Microsatellite Instability in Colorectal Cancer: Overview of Its Clinical Significance and Novel Perspectives

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Abstract: Microsatellite instability (MSI) is a key biomarker in colorectal cancer (CRC), with crucial diagnostic, prognostic, and predictive implications. Testing for mismatch repair deficiency (MMR-D)/MSI is recommended during screening for Lynch syndrome, an autosomal-dominant hereditary disease that is characterized by germline mutations in the MMR genes and associated with an increased risk for several types of cancer. Additionally, MSI-high (MSI-H) status is associated with a better prognosis in early-stage CRC and a lack of benefit from adjuvant treatment with 5-fluorouracil in stage II disease. More recently, MSI has emerged as a predictor of sensitivity to immunotherapy-based treatments. The groundbreaking success of checkpoint inhibitors in MMR-D metastatic CRC has opened a new therapeutic scenario for patients with these tumors. MSI-H CRC, in both the sporadic and hereditary settings, is characterized by distinctive molecular and clinicopathologic features and represents a unique subset of CRC that is the object of growing interest and fervent research efforts. This article, an overview of the expanding role of MSI in CRC, covers its clinical significance, the available data on molecular profiling, novel perspectives on MSI testing, biomarkers in MSI-H CRC, immunotherapy resistance, and novel immunotherapy strategies.

Introduction

The role of mismatch repair deficiency (MMR-D) in colorectal cancer (CRC) has been explored since the early nineties, when evidence first linked the loss of MMR function to familial CRC.[1] Moving forward, elucidation of the molecular mechanisms of MMR-D in CRC in the hereditary and sporadic settings has led to the characterization of a subset of CRC with distinctive molecular and clinicopathologic features. This subset of CRC has been gaining increasing attention in response to the groundbreaking success of checkpoint inhibitors in MMR-D metastatic CRC (mCRC). Notably, microsatellite instability (MSI) status is one of the few biomarkers that have been approved for clinical use in CRC by regulatory authorities. Testing for MSI is

currently recommended for most patients after a diagnosis of CRC, both for hereditary syndrome screening and for the prognostic and treatment implications.[2,3]

Epidemiology, Etiology, and Clinicopathologic Features of MMR-D

MMR is a highly conserved DNA repair mechanism through which the erroneous insertion and deletion and the misincorporation of bases during DNA replication and recombination are recognized and corrected to ensure genomic integrity. The loss of activity of MMR proteins translates into an accumulation of DNA replication errors characterized by a high frequency of frameshift mutations in microsatellite DNA, referred to as microsatellite instability (MSI). MSI leads to a large somatic mutational burden in MMR-D cells, which is known as a mutator phenotype.[4,5]

Notably, the likelihood of MSI in CRC varies according to the stage of the disease, with a higher incidence in the early stages (approximately 20% in stages I and II and 12% in stage III) and a lower incidence in the metastatic setting (4%-5%). Lynch syndrome (LS), an autosomal-dominant hereditary disease characterized by germline mutations in MMR genes (ie, *MLH1*, *MSH2*, *MSH6*, *PMS2*, or *EPCAM* deletions leading to epigenetic inactivation of *MSH2*),[6] is responsible for approximately one-quarter of cases of MMR-D CRC. The remaining cases are defined as *sporadic*. The vast majority (80%-90%) of sporadic cases of MMR-D CRC result from epigenetic silencing of the *MLH1* gene promoter by hypermethylation,[7-9] a phenomenon associated with a high CpG island methylation phenotype (CIMP+).[10,11] Indeed, based on the recent consensus molecular subtypes (CMS) classification of CRCs, MSI is associated with CMS1 (MSI immune subtype), which is characterized by a CIMP+ status.[12,13] The concomitant presence of a *BRAF* V600E mutation, another common feature of CMS1, can be identified in approximately 30% of MMR-D cases and is limited to sporadic MSI.[10] The remaining sporadic cases are defined as "*double somatic*" *MSI*, characterized by multiple somatic mutations in MMR genes without identifiable germline mutations[14] and associated with a higher frequency of somatic mutations in *PIK3CA*.[15]

The MSI-high (MSI-H) phenotype is characterized by clinical and pathologic features distinct from those observed in microsatellite stable (MSS) CRC, such as poor differentiation and mucinous histology, prominent lymphocytic infiltration, right-sided colon location, and early stage at diagnosis.[16] In the metastatic setting, MSI-H CRC is more frequently diagnosed in women and the elderly, and it often presents with synchronous metastases involving the lymph nodes and peritoneum rather than the liver. Additionally, recent studies have highlighted distinct patterns characterizing sporadic and inherited cases of MSI-H mCRC, suggesting a substantial heterogeneity among these tumors based on MSI etiology.[17,18]

MSI Testing in Colorectal Cancer

MSI is currently detected with 2 different approaches: immunohistochemistry (IHC)- and polymerase chain reaction (PCR)–based methods.[3,19] IHC looks at MLH1, MSH2, MSH6, and PMS2 staining on tumor samples to identify the loss of protein expression that characterizes MMR-D as a surrogate for MSI. Molecular DNA testing through a PCR-based approach evaluates a specific panel of microsatellite markers—ie, a 5-marker panel that includes 2 mononucleotide (BAT25/26) and 3 dinucleotide markers (D2S123, D5S346, and D17S250)—to identify instability in these selected loci. Tumors are classified as MSI-H if 30% or more of the loci show instability, as MSS if none of the microsatellite markers shows instability, and as MSI-low (MSI-L) if fewer than 30% of the markers are unstable. If either MSI (PCR-based method) or MMR-D (IHC-based method) is detected, further evaluation is recommended to identify carriers of germline MMR gene mutations. Testing for the *BRAF* V600E mutation and *MLH1* promoter methylation can differentiate sporadic tumors from LS-associated ones in this setting. Both PCR-based testing and IHC are sensitive and specific for MSI detection and have a high concordance rate (>92%). Either test may be performed individually, or the 2 tests may be used in a complementary approach to increase rates of detection in those cases that may be missed by either test alone (the false-negative rate is approximately 5% to 10% for each). Of note, IHC can guide germline testing to the corresponding gene coding for the defective protein in the tumor specimen.

