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A comprehensive overview of promising biomarkers in stage II colorectal cancer

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Highlights

- Adjuvant chemotherapy in stage II CC patients is guided by clinicopathologic findings
- Novel biomarkers to lead to more accurate treatment decisions are an unmet clinical need
- Clinical implications of prognostic biomarkers that need to be evaluated are reviewed
- Immunoscore, ctDNA, and AI are the most promising markers, however these still require further validation

Abstract

Colon cancer (CC) has the highest incidence rate among gastrointestinal cancers and ranks the third in mortality among all cancers, which contributes to the current CC burden and constitutes a major public health issue. While therapeutic strategies for stage I, III, and IV CC are standardized, those for stage II CC remain debatable. The choice of adjuvant chemotherapy for patients with stage II CC depends on stage (pT4) and grade (high) of the disease, the presence of venous, perinervous, and/or lymphatic emboli, or the need of suboptimal surgery (tumor with initial occlusion or perforation needing emergency surgeries, <12 lymph nodes harvested). Several prognostic factors that have been validated in retrospective studies can potentially define a population of CC patients at low and high-risk for recurrence. The role of biomarkers is becoming increasingly important for the future personalized treatment options. We conducted a systematic overview of potential prognostic biomarkers with possible clinical implications in stage II CC.

Keywords: Colorectal cancer, Adjuvant chemotherapy, Artificial intelligence, Circulating Tumor DNA, Carcinoembryonic antigen, Immunoscore

Introduction

Colorectal cancer ranks as the third most common cancer worldwide [1]. Alone it accounts for approximately 10% of all diagnosed cancer (stage I or II: 10%-35%, stage III: 35%, stage IV: 20%) [2]. While the management of patients with stage I, III, and IV is standardized, therapeutic strategy for stage II colon cancer (CC) is not straightforward and remains debatable as a one-size-fits-all strategy is not suitable in this setting.

Patients with stage II CC (T3-T4N0M0) present heterogeneous group with a very different prognosis; the 5-year overall survival ranging from 87.5% in stage IIA to 58.4% in stage IIC [3]. Moreover, some patients with stage II CC may have a worse prognosis than patients with low-risk stage III CC (T1-T3N1) [3]. Pathological features that have been associated with high-risk recurrence include tumor stage (T4) and grade (high), perforated tumors, the presence of venous, perinervous, and/or lymphatic emboli, or suboptimal surgery (initial occlusion or perforation needing emergency surgeries, <12 lymph nodes harvested). If any of these risk factors is present, patients can be considered low-risk [4]. Although there are several prognostic factors described in CC, evidence regarding their aptitude for categorizing patients into high and low-risk subsets is weak and limited to retrospective studies. More recently, patients with stage II mismatch repair-deficient (dMMR) CC (approximately 15% of all CC cases) have been shown to have significantly lower CC recurrence risk [5,3,6].

Advances in the molecular characterization of CC will contribute to the better identification of stage II CC patients who are more likely to benefit from adjuvant chemotherapy and have a higher chance of cure by surgery alone. In stage II CC, standard adjuvant chemotherapy with 5-fluorouracil and leucovorin improves the 5-year survival rate by only 2-5% [7,8]. Therefore, adjuvant treatment in this setting is indicated according to tumor-related prognostic factors and balanced with patients' comorbidities and life expectancy. Over the last years, enhanced accuracy of staging in clinical trials of CC significantly decreased recurrence rates in patients

with stage II disease receiving nearly identical treatment, which reflects stage migration over time [9].

This paper aims to provide an overview of prognostic markers in stage II CC that may have clinical implications in the near future.

Methods

The conduct and reporting of this review is based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [10]. We addressed the following question using the PICO (Population, Intervention, Comparator and Outcomes) structure: “What are the promising biomarkers or prognostic factors in patients with stage II CC? Could these new factors help us to propose an adjuvant chemotherapy to those patients?”

Data Sources

A literature search was performed via the MEDLINE database. We also searched conference abstracts from the meetings of the American Society of Clinical Oncology (ASCO) and the European Society of Medical Oncology (ESMO). The literature search was limited to works published between July 2016 and July 2019 in order to highlight the emerging and relevant biomarkers that may provide a useful tool in personalized medicine. The risks of omitting promising biomarkers by choosing a 3-year window is lower than through a longer period of time and ending up with a larger set of biological, histopathological, or molecular markers that have not been validated or show contradictory results. Biomarkers that have been validated outside of our time frame, are quickly reviewed.

The search was carried out using combinations of the following keywords "colorectal cancer" AND "stage II", AND "prognostic factor". Additionally, reference lists of selected articles were reviewed to identify potentially relevant studies.

Studies selection

The inclusion criteria were as follows: (1) stage II CC (according to TNM staging), (2) potential prognostic marker, (3) studies with any of the following outcomes: overall survival (OS), disease-free survival (DFS), recurrence-free survival (RFS), or cancer-specific survival (CSS), (4) original studies and meta-analysis. The exclusion criteria were as follows: (1) studies assessing the commonly used risk factors such as tumor stage, analysis of <12 lymph nodes, venous emboli, perineural and/or lymphatic invasion, cell differentiation, absence of perforation, mismatch repair status, (2) studies involving cell lines or animal models, (3) case reports, (4) and non-English studies.

