devoid of these immune cells ("cold" tumors). Non-infiltrated tumors can be further categorised depending on the presence of lymphocytes in the invasive margin (suggesting that immune cells can be lured by the tumor but unable to penetrate), placing the patient in an intermediate risk status. Hence, malignancies are characterised as low, middle and high Immunoscore (with low Immunoscore being non-infiltrated and placing patients at hazard). A recent worldwide analysis, done on a sample of 3539 patients, verified the grading method. Interestingly, despite most MSI tumours are invaded, the Immunoscore has been proved to be a better predictor than MSI alone. However, connecting the Immunoscore to all already known clinical data suggested just a tiny, but considerable, increase in predictivity [96]. Additionally, the efficacy of the Immunoscore in predicting response to immunotherapy medications has not yet been shown [97].

Stromal Density

The tumor-stroma percentage has been confirmed as a predictive factor in stage II and III CRC (VICTOR trial). OS and DFS were much lower in patients with a large percentage of tumor stroma [98]. In addition to the quantity of tumor stroma, its composition may be a critical determinant of cancer behavior. For example, the presence of cancer-associated fibroblasts (CAFs) effectively predicts tumor recurrence in CRC patients [99]. Recently, an immune-histochemical score based on the expression of two proteins unique for CAFs has additionally been able to predict the response to neoadjuvant treatment in rectal cancer [100]. Nevertheless, CAFs have been established to be very heterogeneous and improved markers are still needed to characterise subtypes and shed light on their potential prognostic/predictive significance in CRC [101]. The integration of stroma measurement with a functional activity assessment of CAFs might lead to an upgraded stroma-based tool for patient stratification [102].

CpG Island Methylator Phenotype (CIMP)

CpG islands are genomic locations that contain a substantial number of cytosines and guanine nucleotides, which are found in 5' regulatory promoter regions. The CpG island methylator phenotype (CIMP) has been recognized as one mechanism of CRC carcinogenesis. The methylation of CpG islands in the promoters of genes important in malignant transformation leads to the CIMP phenotype, which is present in roughly 18% of CRC patients. The frequent molecular alterations KRAS, BRAF and TP53, as well as the MSI status, are often connected with CIMP. The hypermethylation of at least three out of five pre-defined markers identifies CIMP. There are only a limited number of studies that have evaluated the prognostic importance of CIMP: two retrospective monocentric studies [103, 104] and one post hoc analysis of the CALGB 89803 prospective trial [105]. While these three studies show that CIMP+ malignancies had lower survival compared to CIMP tumors, additional research are needed, especially when CIMP status seem to overlap with BRAF mutations and MMR status. Thus, the independent prognostic importance of CIMP needs to be validated.

PI3KCA

Approximately 14%–18% of CRC patients exhibited mutations in the PI3KCA gene [106]. PIK3CA mutant hot spots are discovered at five places in exons nine and 20,

which are carcinogenic in CRC models [107]. A recent meta-analysis combining twenty-eight trials involving 12,747 patients did not suggest a large predictive value of PIK3CA mutant status in CRC [108, 109]. However, several findings demonstrate that PI3KCA mutations, specifically in exon 20, are connected to clinical resistance of anti-EGFR therapies [110] and to first-line chemotherapy [111]. It is difficult to evaluate the usefulness of PIK3CA as an independent prognostic marker as PIKC3A mutations usually co-occur with RAS or BRAF mutations. Large cohorts, including patients with mutations in PIKC3A but not in RAS or BRAF, are needed in order to shed light on the efficacy of PIKC3A as a biomarker in CRC. Therefore, routine testing for PIK3CA is currently not indicated. Importantly, PIK3CA inhibitors currently show promise in the therapy of different hematologic and breast cancer, but not in colorectal cancer.

TP53

TP53 is the most frequent somatic gene mutation in all cancer types. The mutational status of TP53 has been associated with a good response to adjuvant 5-fluorouracil therapy in stage III CRC patients [112]. Interestingly, in metastatic CRC, patients with TP53 mutations following adjuvant therapy displayed poorer survival rates [113]. More studies are need in order to discover the role of TP53 as a potential prognostic and predictive biomarker in CRC.

miRNAs

miRNAs are small, non-coding RNA molecules which play a crucial role in the regulation of intracellular processes via the post-transcriptional regulation of gene expression [114]. miRNAs are considered to be remarkable biomarkers due to their involvement in numerous physiological processes and their durability in paraffin-embedded (FFPE) tissues, which is a major criteria for the translation of biomarkers into the clinics. Recently, miR-31-3p expression has been proposed as a promising predictive biomarker for anti-EGFR therapy in KRAS wild-type patients treated with adjuvant chemotherapy. Low expression of miR-31-3p in patients treated with chemotherapy and cetuximab has been related with longer progression-free survival when compared with patients expressing high levels of miR-31-3p [115, 116, 117]. After multiple validation studies including over 850 patients from nine independent patient cohorts [118, 119], a qPCR-based diagnosis, termed miRpredX, has been developed by IntegraGen.

Biomarkers in Early-Stage CRC Patients

Surgical resection is the treatment of choice for early CRC stages. In stage II patients, the use of adjuvant chemotherapy remains exceedingly controversial, as surgical resection is often adequate to limit recurrence in most CRC patients. Therefore, treatment of stage II CRC patients with chemotherapy is often considered to be a "overtreatment" as only a subset of patients will benefit from it. Although adjuvant treatment increased overall survival of stage II CRC patients in the QUASAR trial, the absolute improvement in overall survival (OS) was limited (about 3.6% [120]. Nevertheless, up to 30% of stage II patients will relapse following surgery and many of these individuals will succumb to their condition. It is accordingly vital to identify these high-risk patients and treat them with proper medicines. Additionally, it is also necessary to identify who do not require these treatments and who can be treated with other, less onerous and costly, ways.