Here, we present an overview of the clinical value of MSI testing in CRC and evolving novel perspectives.

Hereditary Nonpolyposis Colorectal Cancer: Clinical Implications

MMR-D is the hallmark of hereditary nonpolyposis colorectal cancer, or LS.[20] This disorder is associated with a lifetime risk for CRC of up to 80% depending on the underlying germline mutation; the risk is higher for *MLH1* and *MSH2* mutation carriers and lower for those with *MSH6* and *PMS2* mutations.[21] Additionally, patients with LS are at higher risk for the development of extracolonic cancers. These extracolonic cancers include endometrial carcinoma (the second most frequent LS-related cancer, with an estimated lifetime cumulative risk of up to 60%), and cancers of the ovary, stomach, small bowel, bladder, kidney, brain, gallbladder, and biliary tract.

A timely diagnosis of LS is essential because patients need to undergo surveillance for LS-related cancers when appropriate, according to current screening guidelines. In addition, at-risk relatives should be referred for genetic counseling to identify additional carriers of germline mutations.[21] National Comprehensive Cancer Network (NCCN) guidelines recommend universal screening for all patients with a new diagnosis of CRC to maximize sensitivity in identifying individuals with LS. However, an alternate suggested option is to limit screening to individuals with CRC diagnosed before the age of 70 years, and those aged 70 years or older who meet the Bethesda guidelines.[21]

Details on LS screening and surveillance are reviewed elsewhere.[22] Distinctive features of LS-related CRCs are compared with those of sporadic MSI-H CRCs in the following sections.

Prognostic Value of MSI in Colorectal Cancer

In addition to its role in LS screening, MMR status provides important prognostic and predictive information in patients with early-stage (particularly stage II) CRC. It is widely recognized that MMR-D is associated with both a good prognosis (ie, a significantly lower risk for recurrence) and a lack of benefit from fluorouracil treatment in this setting, although data regarding the predictive value of MSI status for adjuvant chemotherapy have been controversial (further discussed in the next section).[23-27] A large analysis of data from 17 different trials in the ACCENT (Adjuvant Colon Cancer End Points) database investigated how MSI status affected outcome in patients with stage II or III CRC undergoing surgery alone or surgery followed by 5-fluorouracil (5-FU)–based adjuvant treatment. The results showed that outcomes with surgery alone were better for the patients with MSI-H tumors than for those with MSS tumors (hazard ratio [HR] for overall survival [OS] in those with stage II disease, 0.27; *P*=.01). A retrospective subgroup analysis of the adjuvant QUASAR study (Quick and Simple and Reliable) confirmed the positive prognostic value of MSI status in early-stage CRC; the recurrence rate for MMR-D tumors was half that for MMR-proficient tumors.[24] However, the significance of MSI-H status in combination with various risk factors in high-risk stage II CRC (ie, stage II CRC presenting with a T4 primary tumor, lymphovascular invasion, perineural invasion, bowel obstruction or perforation, inadequate lymph node sampling [<12 resected nodes], or close/undetermined/positive margins), particularly in the presence of multiple risk factors, has not been extensively investigated and remains unclear. Notably, the presence of a *BRAF* V600E mutation does not appear to confer a negative prognosis in early-stage MSI-H CRC, as it does in an MSS tumor.[28] In the event of disease recurrence, on the other hand, the *BRAF* V600E mutation has been associated with worse survival after relapse,[29] and distinctive recurrence patterns (ie, peritoneal and lymph node recurrence) related to MSI-H status and *BRAF* mutations have been described in these patients.[30]

Unlike in early-stage disease, no clear evidence is available regarding the prognostic value of MSI-H status in mCRC. Results from recent series suggest a lack of statistically significant differences between OS for MSS mCRCs and OS for MSI-H mCRCs, with some data showing a trend toward a worse OS for MSI-H status.[31-33] The reason underlying the difference between the prognostic value of MSI status in localized CRC and that in mCRC is unknown. Some studies suggest that the frequent association of MMR-D with *BRAF* mutations could be a confounding factor in assessing the effect of MSI-H status on survival in mCRC.[34,35] Nevertheless, the prognostic value of *BRAF* mutations in this setting is still debated. In a recent analysis by Cohen and colleagues, the survival of patients with MSI-H mCRCs carrying a *BRAF* V600E mutation was not worse than that of patients with *BRAF* wild-type tumors.[17]

Complex immune-related mechanisms appear to take part in influencing the prognosis of patients with MSI-H CRCs. For example, immune checkpoint expression has recently been reported to affect prognosis negatively by counterbalancing the positive effect of tumor-infiltrating cytotoxic T lymphocytes in these tumors.[36] Additionally, better outcomes have been reported for LS-associated CRCs than for sporadic MSI-H CRCs.[18]