Data Extraction

Two reviewers (PP and AT) independently performed studies selection based on titles and abstracts. Relevant study characteristics (year of publication, study primary author, and trial design, prognostic factors, and outcomes) were extracted and entered into an excel sheet. Extracted data were double-checked by a third reviewer (RC). Discrepancies were resolved through discussion.

Results

Study selection

The PRISMA flow chart describing the study selection process is shown in Figure 1. There were 616 original studies and 10 records from other sources identified through our search after eliminating duplicates. After screening, irrelevant studies were excluded, leaving 159

articles (25%) for full-text review. Of these, 56 remained for the final analysis (Table S1). A total of 9 negative studies were identified.

Emerging biomarkers

Anatomical laterality

The laterality of the primary tumor has prognostic implications in patients with metastatic CC. Patients with left primary tumors have better OS compared to those with tumors arising in the right colon [11–13]. In a meta-analysis of 66 studies (included 1.437.846 patients with a median follow-up of 65 months) left-sided primary tumor location was associated with a significantly reduced risk of death (HR 0.82, 95% CI: 0.79-0.84; $P < 0.001$) [12].

The effect of laterality on prognosis is not straightforward in early-stage CC. In a prospective phase III study by Taieb et al. [14], DFS was not influenced by laterality in the whole population, but a shorter survival after relapse was observed in right-sided stage III CC patients. Retrospective studies have shown that patients with proximal stage II CC have a decreased risk of recurrence compared to those with distal stage II disease [15–20]. A pooled analysis of the MOSAIC and CALGB trials with 2.318 CC patients (1.861 stage II CC) showed that patients with right-sided tumors had a statistically significant longer DFS compared to those with left-sided tumors when treated with surgery only (HR 1.23, 95% CI: 1.04-1.47) [18]. However, laterality did not affect DFS and OS of patients who received adjuvant fluoropyrimidines. Unfortunately, the available published data do not consider the dMMR status, which may constitute a potential confounding factor as it is often associated with a good prognosis and the right-sided CC predominance.

Carcinoembryonic antigen (CEA) and 19-9 antigen carbohydrate (CA 19-9) biomarkers

The use of CEA as a prognostic factor for stage II colon cancer patients has been recently refined. CEA is a membrane-associated glycoprotein expressed mainly in the gut epithelium that is upregulated in tumor cells. It is implicated in cell adhesion and differentiation. Several studies have reported the prognostic value of CEA levels in CC. Retrospective series have shown that patients with non-metastatic CC and high preoperative CEA levels often have a poor prognosis [21–27]. For example, the 3-year OS of stage I/II CC patients not treated with adjuvant chemotherapy was 79.7% and 64.5% for those with a CEA level of < 2.35 and > 2.35 ng/mL, respectively [27]. The MOSAIC post-hoc analysis of 899 high-risk stage II CC patients who received adjuvant chemotherapy showed that those with a postoperative CEA level of > 2.35 ng/mL had shorter OS (HR 1.90, 95% CI: 1.33-2.72) and DFS (HR 1.49, 95% CI: 1.10-2.09) [21]. Another retrospective analysis including 623 CC patients (40% stage II CC) showed that those with a CEA level of ≥ 5 ng/mL (HR 5.33, 95% CI: 1.59-17.9) and between 2.5 ng/mL and 5 ng/mL (HR 3.05, 95% CI: 1.27-7.32) had an increased risk of recurrence [22].

CA 19-9 is a glycoprotein with a transmembrane protein skeleton synthesized by normal human pancreatic and biliary ductal cells and by gastric, colon, endometrial, and salivary epithelia. In clinical practice, CA19-9 represents less interesting tumor marker. A retrospective study of 384 patients with stage II CC showed that preoperative CA19-9 above the normal range was associated with a higher risk of recurrence (HR 1.96, 95% CI: 1.02-3.58) [28].

CDX2 expression

CDX2 is a transcription factor that plays an important role in colonic epithelial tissue regulation and differentiation. Loss of CDX2 expression that was found in 7%-16% CCs seems to identify a subgroup of patients with high-risk stage II disease [29,30]. Several

studies indicated that CDX2 expression is associated with a poor prognosis in this setting. In study by Pilati et al, CDX2-negative CCs (i.e. expression ≤ 6.5) represented 10.1% (8/79) of an independent cohort of GSE33113 study population (independent dataset of 90 patients with stage II CC). CDX2 expression was an independent prognostic factor (OS: HR 2.02; 95% CI: 1.27-3.23; $P = 0.003$; RFS: HR 1.73; 95% CI: 1.06-2.82; $P = 0.027$) in multivariate analysis adjusted for chemotherapy, TNM stage, gender, age, microsatellite instability (MSI) status, and tumor location, as previously reported by Dalerba et al [29]. In that retrospective analysis of the validation cohort including 265 patients with stage II or III CC, the rate of DFS was shorter among the patients whose tumors lost expression of CDX2 (HR 2.42; 95% CI: 1.36-4.29) [29]. These negative data are consistent with the findings by Hansen et al., who had reported a negative prognostic impact of CDX2 on DFS in two independent cohorts of patients with stage II CC [31]. The recent prospective single-center cohort analysis of patients with stage I-III CC by Llaverro et al. also confirmed the negative prognostic value of the loss of CDX2 protein expression ($P = 0.004$) [32]. The association of CDX2 expression with survival was also investigated by Tomasello et al. in their meta-analysis of 16 studies including 6,291 patients from all stages (85% stage II-III CC) [33]. The study showed a 50% increased risk of death when the expression of CDX2 was lost. Based on the analysis of four studies, which included exclusively stage II and III CC patients, the CDX2 expression was associated with a 70% reduction in the risk of death (HR 0.3, 95% CI: 0.12-0.77) [33].