In recent years, a considerable amount of study has been focused on the identification of high-risk stage II patients who might benefit from adjuvant chemotherapy. Both NCCN (https://www.nccn.org) and ESMO (https://www.esmo.org) guidelines have identified several clinical factors that predict poor patient prognosis, including emergency presentation (tumor obstruction, perforation), the inadequate number of assessed lymph nodes (<12), T4 tumors, poor histological differentiation, lymphovascular invasion, perineural invasion, and the presence of positive resection margins. Three prognostic scores, MSKCC, ACCENT and Numeracy, have been established on the basis of these clinical and pathological characteristics. Additionally, as noted previously, primary tumor site seems to operate as a substantial prognostic factor, recently addressed in Gallois *et al.* [121]. However, these clinicopathological factors are insufficient and the identification of early-stage CRC patients at high risk of relapse is an unmet therapeutic need.

Tests Based on Gene Expression

Table 3: List of industrially developed or currently available commercial prognostic gene signatures for CRC

Name	Company	Genes/Gene Signature	Targeted Population (Stage)	Actual Status	Ref.
ColoPrint	Agendia	*MCTP1, LAMA3, CTSC, PYROXD1, EDEM1, IL2RB, ZNF697, SLC6A11, IL2RA, CYFIP2, PIM3, LIF, PLIN3, HSD3B1, ZBED4, PPARA, THNSL2,* and *CA438802*	II	Not FDA approved	[127,139,140]
OncotypeDX	Genomic Health	*BGN, MKI67, MYBL2, GADD45B, FAP, INHBA,* and *C-MYC*) and five reference genes (*ATP5E, GPX1, PGK1, UBB,* and *VDAC2)*	II/III	Available test Not FDA approved	[124,130,131,132,133,134,135,136]
ColDX/GeneFX	Almac/Helomics Therapeutics	634 probe set	II/III	Available test Not FDA approved	[129]
OncoDefender	Everist Genomics	*BMI1, ETV6, H3F3B, RPS10,* and *VEGFA*	I/II	Available test Not FDA approved	[128]
ColoGuideEx	inven2	*PIGR, CXCL13, MMP3, TUBA1B, SESN1, AZGP1, KLK6, EPHA7, SEMA3A, DSC3, CXCL10, ENPP3, BNIP3*	II	Unknown status	[130]
ColoGuidePro (derived from ColoGuideEx)		*OLFM4, CXCL9, DMBT1, UGT2B17, SEMA3A, NT5E,* and *WNT11*	III	Unknown status	[131]

Recently, various biomarkers based on single or multigene expression patterns have been proposed for diagnosing high-risk subgroups in early-stage CRC patients. A limited number of these molecular markers are presently commercially available for oncologists (Table 3). OncotypeDX, developed by Genomic Healthcare, uses a 12-gene test, which incorporates seven cancer-related genes and five reference genes, found by RT-PCR on formalin-fixed, paraffin-embedded tumoral tissues [122]. The second genome classifier, ColoPrint, is an 18-gene expression profile produced by Agendia, a molecular diagnostic business [123]. ColoPrint uses patient RNA profiles acquired from fresh/snap-frozen specimens or samples kept in particular preservation solutions, such as RNA later. Both genome classifiers have been found to be capable of predicting the development of distant metastasis in stage II CRC patients and to identify patients who may be safely managed without chemotherapy, independently of other clinical risk factors. Other commercial kits, such as OncoDefender [124] and GeneFX [125] (Table 3), are also available, but possess a substantially lesser market share than OncotypeDX. ColoGuideEx [126] and ColoGuidePro [127], initially published in 2012, are two predictive gene expression signatures for stage II and III CRC, respectively. However, no commercial test is currently available for these two classifiers, suggesting that the translation from a microarray-based platform to a PCR-based platform is hard for bigger multi-gene signatures. In addition, several recent investigations have highlighted the poor performance of commercially available gene classifiers, the only exception being OncotypeDX [128, 129, 130]. Recently, a big meta-analysis encompassing 2166 samples from 12 independent datasets was put up in order to assess various molecular gene signatures for their capacity to predict patient survival. While ColoGuideEx, ColoGuidePro, Oncodefender, and Coloprint did not indicate any substantial association with survival, OncotypeDX was able to significantly predict survival ($p = 6.6\times10-2$, HR = 2.05). However, when comparing the predictive performance of the kit to that of MSI status, gender, KI67, and CDX2 expression, OncotypeDX lost its independent prognostic significance. Furthermore, OncotypeDX has a number of serious limitations, notably the exorbitant cost of the test [131] and the difficult scoring process [132]. More specifically, OncotypeDX employs three categories: low, moderate, and high. This leaves clinicians with a high number of patients for whom treatment decision remains questionable, as an intermediate category can affect up to 39% of the patients [133], leading to a small degree of discrimination between the low- and high-risk groups (22% of recurrence in the high-risk group vs. 12% in the low-risk group. In addition, OncotypeDX appears to be a better predictive marker for stage III (which is usually indicated for adjuvant treatment) than for stage II. In this stage, easily available criteria such as T4 and MSI status appear as stronger predictors of recurrence [134], making the added benefit of OncotypeDX less rigorous. Therefore, OncotypeDX is unlikely to play a substantial role in determining treatment in stage II CRC patients.

Authors contributions

AC and CMP collaborated on the paper's conception and wrote the paper. AC,CMP, NB and VG reviewed the paper and approved the final version of the article to be published.

Funding

This work had no funding.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.
Settings.

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