Predictive Value of MSI Status in Colorectal Cancer

As mentioned earlier, MSI status has been shown to have a predictive value in terms of lack of benefit from 5-FU–based adjuvant treatment in stage II CRC.[23,24,26] Data from retrospective subgroup analyses of several studies have consistently reported a lack of efficacy of 5-FU treatment in this patient population. The most solid evidence comes from the analysis of adjuvant trials in the ACCENT database, in which no benefit and even a trend toward worse survival was observed in patients with MSI-H stage II tumors treated with adjuvant 5-FU.[26] However, in the same study, patients with stage III tumors showed a significant survival benefit from adjuvant fluoropyrimidine-based treatment regardless of MSI status. The benefit of oxaliplatin-based adjuvant chemotherapy, on the other hand, appears to be independent of MSI status, according to consistent data from several retrospective analyses, including updated results at 10 years of follow-up of the MOSAIC trial (Multicenter International Study of

Oxaliplatin/5FU-LV in the Adjuvant Treatment of Colon Cancer).[29,37,38] Interestingly, a previous retrospective study by Sinicrope and colleagues suggested that the etiology of MMR-D CRC (ie, sporadic vs germline mutation) might affect the efficacy of fluoropyrimidine in the adjuvant setting; disease-free survival with 5-FU was better in individuals with MSI-H disease due to a germline mutation than in those with sporadic MSI-H tumors.[39] To date, treatment guidelines do not recommend adjuvant chemotherapy for patients with low-risk stage II MSI-H CRC owing to their excellent prognosis and evidence of a lack of benefit from 5-FU treatment. In contrast, adjuvant treatment is recommended for patients with stage III disease regardless of MSI status, and oxaliplatin-based regimens are favored.[2]

In the metastatic setting, recent data from Tougeron and colleagues suggest a greater activity of irinotecan-based chemotherapy in MSI-H mCRC—in terms of both progression-free survival (PFS) and OS—than of oxaliplatin-based chemotherapy. In their MSI-H series, the same authors report a significant improvement in PFS and objective response rate favoring bevacizumab (Avastin, Genentech) vs anti–endothelial growth factor receptor (EGFR) agents.[40] A longer OS following bevacizumab than following cetuximab (Erbitux, Lilly) in MSI-H CRC was also observed in a subgroup analysis of data from the Cancer and Leukemia Group B (CALGB)/Southwest Oncology Group (SWOG) 80405 trial (Cetuximab and/or Bevacizumab Combined With Combination Chemotherapy in Treating Patients With Metastatic Colorectal Cancer).[31] Vascular endothelial growth factor (VEGF) is known to be involved in the mechanisms of immunomodulation in the tumor microenvironment. The combination of immunotherapy and antiangiogenic treatment is a novel strategy that is currently under study, with promising results.[41]

The prominent predictive value of MSI status in CRC, however, has recently emerged following the unprecedented results of immunotherapy with checkpoint inhibitors in MMR-D mCRC, which have opened a new era in the treatment of these tumors. The relevant checkpoint inhibitors are categorized as inhibitors of either cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) or programmed death 1/programmed death ligand 1 (PD-1/PD-L1).

Activity of the anti–PD-1 agent pembrolizumab (Keytruda, Merck) was demonstrated in 28 patients with chemorefractory MSI-H mCRC in the phase 2 KEYNOTE-016 trial (Phase 2 Study of MK-3475 in Patients With Microsatellite Unstable Tumors). In this study, pembrolizumab significantly improved the response rate, disease control rate (DCR), median PFS, and OS in the MSI-H group compared with the MSS group (response rate, 50% vs 0%; DCR, 89% vs 16%; hazard ratio [HR] for PFS, 0.135; P<.001; HR for OS, 0.247; P=.001).[42,43] Shortly after, the phase 2 CheckMate 142 trial (A Phase 2 Clinical Trial of Nivolumab, or Nivolumab Combinations, in Recurrent and Metastatic Microsatellite High and Non-MSI-H Colon Cancer) investigated the combination of the anti–CTLA-4 agent ipilimumab (Yervoy, Bristol-Myers Squibb) and the anti–PD-1 agent nivolumab (Opdivo, Bristol-Myers Squibb). This trial showed statistically significant results in the same setting. Recently updated results of this trial reported a response rate of 31% (95% CI, 20.8-42.9), with a 12-month PFS rate of 50% and a 12-month survival rate of 73% in the patients receiving nivolumab monotherapy (n=74). Results with the combination of ipilimumab plus nivolumab (n=119) were even more remarkable, with a response rate of 55% (95% CI, 45.2-63.8) and 12-month PFS and survival rates of 71% and 85%, respectively.[44,45] The toxicity profiles of both pembrolizumab and ipilimumab/nivolumab were manageable; expected immune-mediated adverse events (ie, colitis, endocrinopathies, hepatitis, nephritis, and pneumonitis) were responsive to the use of protocol-specified management algorithms. Of note, efficacy in the CheckMate 142 trial was maintained in the 13% of patients on combination therapy who discontinued treatment because of adverse events (response rate, 63%; DCR, 81%).[45] Moreover, on-treatment improvements occurred overall in key patient-reported outcomes.[45] Complete radiologic responses and long-term durable responses were observed with both pembrolizumab and ipilimumab/nivolumab, suggesting an unprecedented rate of long-term survival among heavily pretreated patients with chemorefractory mCRC. Notably, the responses in CheckMate 142 were unrelated to tumor *BRAF* mutational status, immune cell PD-L1 expression, and clinical history of LS. On the basis of these striking results, the US Food and Drug Administration granted approval for use of the checkpoint inhibitors pembrolizumab[46] and nivolumab[44] in the treatment of MSI-H or MMR-D mCRC. More recently, accelerated approval was granted to ipilimumab in combination with nivolumab for the treatment of patients with MSI-H or MMR-D mCRC and disease progression following treatment with a fluoropyrimidine, oxaliplatin, and irinotecan.[47] Thus, MSI status has become a crucial biomarker to define patients' therapeutic options in the metastatic setting. Several trials are currently investigating immune checkpoint inhibitors in various settings, including earlier lines of treatment and different combination strategies with or without chemotherapy or other targeted agents (Table). Results from these studies are eagerly awaited and are expected to change the treatment algorithm for MSI-H CRC.