Pathology markers

- Tumor budding (TB)

Tumor budding has been defined as a single tumor cell or a cell cluster of up to four tumor cells at the invasion front [34]. The International Tumor Budding Consensus Conference (ITBCC) guidelines with a recommendation for assessment of tumor budding in CC have

been included in the recent College of American Pathologists (CAPs) Colorectal Cancer Protocol. These are expected to allow a uniform reporting of TB data. However, the interpretation of such results may be challenging especially in the setting of intense inflammation, fibrosis, or gland fragmentation and therefore should be addressed in the future CC guidelines.

TB may be considered a surrogate for epithelial-to-mesenchymal transition (EMT). It appears that TB has a prognostic value in patients with stage II CC as those with low TB have longer 5-year DFS (89%-91% versus 52-74% in high TB tumors; $P = 0.001$) [35,36]. The importance of TB appears to be increased in patients with stage II and III dMMR tumors [37].

- Isolated tumor cells

Isolated tumor cells (ITC) are defined as tumor cells in the sinus glands of ≤ 0.2 mm. More than 50% of pN0-CC patients have ITC in transit lymph nodes and have a significantly higher risk of recurrence [38].

- Stromal tumor cells

Another biomarker currently being investigated in stage II CC is stromal tumor cells. However, its role as a prognostic factor is presently not clear. In a nationwide population-based study by Eriksen et al. prognostic value of tumor stroma ratio (TSR) and TB were evaluated [39]. Low TSR was associated with worse RFS (HR 1.34, 95% CI: 1.01-1.79) and OS (HR 1.38, 95% CI: 1.02-1.86). The authors suggested that TSR provides valuable prognostic information and adding TSR to the current risk stratification may contribute to better patient selection. TSR was scored as low when $> 50\%$ of the stroma was present. Danielsen et al. found in their study that the combination of a non-diploid and a high stromal tumor cell fraction was associated with poorer specific cancer survival (HR 2.95, 95% CI: 1.73-5.03) in stage II CC patients [40].

- Other markers

Other specific markers of EMT have been described such as loss of E-cadherin, beta-catenin, or SNAIL transcription factor expression [41]. The combined score of five immunohistochemical EMT markers within the tumor center was correlated with cancer-specific survival in stage II and III CC patients [42].

Yamamoto et al. showed in their prospective study that micrometastasis volume determined by qRT-PCR of carcinoembryonic antigen (CEA) mRNA in lymph nodes is a useful marker in identifying patients who are at high or low risk for recurrence of stage II CC [74]. High micrometastasis volume was associated with a lower 5-year DFS in multivariable analysis (risk ratio = 2.38, 95% CI: 1.31-4.34) [43].

Immune biomarkers

- Circulating immune cells

Recently described hallmarks of cancer have highlighted a role of an immune system in carcinogenesis. The neutrophil to lymphocyte ratio (NLR) is a better surrogate of the balance between pro and anti-tumoral inflammation and its prognostic significance has been extensively studied in several solid tumors. Dimitriou et al. disclosed that an elevated NLR (> 4.7) was related to a lower median OS (HR 3.62; 95% CI: 1.33-4.82) and DFS (HR 2.76; 95% CI: 1.07-7.13) in stage II CC patients (63% of the study population) [44]. The systemic inflammation preoperative score that combines the albumin levels and lymphocyte to monocyte ratio, was reported as a new prognostic indicator. Using this score, patients (244 with stage II CC) can be categorized into good (risk factor 0), intermediate (risk factor 1), and poor-risk (risk factor 2) subsets, which seems to have prognostic impact on OS [45, 46].

- Peritumoral immune infiltrate

High tumor-associated neutrophil counts appears to be associated with improved cause-specific survival and OS [47,48].

The Immunocore (IS) [49], which relies on the lower recurrence rate of patients presenting cytotoxic T cells at the peritumoral level, aims to evaluate the peritumoral immune microenvironment by assessing T lymphocytes, namely CD3+ and CD8+, at the tumor invasion margin and intra-tumor level. The IS has been internationally validated in a study of 2,681 patients (a high IS had the lowest risk of recurrence in 5 years: high HR versus low HR 0.20; $P < 0.0001$). The prognostic impact of IS on DFS, independent of age, sex, T and N stage, and MSI, was maintained in the subgroup of 1,434 stage II CC (HR 0.33; 95% CI: 0.21-0.52). The IS had the highest relative contribution to the risk of recurrence among all clinicopathological parameters [50].

In another retrospective study reported by Okada et al., which included 320 stage II CC patients, the size of locoregional lymph nodes was correlated with OS. The number of CD56-positive cells (NK-cell marker) in locoregional lymph nodes was significantly correlated with RFS (HR 0.22; 95% CI: 0.07-0.56) [51]. The majority of tumor-associated macrophages (TAMs), derived from circulating monocytes, have the "M2" phenotype, which promotes tumor growth by causing immunosuppression and angiogenesis. Shibutani et al. showed that the peripheral monocyte count was associated with the TAMs density and a high density of TAMs with a poorer prognosis [52].