The high level of activity of immune checkpoint inhibitors in patients with MSI-H disease might be explained by the high burden of somatic mutations identified in MSI-H tumors that can be recognized by

the patient's immune system. MSI-H tumors are characterized by a dense Th1 lymphocytic infiltration and a cytokine-rich environment that is associated with the highly upregulated expression of multiple immune checkpoints.[48] Not all patients with MSI-H tumors, however, respond to immunotherapy, suggesting that a deeper understanding of immune-related mechanisms in MSI-H CRC is required. Emerging mechanisms of immune escape and novel immunotherapy strategies are discussed in the following sections.

Molecular Heterogeneity of MSI-H Colorectal Cancer

Remarkable genetic diversity and molecular heterogeneity exist in CRC with MSI, a fact that has led to further subclassification and prognostic variations. Intrinsic DNA features, such as motif size and sequence, as well as tract length, are responsible for diversity in the microsatellite markers used to detect MSI.[49]

Loss of MSH2 and MLH1, major mismatch repair proteins, results in the widespread destabilization of microsatellite motifs. This leads to the MSI-H phenotype[49] with a high tumor mutational burden (TMB).[50] However, molecular heterogeneity can be found even within the MSI-H phenotype. Recent next-generation sequencing (NGS) panel studies have shown a higher TMB in MSI-H tumors with MSH2 and MSH6 comutations than in those with MLH1 or PMS2 comutations.[50] Of note, inactivation of PMS2 or MSH6 accounts for a minor proportion of MSI-H cases.[51] Additionally, some studies have shown high rates of *NTRK* gene rearrangements in MSI-H CRC, potentially highlighting a new subgroup in this category.[52]

In another classification, known as the alternative MSI (A-MSI) phenotype, different genome maintenance pathways are disrupted.[49] These include loss of MSH6, MSH3, and PMS2, resulting in a higher rate of instability at dinucleotide repeats. In vitro studies show that tumors with increased expression of DNA polymerase beta (pol-β) are expected to display A-MSI restricted to mononucleotides, whereas tumors with decreased expression of polymerase kappa (pol-κ) are expected to display A-MSI restricted to dinucleotide and tetranucleotide repeats.[49]

About 3% to 15% of patients who have CRC with MSI have an MSI-L phenotype, which is defined, as previously mentioned, as an instability at 1 microsatellite marker when the Bethesda consensus panel is used, or instability at fewer than 30% of loci if more than 5 markers are analyzed.[49] Instability in dinucleotide repeats resulting from pol-κ mutations has been used to detect the MSI-L phenotype. Some studies have shown that MSI-L CRCs have a different onset and frequency of *KRAS* mutations,[53] with different levels of loss of heterozygosity at 1p32, 8p12-22, 5q, and 18q. MSI-L tumors also display a low level of expression of O-6-methylguanine DNA methyltransferase owing to promoter hypermethylation, which is associated with both *KRAS* and *TP53* mutations.[54]

The microsatellite markers primarily used to diagnose LS are mononucleotide repeats.[49] Similar to the CRCs with other MSI classifiers, LS CRC displays significant molecular heterogeneity. For instance, LS with loss of expression of MSH2 is subclassified as the "MSH2 epimutation" phenotype.[51] Binder and colleagues[55] conducted genome-wide DNA and RNA sequencing on tumor samples from patients with LS and divided them into 2 groups on the basis of their genomic characteristics: G1 and G2. G1 LS tumors displayed higher mutation rates, a larger fraction of recurrent frameshift mutations, and greater microsatellite slippage, whereas G2 LS tumors showed weaker MSI and fewer mutations overall. Frequently mutated genes in G1 tumors were *ACVR2A*, *TGFBR2*, *CDC27*, *AIM2*, and *PDS5B*, which have also been reported in sporadic MSI-H CRC. G1 tumors uncommonly had *BRAF* mutations, in contrast to sporadic MSI-H CRCs.[17] *MLH1* mutations were present in 83% of G1 tumors and 40% of G2 tumors,[55] whereas loss of MLH1 expression through hypermethylation was more frequent in sporadic MSI-H CRCs.[17] RNA sequencing showed that the G1 tumor microenvironment had an enrichment of genes related to inflammatory processes, which were not prominent in G2 tumors, possibly contributing to the high degree of immunogenicity observed in cases of LS.[55] The immune-related signature in the G1 microenvironment was characterized by overexpression of CD3, CD4, and CD19. Furthermore, a heat map of genes in G1 tumors showed that a large number of mutations led to reduced mRNA expression, similar to that in sporadic MSI-H CRC. Interestingly, G1 tumors were also similar to *KRAS*-mutated MSS CRCs; recurrent somatic mutations of genes associated with dysregulated KRAS signaling were noted in the G1 subgroup.[55,56] Overall, the mutation spectra and microsatellite length distributions in G1 LS CRC are similar to those in sporadic MSI-H CRC, whereas the G2 LS CRC mutational profile is similar to that of sporadic MSS CRC.

Novel Perspectives on MSI in Colorectal Cancer

MSI Testing: Moving Forward to Next-Generation Sequencing and Liquid Biopsy

Novel approaches to MSI testing are being developed that incorporate high-throughput technologies and are changing the landscape of pharmacogenomics and tumor profiling. Although both IHC- and PCR-based approaches are currently considered the standard for MSI

detection, several studies have proposed other methods, based on the evaluation of NGS data, as tools for MSI assessment.[57-60] These computational tools evaluate the length of microsatellites in target loci, pairing tumor and normal-sequence data to detect somatic microsatellite changes with a high degree of sensitivity and specificity. NGS testing to guide precision oncology is currently being implemented in clinical practice, hence this approach appears to be a useful and cost-effective resource.