~~Emerging~~ Molecular markers

Genes expression analysis

- Consensus molecular subtypes (CMS)

Colon cancer can be categorized into four main subgroups (CMS-1 to CMS-4) according to gene expression detected by transcriptomics analyses [53]. CMS-1 (genomic instability) subtype with enrichment in *BRAF* mutations, CpG island methylator phenotype, and hypermutations reflecting infiltration of immune cells is found in 14% of CC. This molecular subtype is associated with a better prognosis in the early stage while has a poor prognosis at relapse. [53]. CMS-2 (epithelial) subtype, representing 37% of patients, is characterized by WNT and MYC signaling pathways activation [54]. Patients with early-stage CMS-2 tumors (including mostly stage II CC) benefit significantly from adjuvant chemotherapy [55,56]. In CMS-3 (metabolic) subgroup, defined by a deregulation of metabolic pathways, *KRAS* mutations, and the presence of MSI, only patients with stage III seems to profit from adjuvant treatment (HR 0.16: 95% CI: 0.005-0.54) [56]. CMS-4 (mesenchymal), observed in 23% of colorectal tumors, is defined as high stromal invasion, TGF- β signaling activation, and poor-prognosis subtype [53].

An analysis of 469 stage II and III CC reported by Pilati et al. [30] suggested that the consensual CMS classification and lack of CDX2 expression could be a useful marker to identify CMS4/CDX2-negative patients with a very poor prognosis. This combination was highly prognostic for both RFS (HR 2.58: 95% CI: 1.35-4.96) and OS (HR 2.53; 95% CI: 1.29-4.99). Similar results, however without reaching statistical significance due to a lack of power, were obtained in a validation cohort of 90 stages II CC patients [30].

In daily clinical practice, tumors are not grouped according to the CMS classification due to technical difficulties. Trinh et al. [57] developed a CMS immunohistochemistry-based classifier based on five antibodies (CDX2, FRMD6, HTR2B, ZEB1, and KER) to validate the prognostic and predictive value of molecular CC subtyping. The classifier in addition to four

mismatch-repair (MMR) proteins demonstrated a 87% concordance with the gold-standard transcriptome-based classification.

Sadanandam et al. [58] identified five distinctive subtypes including goblet-like, enterocyte, stem-like, inflammatory, and transit-amplifying using a 786-gene expression signature. This classification is highly similar to that of CMS. Recently, different studies reported on how to transcriptomics can be more practical and applicable to clinical routine. Ragulan et al. [59] proposed analytical development and validation of a custom NanoString nCounter platform-based biomarker assay (NanoCRCA) for stratification of CC into subtypes to facilitate their prospective validation in clinical trials and beyond. They applied NanoCRCA to 413 fresh frozen or formalin-fixed paraffin-embedded (FFPE) samples from 355 CC patients samples and classified them into five CRCA molecular subtypes. Predicted subtypes showed the expected association with the CMS subtypes [59].

- Genomic scores

Several genomic scores have been developed to predict the risk of recurrence. The Oncotype DX Breast Recurrence Score quantifies the risk of distant recurrence after surgery in breast cancer patients. The Oncotype DX Colon Cancer 12-gene assay has been clinically validated to predict the probability of recurrence following resection of stage II and stage III CC [60-62]. In the SUNRISE study of 597 patients [63], the 12-gene Recurrence Score assay has been validated in resected stage II CC patients (n = 247) treated with or without chemotherapy and in resected stage III CC (n = 350) treated with chemotherapy. In low, intermediate, and high-risk Recurrence Score risk groups the risk of recurrence at 5 years was 9%, 14%, and 19%, respectively. Patients with stage II disease in the high-risk group had the 5-year risk of recurrence similar to that of patients with stage IIIA-IIIB disease in the low-risk group (19% versus 20%). The Recurrence Score correlated with RFS (HR 1.77; 95% CI: 1.27-2.47), DFS

(HR 1.90; 95% CI: 1.38- 2.63), and OS (HR 2.02; 95% CI: 1.35-3.01) [63]. Another score, a microarray-based ColDx gene expression score was validated using CC specimens collected as part of the phase III Alliance C9581 trial [64]. The ColDx risk score showed a recurrence-free probability of 82% at 5 years in high-risk CC patients versus 91% in low-risk CC patients ($P = 0.01$). In Low et al. study [65] a formalin-fixed and paraffin-embedded-based prognostic signature, Colomet19 (based on the mRNA expression of 19 genes) predicted an increased risk of developing metastases (67%) in early-stage, clinically-defined low-risk, microsatellite-stable CRC patients.

RAS/BRAF mutations

KRAS and *BRAF* mutations uncouple cell proliferation signalling from the epidermal growth factor receptor (EGFR) pathway. *KRAS* mutations occur in 36%-46% of stage II CC. No statistically significant association between *KRAS* mutations and patient outcomes has been observed [66–68]. In two retrospective studies by Shen et al. [67] and Li et al. [68] including 124 and 160 patients with stage II CC, respectively, no *KRAS* mutation did not have prognostic value. Unlike in these studies, a prospective cohort of 764 stage II ($n = 266$) and stage III ($n = 498$) CC patients reported by [69] showed that *KRAS* mutation was associated with poor prognosis (HR 1.99; 95% CI: 1.37-2.91). *BRAF*-mutated CC had a worse prognosis (HR 2.46; 95% CI: 1.51-4.03). Although, patients with *BRAF*/dMMR tumors may have better prognosis related to *MLH1* promoter hypermethylation. Regardless of disease stage, *BRAF V600E* mutation that is found in 7.6% of stage II CC patients [70] has been correlated with worse prognosis. Chen et al. [71] in their retrospective study of early-stage CC (123 stage I and 60 stage II patients) showed no significant association between *BRAF V600E* mutation and OS. Interestingly, in a meta-analysis of 1,035 patients with stage II and

III CC treated with adjuvant chemotherapy these with BRAF wild-type tumors had a favorable OS outcome compared to those with *BRAF V600E*-mutated tumors [72].

HER2 overexpression

HER2, a transmembrane glycoprotein that belongs to the EGFR family, is activated after dimerization and implicated in cell growth, proliferation, and differentiation. HER2 overexpression was observed in 25 of 1,941 (1.3%) stage II and III CC patients [73].

The prognostic role of HER2 is not clear with limited data available. In a retrospective analysis of 1,914 samples of stages II and II CC patients reported by Richman et al., recurrence and OS did not differ between patients with *HER2* overexpression and their non-overexpressing counterparts. No significant correlation between *HER2* overexpression and response to the 5-fluorouracil regimen was observed [73]. Contrary to this study, Feng et al. found an association between *HER2* overexpression and better OS (100% versus 69%; $P = 0.027$) and RFS (100% versus 64%; $P = 0.025$) at 5 years in stage II CC patients treated with 5-fluorouracil-based adjuvant chemotherapy compared with chemo-naïve patients. HER2 was not a prognostic factor for OS or RFS ($P = 0.92$ and $P = 0.43$, respectively), [74]. Despite the evident limitations of this study, the role of *HER2* overexpression in localized CC remains to be defined in clinical trials, although it appears to be a prognostic and resistance factor to anti-EGFR therapies in metastatic CC.

Role of microRNAs (miRNAs)

MicroRNAs are short strands of non-coding RNA that affect the regulation and post-transcriptional expression of genes. These molecules are stable and therefore particularly interesting to detect in plasma or at the tumor level given that their deregulation promotes carcinogenesis. Yamazaki et al. [75] showed in a cohort of 147 stage II CC patients that high expression of miR-181 is associated with an increased risk of recurrence (HR 7.46; 95% CI:

1.97-28.57). Two other cohort studies of patients with stage II CC observed that those with high miR-21 expression showed a significantly worse OS than patients with low miR-21 expression [76-77]. Currently, working groups are developing signatures for a panel of potentially prognostic and/or predictive miRNAs for CC chemotherapy response [78].

Circulating tumor DNA (ctDNA)

Several prospective studies have shown that ctDNA is a prognostic factor in resected stage II and III CC [32,79,80]. The assessment of a post-operative ctDNA level, either by mutation tracking or by methylated markers searching, can represent an evaluation of minimal residual disease [32,79,81]. With the increased sensitivity of current next-generation sequencing techniques (different academics or commercial tests available), ctDNA can be detected with more sensibility and specificity. However, there is still a lack of consensus in technical approaches of choice. A prospective study of 230 patients with stage II CC by Tie et al. [80] showed postoperative ctDNA in 14 of 178 (7.9%) patients, 11 of whom had an increased risk of recurrence in comparison to those without identifiable ctDNA (16 of 164 patients). Moreover, RFS was decreased in ctDNA-positive patients treated (HR 11; 95% CI: 1.8-68) or not (HR 18, 95% CI: 7.9-40) with adjuvant chemotherapy. One of the ongoing challenges is to better assess the value of ctDNA in clinical practice for stage II CC, including the detection rate and the need for adjuvant chemotherapy. In the IDEA France study [82] investigating 3 versus 6 months of oxaliplatin-based adjuvant chemotherapy for patients with stage III CC, 805 patients had blood samples analyzed for ctDNA before starting chemotherapy. Of these patients, 109 (13.5%) were ctDNA-positive. In multivariate analysis, the presence of ctDNA was as an independent prognostic marker (adjusted HR 1.85; 95% CI: 1.31-2.61; $P < 0.001$). Stage III patients (T1-3, N1) with ctDNA had a poor prognosis. Authors concluded that the 3-month regimen should be reserved for low-risk stage III patients without ctDNA and the 6-

month for other patients. In a prospective monocentric cohort of 150 patients followed for stage I-III colon cancer, Tarazona et al. [83] reported that the presence of post-operative ctDNA is prognostic and longitudinal ctDNA monitoring (during follow-up using a panel of 29 genes) increases the sensitivity and specificity of detection. ctDNA appeared detectable on average 11.5 months before the radiological diagnosis of metastatic recurrence, but it is unclear whether or not this tool might be beneficial for the patients. There might be here a window of opportunity for clinical trials for patients with biological relapse. Trials evaluating the prognostic impact of ctDNA and the effect of adjuvant chemotherapy on ctDNA-positive stage II CC patients are currently underway (DYNAMIC II [NCT03737539], IMPROVE IT [NCT03748680], and PRODIGE70-CIRCULATE [EudraCT no. 2019-000935-15]).

Other relevant molecular markers

A retrospective study of 246 early-stage CC patients (128 stage II CC) treated with surgery followed by 5-fluorouracil-based adjuvant chemotherapy by Li et al. [84] showed that the concurrent occurrence of *TP53* and *PI3KCA* mutations was associated with a shorter OS (HR 2.02; 95% CI: 1.04-3.91) in stage II-III CC compared with *PIK3CA* and *TP53* wild-type tumors.