Another evolving scenario for the molecular profiling and dynamic monitoring of tumors is liquid biopsy. This technology has been undergoing rapid development in the last few years as a less invasive and more comprehensive method to capture the molecular heterogeneity of different tumor subclones in the same patient.[61] Additionally, seriation analysis allows early detection of the emergence of resistance to targeted therapies in the metastatic setting and thus can be used to tailor treatment.[62,63] On the basis of this evidence, increasing interest has been directed toward the possibility of detecting MSI status by means of liquid biopsy. In a small series of patients, Kasi tested use of the mutational burden in circulating cell-free tumor DNA (cfDNA) as a surrogate marker for MMR-D or MSI in patients with CRC and was able to identify a threshold for discriminating between MMR-D and MMR-proficient tumors.[64] More recently, Barzi and colleagues analyzed MSI markers in cfDNA by comparing microsatellite alterations in circulating free DNA with those in genomic DNA extracted from buffy coats from the same patient. This method effectively discriminated between MSI-H and MSS tumors. Additionally, the authors reported that patients who had MSI detected in cfDNA and received immunotherapy exhibited a decline in the relative amounts of tumor-derived microsatellite alleles in subsequent specimens and responded to immunotherapy, whereas a patient with an MSI-H primary tumor but no MSI detected in cfDNA experienced a rapid progression on immunotherapy.[65] Thus, the detection of MSI in cfDNA appears to be feasible and may reflect treatment response and disease burden. Larger and prospective studies to validate these findings are warranted.

Novel Biomarkers in MSI-H Colorectal Cancer

Understanding the mutational landscape of MSI-H CRC is crucial for discovering novel surrogate biomarkers and explaining the prognostic variations observed in patients with MSI. Several studies have identified target gene mutations in MSI CRC that have no prognostic significance or validation in larger cohorts. Among these, certain genetic mutations have shown consistent results across studies and could be used as promising prognostic biomarkers in MSI-H CRC in the future.

The protein ß2-microglobulin (B2M) is one of the few novel prognostic biomarkers in patients with MSI CRC. B2M associates with major histocompatibility complex I (MHC-I)/human leukocyte antigen I (HLA-I) on the cell surface and is crucial for antigen presentation.[66] Up to 30% of MSI CRCs display mutations in the *B2M* gene,[51] with a consecutive loss of HLA class I antigens that prevents antigen recognition and tumor cell cytotoxicity. *B2M* mutations likely evolved as a mechanism of immune evasion, providing a selection advantage for MSI tumor cells.[67] This is reflected by the significantly elevated numbers of PD-1–positive T cells observed in *B2M*-mutant MSI CRCs in comparison with *B2M* wild-type MSI CRCs.[68] Therefore, these mutations are expected to contribute to PD-1 inhibitor resistance, as already observed in patients with melanoma.[68] *B2M* mutations are also more frequent in LS-associated cancers than in sporadic MSI-H cancers.[68] This could explain why LS-associated cancers display significantly elevated immune cell infiltration compared with sporadic MSI cancers, in line with previous findings.

One of the most important aspects of using B2M as a biomarker is related to its role in predicting metastases in MSI-H CRC in a stage-dependent manner. Barrow and colleagues[67] investigated 285 patients with MSI-H CRC and found a *B2M* mutation rate of 24.2%. In this study, none of the patients who had stage II disease with a *B2M* mutation experienced a recurrence, whereas 18.2% of the patients with *B2M* wild-type tumors had a recurrence. Furthermore, Kloor and colleagues[69] studied 104 patients with MSI-H CRC and detected no *B2M* mutations in those with distant metastases. This finding was confirmed by Tikidzhieva and colleagues,[70] who studied the association in 34 patients with MSI-H CRC; they found no cases of relapse in the patients with *B2M* mutations during the 12-month follow-up period compared with a 25% relapse rate in the patients who had *B2M* wild-type tumors. Koelzer and colleagues[71] analyzed B2M expression in 98 samples of MSI-H CRC and found that none of the carriers of a *B2M* mutation had a relapse during a 5-year period, whereas 17.7% of the *B2M* wild-type carriers had a relapse. They also found that the presence of a *B2M* mutation increased the 5-year OS rate from 72.1% to 91.7%. These studies highlight the important role of B2M in the metastatic process, affecting clinical outcomes among patients with a high degree of MSI. The *B2M* mutation could serve as a vital biomarker for metastases and checkpoint inhibitor resistance in MSI-H CRC, thereby guiding treatment strategies in the future.

Loss of expression of another protein, heat shock protein 110 (HSP110), is also associated with an improved prognosis.[72] HSP110 is a molecular chaperone protein induced in cells under stressful conditions.[73] Its overexpression in malignant cells enhances their survival.[73] In CRC, HSP110 is associated with nodal metastases and an advanced stage.[72] Increased expression

of HSP110 is also associated with the infiltration of protumoral macrophages, whereas loss of HSP110 leads to tumor-suppressive macrophage infiltration.[74] Dorard and colleagues[73] identified a T17 mononucleotide repeat in intron 8 of the *HSP110* gene that was shortened in MSI CRC cell lines, leading to an aberrant *HSP110* transcript. This mutant *HSP110* transcript encoded a truncated isoform of HSP110, known as HSP110ΔE9. The mutant HSP110ΔE9 protein increased the sensitivity of MSI CRC cell lines to oxaliplatin and 5-FU.[73] Furthermore, in a recent study by Oh and colleagues,[72] increased expression of wild-type *HSP110* was associated with poor outcomes in patients with MSI-H CRC and could serve as a biomarker to stratify patients with MSI-H CRC according to prognosis. Additionally, HSP110 could be a target of immunotherapy according to the results presented by Sawada and colleagues,[75] who identified cytotoxic T cells secreting interferon-γ by detecting specific HSP110-derived epitopes in the peripheral blood of patients with CRC. Thus, the role of HSP110 as a biomarker for chemotherapy sensitivity and response to immunotherapy warrants further validation in larger trials.

Immune checkpoint ligand expression on infiltrating T cells could serve as another prognostic biomarker in CRCs with MSI. Lee and colleagues[76] investigated the prognostic significance of immune checkpoint molecules, including CD274, LAG3, and IDO1, in both tumor cells and infiltrating T cells of MSI-H CRC. CD274, LAG3, and IDO1, which are frequently upregulated in MSI-H CRC, are currently the targets of immune checkpoint inhibitors.[76] CD274 binds to its ligand PDCD1, forming a pair of negative costimulatory molecules that suppress T-cell function and mediate immune evasion. LAG3 also mediates T-cell function activation and homeostasis, leading to an immunosuppressive tumor microenvironment.[77] IDO1 induces immunosuppression through the breakdown of tryptophan in the tumor microenvironment and tumor-draining lymph nodes.[76] However, Lee and colleagues discovered that the expression of these ligands on T cells, and not on tumor cells, was related to a lower risk for recurrence after curative surgery in patients with MSI-H colon cancers. They postulate that T-cell overexpression of these ligands could contribute to adaptive resistance, in which activated T cells trigger a negative feedback mechanism in the tumor microenvironment that results in an immune equilibrium. Thus, CD274, LAG3, and IDO1 expressed on infiltrating T cells could serve as important prognostic indicators in MSI-H colon cancers.

Loss of tumor suppressor genes, such as *SMAD4*, has a pronounced effect on outcome in MSI-H CRC in comparison with MSS CRC.[51] Mutations in *SMAD4* are associated with juvenile polyposis syndrome, in which increased growth of polyps in the gastrointestinal tract leads to a higher risk for CRC.[78] SMAD4 is a downstream intracellular mediator of the transforming growth factor beta (TGF-β) superfamily signals that regulate cell growth. After activation, it enters the nucleus and upregulates the transcription of responsive genes.[51] Isaksson-Mettavainio and colleagues studied the prognostic significance of *SMAD4* in MSI-H CRCs; they found that loss of *SMAD4* was associated with shorter cancer-specific survival in patients who had MSI-H tumors (HR, 1.99; 95% CI, 1.13-3.51; $P<.003$) but was not significantly associated with survival in those who had MSS tumors,[79] even though *SMAD4* loss was more frequent in MSS tumors (31.5%) than in MSI tumors (14.1%). Thus, the frequency of mutations does not always correlate with their prognostic significance, and low-frequency mutations, such as *SMAD4* mutations, can be important prognostic biomarkers used to stratify patients with MSI.

Pathways of Immune Evasion and Novel Immunotherapy Strategies in Colorectal Cancer

The strength and timing of the immune anticancer response is modulated by a dynamic, complex set of tumor, host, and environmental factors. In this challenging scenario, the identification of novel predictive biomarkers, in addition to a deeper understanding of the mechanisms of primary and acquired resistance to immune checkpoint inhibitors, is mandatory to improve treatment outcomes, develop new actionable strategies for patients with MSI-H tumors, and potentially extend the benefit of immunotherapy to a wider population of patients.

A recent large study from Grasso and colleagues analyzed 1211 primary CRC samples, including 179 MSI-H tumors, to identify the genetic drivers of immune recognition and evasion in CRC. This multiomic analysis showed that MSI-H CRC has a high proportion of significantly mutated genes in important immune-modulating pathways and in the antigen-presenting machinery, including biallelic losses of *B2M* and *HLA* genes. Additionally, Wnt/β-catenin signaling genes were significantly mutated in all CRC subtypes, and activation of the Wnt/β-catenin signaling cascade correlated with the absence of T-cell infiltration. These data highlight the immune-editing processes that MSI-H tumors undergo, which result in genetic events that allow immune escape despite the high mutational load and frequent lymphocytic infiltration characterizing MSI-H CRC. The authors suggested that Wnt signaling inhibitors might be used to reverse immune exclusion in immunotherapy-resistant tumors.[80]

Another study, from Albacker and colleagues, identified *JAK1* frameshift mutations, leading to a loss of the JAK1-mediated interferon response, as a potential form of pancancer adaptation to the immune response against MSI tumors.[81]

Table. Main Ongoing Trials With Immune Checkpoint Inhibitors in Colorectal Cancer