Metastasis associated in colon cancer 1 (*MACC1*) gene is involved in cell proliferation, EMT, migration, and tumor progression in mice and is an independent prognostic indicator of metastasis occurrence in humans. In stage II CC patients, *MACC1* is associated with a decreased risk of metastasis. Moreover, patients with proficient MMR (pMMR)/*MACC1*-low tumors have a similar favorable prognosis to those with dMMR tumors [85], which highlights the potential implication for adjuvant therapy. Rohr et al. [85] evaluated the prognostic value of *MACC1* and pMMR status for recurrence risk prediction in two validation cohorts of 139

stage II CC patients. In the pooled BIOGRID datasets, 6.5% of chemotherapy-naïve patients were pMMR/MACCC1-low with no disease recurrence resulting in a 17% higher 5-year RFS versus pMMR/MACCC1-high patients ($P = 0.037$).

Role of Artificial Intelligence (AI)

Data mining from large databases may also be considered to develop algorithmic patterns based on clinical, pathological, and molecular prognostic markers. A large-scale Pan-Cancer study reported by Kleppe et al. [86] has defined and validated a novel prognostic biomarker for the automatic detection of chromatin organization called Nucleotyping using Machine-Learning technologies in 461,000 images of tumor cell nuclei in 390 patients (discovery cohort) treated for stage I or II CC. The approach is based on the rationale that the organization of chromatin can affect gene expression while its disorganization could contribute to carcinogenesis. Chromatin heterogeneity was a significant predictor of specific cancer survival in dMMR (HR 2.9; 95% CI: 1.0-8.4) and pMMR (HR 1.8; 95% CI: 1.2-2.7) stage II patients, while MSI was not a significant predictor of homogeneity (HR 1.3; 95% CI: 0.7-2.4) or heterogeneity of chromatin (HR 0.8; 95% CI: 0.3-2.0). Clinical trials are needed to assess the cost-effectiveness and the potential use of the Nucleotyping to guide therapeutic decisions in patients with stage II CC before and its applicability to clinical practice.

Recently, a clinically relevant prognostic biomarker was developed using deep learning allied to digital scanning of conventional haematoxylin and eosin stained tumor tissue sections in several cohorts of patients with resectable tumors [87]. More than 12 000 000 image tiles from patients with a distinctly good or poor disease outcome from four cohorts were used to train a total of ten convolutional neural networks, purpose-built for classifying supersized heterogeneous images. This AI biomarker stratified stage II and III patients into distinct prognostic groups that potentially could be used to guide selection of adjuvant treatment by

avoiding therapy in very low-risk groups and identifying patients who are more likely benefit from more intensive treatment regimes.

Discussion

The management of stage II CC is not straightforward and is often a matter of controversy at multidisciplinary meetings.

Patients with CC commonly receive adjuvant chemotherapy when present with high-risk features and pMMR tumors [4]. However, the MOSAIC trial [88] did not show a survival benefit of adjuvant chemotherapy with FOLFOX4 over LV5FU2 in patients with stage II CC patients and stage II CC patients with high-risk features. In the IDEA study of patients with stage II CC [89], T4 was the major prognostic factor to determine the duration of adjuvant chemotherapy.

Besides MSI/dMMR, which is predictive of the adjuvant fluoropyrimidines-based chemotherapy non-efficacy, reports on biomarkers that orient treatment decisions in CC patients are scarce (Table 1).

Historical biomarkers may be revisited such as CEA, which a threshold value to identify recurrence has been reconsidered at 2.35 ng/mL. More recent biomarkers such as ctDNA and Immunoscore have shown promising results, with the latter showing considerable efficacy for predicting the risk of recurrence in comparison to other clinicopathological parameters [50].

In the IDEA France cohort [87], low Immunoscore was associated with lower chemosensitivity in patients with high-risk stage III CC (pT4 and/or N2) versus high Immunoscore that tended to provide more benefit when patients were treated with chemotherapy. This approach still requires prospective validation in stage II CC patients. The role of other molecular tools such as genomic scores or CMS is yet to be evaluated. The combination of CMS with other poor prognostic factors such as the loss of CDX2 expression

becomes an interesting approach [30]. Similarly, AI combining radiological (from CT scans) and anatomopathological, or molecular data, could become an interesting biomarker, though necessitate validation [86-87].

Clinically relevant potential biomarkers in stage II CC are summarized in Table 2.

Prognostic biomarkers are often identified from observational data and are regularly used to identify the likelihood of a clinical event, which is mainly disease recurrence for the purpose of this review. These biomarkers can help stratify patients entering clinical trial, but are not necessarily predictive factors of adjuvant therapy, limiting their application in routine clinical practice. Particularly, the reproducibility of AI in real life and also the risk of "dispersion" with numerous AI biomarkers warrant further study.