Target	Agent	Phase	Setting (Treatment Line)	Treatment	Identifier
PD-1 (± other agents)	Pembrolizumab	2	First-line	Pembrolizumab + FOLFOX	NCT02375672
		3	First-line, MSI-H mCRC	Pembrolizumab vs FOLFOX or FOLFIRI (± cetuximab or bevacizumab)	NCT02563002 (KEYNOTE-177)
		2	Refractory	Pembrolizumab	NCT01876511 (KEYNOTE-016)
		2	Refractory	Pembrolizumab	NCT02460198 (KEYNOTE-164)
		2	Refractory, advanced solid tumors including MSI-H mCRC	Pembrolizumab	NCT02628067 (KEYNOTE-158)
		1/2	Refractory, *RAS/BRAF* wild-type	Pembrolizumab + cetuximab	NCT02318901
		1/2	Refractory, *RAS* wild-type	Pembrolizumab + cetuximab	NCT02713373
		2	Refractory	Pembrolizumab + radiotherapy or ablation	NCT02437071
		1b	Resectable liver mCRC	Pembrolizumab + stereotactic body radiotherapy	NCT02837263 (KEYNOTE-290)
		2	Refractory	Pembrolizumab + azacitidine	NCT02260440
		1	Refractory, advanced solid tumors including MSI-H mCRC	Pembrolizumab + itacitinib (JAK1 inhibitor)	NCT02646748
		1/2	After first-line, advanced solid tumors including MSI-H mCRC	Pembrolizumab + epacadostat (IDO inhibitor)	NCT02178722 (KEYNOTE-037)
	Nivolumab	2	Refractory, first-line (cohort C3)	Nivolumab ± other agents Cohort 1: Nivolumab Cohort 2: Nivolumab + ipilimumab (anti–CTLA-4) (escalation dose) Cohort 3: Nivolumab + ipilimumab Cohort 4: Nivolumab + ipilimumab + cobimetinib Cohort 5: Nivolumab + BMS-986016 (anti-LAG3) Cohort 6: Nivolumab + daratumumab	NCT02060188 (CheckMate 142)
		1/2	Refractory	Nivolumab + irinotecan or XELIRI	NCT02423954
		2	Refractory, MSS mCRC	Nivolumab + TAS-102	NCT02860546
		2	Refractory	Nivolumab + ipilimumab + radiotherapy	NCT03104439
		1/2	Refractory	Nivolumab + epacadostat (anti-IDO)	NCT02327078
		1/2	Refractory	Nivolumab + varlilumab (anti-CD27)	NCT02335918
		2	Stages I-III	Nivolumab + ipilimumab ± celecoxib	NCT03026140
		1/2	Neoadjuvant therapy, rectal cancer	Chemoradiotherapy followed by nivolumab before surgery	NCT02948348

(Table continues on following page)

Table. *(Continued)* Main Ongoing Trials With Immune Checkpoint Inhibitors in Colorectal Cancer

Target	Agent	Phase	Setting (Treatment Line)	Treatment	Identifier
PD-L1 (± other agents)	Atezolizumab	3	First-line, MSI-H mCRC	mFOLFOX6/bevacizumab ± atezolizumab	NCT02997228
		2	Refractory	Atezolizumab + stereotactic radiotherapy	NCT02992912
		2	Refractory	Atezolizumab + bevacizumab	NCT02982694
		3	Stage III, MSI-H CRC	FOLFOX6 ± atezolizumab	NCT02912559
	Avelumab	2	After first-line, MSI-H mCRC or polymerase ε–mutated	Avelumab	NCT03150706
			Second-line, MSI-H mCRC	Avelumab vs FOLFOX or FOLFIRI (± targeted treatment)	NCT03186326
	Durvalumab	2	Refractory, MSI-H mCRC	MEDI4736 (durvalumab)	NCT02227667
		2	Refractory	MEDI4736 + trametinib (anti–CTLA-4) vs BSC	NCT02870920
		2	Refractory, MSS mCRC	MEDI4736 + trametinib ± radiotherapy	NCT02888743
		2	Refractory, advanced solid tumors including MSS mCRC	MEDI4736 + azacitidine	NCT02811497

BSC, best supportive care; CRC, colorectal cancer; CTLA-4, cytotoxic T-lymphocyte–associated protein 4; FOLFIRI, leucovorin, 5-fluorouracil, irinotecan; FOLFOX, leucovorin, 5-fluorouracil, oxaliplatin; IDO, indoleamine-pyrrole 2,3-dioxygenase; mCRC, metastatic colorectal cancer; MSI-H, microsatellite instability high; MSS, microsatellite stable; PD-1, programmed death 1; PD-L1, programmed death ligand 1; XELIRI, capecitabine, irinotecan.

Epigenetic immunomodulation also plays a key role in tumor immune escape. Several trials are already testing the association between immune checkpoint inhibitors and epigenetic drugs—such as modifiers of histone acetylation or methylation and DNA methylation—in various cancer types, including CRC.[82]

To improve the efficacy of immunotherapy in CRC and prevent immune evasion, a strong rationale supports testing synergic combination therapies in which immune checkpoint inhibitors and other agents target various key immune regulators (eg, LAG3, IDO1, KIR, TIM3, OX-40, and Toll-like receptors), or exploiting additional strategies to increase antigenicity, enhance the immune response, target the immunosuppressive microenvironment, and overcome innate resistance in MSS tumors (reviewed by Le and colleagues[83]). Several novel immunotherapy treatment strategies are under investigation in CRC (see Table and eTable at hematologyandoncology.net). Of note, promising results have been observed with the combination of bevacizumab plus the anti–PD-L1 agent atezolizumab (Tecentriq, Genentech) in MSI-H tumors, with early data showing a disease control rate of 90%.[41] Other promising approaches include the combination of an anti-PD-1 drug with various agents or regimens. These include the following: chemotherapy, such as FOLFOX (leucovorin, 5-FU, oxaliplatin) or trifluridine/tipiracil (Lonsurf, Taiho Oncology); radiotherapy; anti-EGFR agents, such as cetuximab; epigenetic modifiers, such as azacitidine; JAK1 or phosphoinositide 3-kinase delta (PI3Kδ) inhibitors in MSI-H CRC; IDO inhibitors in MSI-H CRC; and anti–colony-stimulating factor 1 receptor (CSF1R) agents, which are active in selectively depleting myeloid-derived suppressor cells in the tumor microenvironment.[84] Finally, approaches based on modulation of the gut microbiome might be able to enhance the effects of immunotherapy in otherwise resistant tumors.[85] Results from ongoing trials will be of pivotal importance for the future development of immunotherapy in CRC.