Data reported herein are often extrapolated from studies including stage II and III CC patients with no stage-specific analyses (i.e., Tomasello et al. for CDX2, Kalberg et al. for tumor budding, Pilati et al. for CDX2/CMS, Chen et al. for BRAF). Although this raises some concerns about the actual value of these biomarkers in stage II patients, these are the only data available at this time. Our study may potentially suffer from publication bias as negative studies were probably less likely reported than positive studies. Moreover, we applied very stringent selection criterion allowing only studies published between 2016 and 2019 that might have excluded fraction of potentially promising biomarkers for stage II CC. Novel biomarkers must be investigated in relation to "historical" biomarkers in order to become part of current clinical practice. For instance, ctDNA is a costly marker with many biological and technical challenges [79], while many studies indicated postoperative CEA as a major prognostic factor [21]. The latter could be used to support decisions on inclusion in strategy trials evaluating the role of ctDNA in stage II CC. The large existing databases (IDEA, ACCENT, MOSAIC, AVANT...) make it possible to test some biomarkers, but only

retrospectively and with the probability that data on these may be missing. One approach will therefore be to design large randomized clinical trials to study novel prognostic factors and stratify them according to already known prognostic factors and to centre. Anyhow it seems mandatory to develop consensual guidelines for clinical and biological characteristics to be systematically collected during clinical trials, as it has been proposed in metastatic setting [97]. It would also be relevant to investigate the impact of the biomarker in population-based trial subgroups to define different prognostic subtypes (good prognosis, intermediate, poor prognosis) in terms of recurrence to guide adjuvant decisions. A complementary approach to define prognostic subgroups could be prognostic scores based on clinical, biological, histological, and molecular data that would permit meaningful joint interpretation of biomarkers.

Conclusion and Perspectives

Over the past few years, several biomarkers have been proposed and encouraging progress has been made in understanding the hallmarks of cancer, especially about the microenvironment and immune system. One of the most useful innovations in guiding treatment decisions includes the measurement of ctDNA to detect residual tumor cells with the subsequent choice to administer adjuvant chemotherapy. Immunoscore and AI also have the potential to guide treatment decision-making, but they require further prospective validation in order to be authorized and reimbursed by health authorities. The validation of biomarkers for clinical use requires evidence from dedicated prospective randomized clinical trials exploring the impact of ctDNA-guided adjuvant therapies with a robust methodology. Such studies are currently underway.

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TA has served in a consulting/advisory role and or received honoraria for, Amgen, Bristol-Myers Squibb, Chugai, Clovis, Halliidx, MSD Oncology, Pierre Fabre, Roche/Ventana, Sanofi, Servier and has received travel, accommodations, and expenses from Roche/Ventana, MSD Oncology, and Bristol-Myers Squibb.

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Figure 1. Flow chart representing the selection process

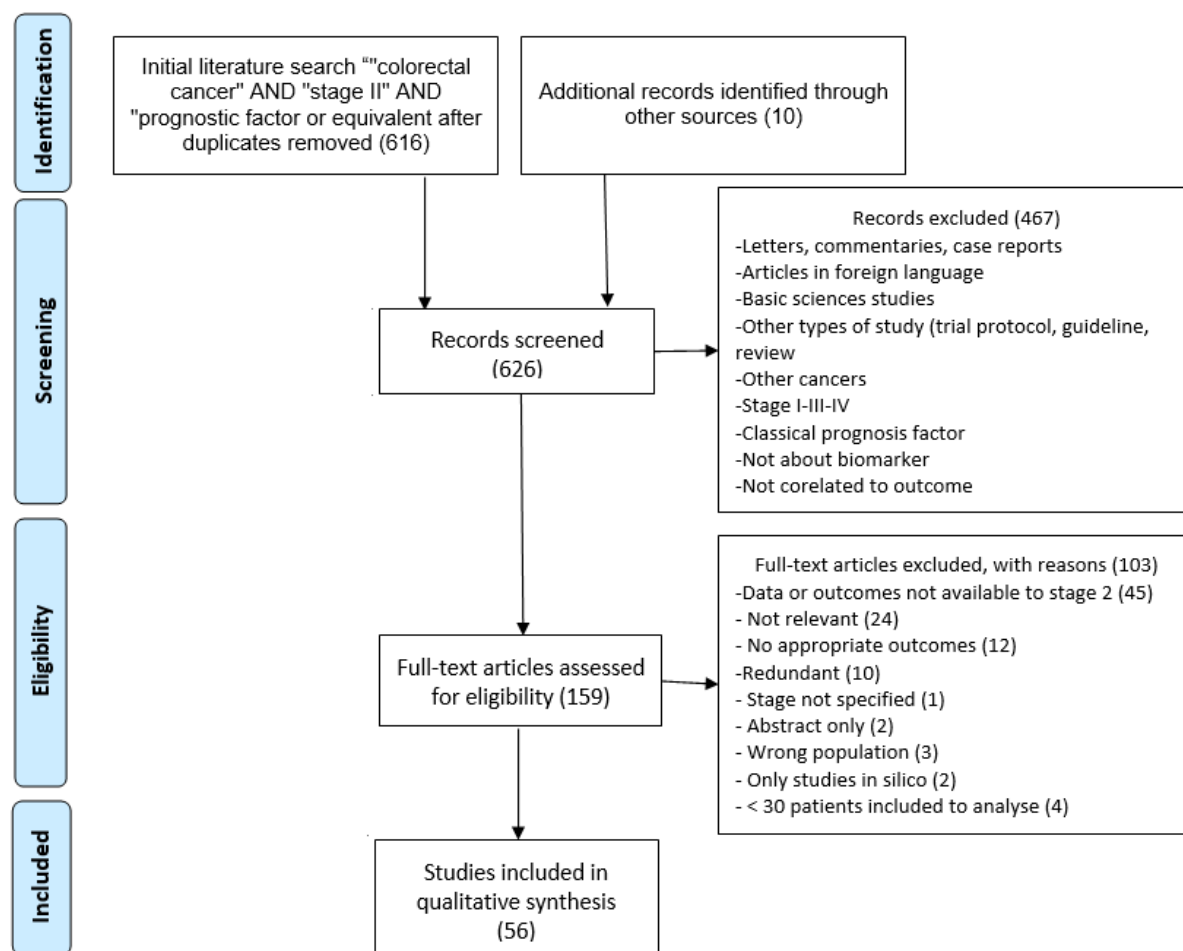


Table 1. Selected studies investigating conventional prognostic biomarkers for stage II colon cancer

Prognostic Factor	Type of study	Incidence % (N/Total)	Multivariate analysis for OS HR (95% CI)	P	Reference
T4	Retrospective	18 (35/195)	5.49 (1.06-28.43)	0.04	[90]
	Prospective	15 (223/1436)	1.87 (1.25-2.81)	0.004	[60]
	Prospective	NA	1.83 (1.23-2.75)	0.005	[91]
Poor differentiation	Retrospective	16 (29/187)	0.41 (0.04-4.52)	0.46	[90]
	Prospective	16 (NA)	2.54 (1.54-4.1)	<0.001	[92]
Perinervous invasion	Prospective	16 (50/302)	2.42 (1.52-3.84)	<0.001	[92]
	Retrospective	4 (1250/32493)	1.14 (1.06-1.23)	<0.001	[93]
Venous invasion	Prospective	14 (200/1436)	1.74 (1.11-2.73)	0.015	[92]
	Prospective	15 (70/465)			[94]
	Retrospective	11 (3704/32493)	1.32 (1.17-1.48)	<0.001	[93]
Occlusion	Retrospective	30 (57/193)	1.77 (0.7-4.47)	0.22	[90]
	Retrospective	10 (48/497)	2.47 (1.34-4.54)	0.004	[95]
Perforation	Retrospective	14 (28/195)	1.19 (0.22-6.25)	0.84	[90]
N of analyzed nodes less than 12	Prospective	35	NA		[96]
	Retrospective	7 (13/195)	3.82 (1-19-12.19)	0.02	[90]
	Prospective	NA	1.47 (1.01-2.14)	0.04	[91]
Micro-satellite instability	Prospective	NA	0.32 (0.15-0.69)	<0.001	[91]
	Prospective	15 (190/1064)	0.16 (0.04-0.64)	0.01	[5]
	Prospective	13 (181/1436)	0.36 (0.17-0.80)	0.04	[60]

Table 2. Potential prognostic biomarkers in stage II CC to be considered in future clinical trials*

Prognostic factor	Design, type of study	N stage II / N population	Multivariate analysis			Reference
			HR (95% CI)	Outcome	P	
Laterality	Retrospective cohort from clinical cancer registry	3,684 (39%) / 9509	0.85 (0.75-0.98)	Right laterality associated with OS	0.02	Brungs et al. [16]
	Retrospective study from clinical trial registry	1,861 (80%) / 2,318	1.23 (1.04-1.47)	Left laterality associated with DFS	0.015	Giryes et al. [18]
Tumor budding	Retrospective monocentric cohort	135 (100%)	3.7 (1.4-9.5)	Tumor budding grade associated with survival	<0.05	Lee et al. [35]
	Retrospective monocentric study	134 (100%)	3.31 (1.51-7.28)	Tumor budding grade associated with DFS	0.003	Du et al. [23]
Immunoscore	Retrospective multicentric international study with three cohorts (training, internal, external validation sets)	1,434 (54%)	0.47 (0.33-0.65) 0.67 (0.52-0.86)	Respectively high and intermediate Immunoscore associated OS (versus low)	0.0014 0.0001	Pagès et al. [50]
Molecular markers						
CDX2	Meta-analysis (all studies except 1 were retrospective cohorts)	NR	0.3 (0.12-0.77)	CDX2 expression associated with OS	0.01	Tomasello et al. [33]
miR-181	Retrospective cohort with training and validation set	80 (100%)	7.46 (1.97-28.57)	High miR-181 associated with RFS	0.003	Yamazaki et al. [75]
Oncotype-Dx (12 genes)	Prospective cohort with retrospective analyses	247 (41%)	2.02 (1.35-3.01)	Recurrence score per 25 units associated with OS	<0.001	Yamanaka et al. [63]
Biological markers						
ctDNA	Prospective multicentric Australian trial	230 (100%)	14 (6.8-28)	ctDNA postoperatively positive status	<0.001	Tie et al. [80]

				associated with RFS		
CEA	Retrospective cohort from clinical cancer registry	45,449 stage I + II	1.56 (1.49-1.64)	Preoperative CEA level ≥ 2.35 ng/mL associated with OS	<0.001	Margalit et al. [27]
	Retrospective analyze from the MOSAIC trial	899 (100%)	1.90 (1.33-2.72)	Postoperative CEA level ≥ 2.35 ng/mL associated with OS	<0.0001	Auclin et al. [21]
Nucleotyping	Retrospective with 1 training and 2 validation cohorts	442 (100%)	1.9 (1.2-3.2)	Chromatin heterogeneity from Gloucester cohort associated with CSS	0.026	Kleppe et al. [86]
DoMore-V1-CRC	Retrospective study with 4 different cohorts	2,272 (40%)	1.9 (1.4-2.6)	Poor prognosis of the DoMore-V1-CRC associated with CSS	<0.0001	Skrede et al. [87]

ctDNA, circulating tumoral DNA; CEA, carcinoembryonic antigen; OS, overall survival; DFS, disease-free survival; RFS, recurrence-free survival; CSS, cancer-specific survival

* Only the most frequently reported prognostic factors with the least contradictory results were considered