Conclusions

MSI is a key biomarker in CRC that has crucial diagnostic (ie, LS), prognostic, and predictive implications. Therefore, testing for MSI status is critical in CRC and should be recommended for all patients with newly diagnosed CRC. The advent of immunotherapy for MSI-H mCRC has changed the therapeutic scenario for patients with these tumors and is one of the biggest practice-changing events in the treatment of mCRC, although its availability is limited to a small subgroup of patients. Because of the

low prevalence of MSI-H mCRCs, research in this setting has been limited. Future efforts should be directed toward a better characterization of these tumors to guide the development of novel treatment strategies and to dissect the mechanisms of resistance to immunotherapy, potentially extending relevant findings to improve the treatment of MSS tumors.

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Supporting Online Material for "Microsatellite Instability in Colorectal Cancer: Overview of Its Clinical Significance and Novel Perspectives"

eTable. Main Ongoing Trials With Novel Immunotherapy Strategies in Colorectal Cancer

Target/Agent	Phase	Setting (Treatment Line)	Treatment	Identifier
PD-1/PD-L1 inhibitors + novel agents	1/2	Refractory	Pembrolizumab + BBI608 (napabucasin, STAT3, and cancer cell stemness inhibitor)	NCT02851004
	1/2	Refractory	Pembrolizumab + poly ICLC (TLR3 agonist)	NCT02834052
	1/2	Refractory	Pembrolizumab + AMG820 (anti-CSF1R)	NCT02713529
	1/2	Refractory, advanced solid tumors	BMS-986015 (KIR inhibitor) + nivolumab ± ipilimumab	NCT01714739
	1	Refractory, advanced solid tumors including mCRC	Nivolumab + enadenotucirev (oncolytic virus)	NCT02636036
	1	Refractory, advanced solid tumors including mCRC	TSR-022 (TIM-3 inhibitor) ± nivolumab	NCT02817633
	1	Refractory, advanced solid tumors including mCRC	Atezolizumab + CPI-444 (adenosine-A2A receptor 2 inhibitor)	NCT02655822
	1	Refractory, advanced solid tumors	Atezolizumab + MOXR0916 (OX-40 inhibitor)	NCT02410512
	1/2	Refractory, advanced solid tumors including MSS mCRC	Durvalumab + olaparib and/or cediranib	NCT02484404
	1	Refractory	Durvalumab + pexidartinib (anti-CSF1R)	NCT02777710
	1	Refractory, advanced solid tumors	MEDI6469 (OX40 inhibitor) + durvalumab or tremelimumab	NCT02705482
Cancer vaccines, cytokines, and adoptive cell transfer	3	Stage II, resectable	Surgery ± OncoVAX	NCT02448173
	2	Stage II/III	CIK	NCT01929499
	2	Refractory	Type 1 polarized dendritic cell (αDC1) vaccine + IFN alfa-2b, rintatolimod, and celecoxib	NCT02615574
	2/3	Refractory	AlloStim + cryoablation vs physician choice	NCT01741038
	2/3	Stage III	DC-CIK + FOLFOX vs FOLFOX	NCT02415699
	2	Resectable, adjuvant	DC-CIK + chemotherapy + radiation	NCT02202928
	3	Adjuvant	Chemotherapy ± CIK	NCT02280278
	1/2	Refractory	DC-CIK + anti–PD-1 antibody	NCT02886897
	1/2	Refractory	DC-CIK and CIK + chemotherapy	NCT03047525
	2	Refractory	Tumor-infiltrating lymphocytes + pembrolizumab	NCT01174121
	1/2	Refractory, MUC1+	Anti-MUC1 CAR-pNK cells	NCT02839954
	1/2	Refractory, MUC1+	Anti-MUC1 CAR-T cells	NCT02617134

(Table continues on following page)

eTable. *(Continued)* Main Ongoing Trials With Novel Immunotherapy Strategies in Colorectal Cancer

Target/Agent	Phase	Setting (Treatment Line)	Treatment	Identifier
TLR	2	Neoadjuvant	CBLB502 (TLR5 agonist)	NCT02715882
	1/2	Resectable CRC	Surgery ± IFN, celecoxib, and rintatolimod (TLR3 agonist) before surgery	NCT01545141
	3	Maintenance, stage IV	MGN1703 (TLR9 agonist)	NCT02077868
Other	2	Neoadjuvant, rectal cancer	Galunisertib (LY2157299, TGF-β receptor inhibitor) + chemoradiation	NCT02688712
	2	Adjuvant, stage III	rhGM-CSF	NCT02466906
	1/2	Refractory	High-activity natural killer	NCT03008499
	1/2	Refractory	IMM-101 + FOLFOX	NCT03009058

CAR, chimeric antigen receptor; CIK, cytokine-induced killer cells; CRC, colorectal cancer; CSF1R, colony stimulating factor 1 receptor; DC, dendritic cell; FOLFOX, leucovorin, 5-fluorouracil, oxaliplatin; IFN, interferon; IDO, indoleamine-pyrrole 2,3-dioxygenase; KIR, killer cell immunoglobulin–like receptor; mCRC, metastatic colorectal cancer; MSS, microsatellite stable; MUC1, mucin 1; PD-1, programmed death 1; PD-L1, programmed death ligand 1; pNK, peripheral natural killer; rhGM-CSF, recombinant human granulocyte-macrophage colony–stimulating factor; TGF-β, transforming growth factor beta; TIM-3, T-cell immunoglobulin and mucin domain 3; TLR, Toll-like receptor.