

# Microsatellite Instability Pathway and EMAST in Colorectal Cancer

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**Abstract** Microsatellite instability (MSI) refers to the biochemical detection of frameshifted microsatellite sequences from genomic DNA. Genesis of MSI is due to defective DNA mismatch repair (MMR) that fails to correct post-DNA replicative slippage mistakes at microsatellites. Most of the estimated 100,000 genomic microsatellites are non-coding; however, ~150–300 microsatellites are coding such that, when frameshifted during the pathogenesis of an MSI tumor, they can generate immunogenic neopeptide antigens that limit the growth of tumor and prolong patient survival. In addition to the immune reaction and longer survival, patients with MSI colorectal cancers tend to have poorly differentiated tumors with mucinous features that are located in the right colon. Patients with MSI tumors are more resistant to 5-fluorouracil-based adjuvant chemotherapy but may be responsive to PD-1 immune checkpoint blockade. Specific defects of MMR function not only drive MSI but also elevate microsatellite alterations at selected tetranucleotide repeats that may further modify patient outcome.

**Keywords** Microsatellite instability · EMAST (elevated microsatellite alterations at selected tetranucleotide repeats) · DNA mismatch repair · Lynch syndrome · Constitutional mismatch repair deficiency syndrome · Sporadic · Familial ·

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Colorectal cancer · Lynch-like syndrome · DNA microsatellites · DNA repair · Patient outcome · Patient survival · *MLH1* hypermethylation · Mononucleotide repeats · Dinucleotide repeats · Trinucleotide repeats · Tetranucleotide repeats · MSH3

## Introduction

One of the most significant discoveries over the past two-and-a-half decades for the understanding of colorectal cancer (CRC) pathogenesis has been the identification of microsatellite instability (MSI), a biomarker from human tumor tissue [1, 2, 3•]. It is now recognized that MSI is likely one of the three pathways (the other two are chromosomal instability and the CpG island methylator phenotype, itself overlapping with MSI pathogenesis) that generates genomic driver mutations for the formation of CRC and is observed in ~15% of all CRCs [1–5]. This discovery in human cancer was made possible by investigations originally made in bacterial systems (yielding the 2015 Nobel Prize for Chemistry) that showed that DNA microsatellite sequence fidelity after DNA replication is maintained by a specific system termed DNA mismatch repair (MMR) [6•]. Once it was recognized in humans, there was rapid association with the inherited cancer syndrome we now call Lynch syndrome, which is defined by a monoallelic germline mutation in a MMR gene [1, 7•, 8]. Furthermore, defective MMR became recognized as the driver pathway in a subset of sporadic CRCs, and additional studies demonstrated different biological behavior for MSI cancers that now informs daily medical practice [1, 2, 3•, 9].

Microsatellite DNA, defined as repeated sequences of 1–6 nucleotides in a repeated contiguous fashion of typically 6 to perhaps more than 40 times (e.g., mononucleotide A<sub>n</sub>,

dinucleotide  $[CA]_n$ , trinucleotide  $[CAG]_n$ , tetranucleotide  $[AAAG]_n$ , where  $n$  represents the number of repeats of the sequence), is present ~100,000 times in the human genome [1, 2]. They are thought to be sites for homologous recombination. The vast majority of microsatellites are in non-coding DNA, with only ~150–300 within coding sequences. Measurement for frameshift of any of these microsatellite sequences might be thought of collectively as microsatellite instability, but MSI is historically defined by frameshifts at mononucleotide and dinucleotide repeats [1]. When one examines a panel of at least 5 mono- and dinucleotide microsatellite markers, the presence of 20% or greater with frameshifts (termed MSI-high) fairly accurately corresponds with a defect in MMR function, particularly from the MMR proteins MSH2 or MLH1. Inactivation of MSH2 and MLH1, the most common proteins affected in Lynch syndrome, causes complete loss of MMR function; MLH1 is also the principal protein affected in sporadic MSI colorectal cancers [1, 2, 3•]. When between 0 and 20% of markers show frameshifts, this is termed MSI-low, and the frameshift is almost exclusively in the dinucleotide markers and not mononucleotide markers [1, 10•, 11]. Although MSH6 dysfunction can generate mono- and dinucleotide frameshifts, the absence of any mononucleotide frameshifts characteristic of MSI-low is most likely associated with isolated MSH3 dysfunction [10•, 11]. Lack of any mono- or dinucleotide markers with frameshifts defines microsatellite-stable (MSS) CRCs, equating to no observable defect in MMR function [1, 10•, 12]. Measurement of frameshifts from tri- or tetranucleotide microsatellites is exclusively a result of MSH3 dysfunction and at present is termed elevated microsatellite alterations at selected tetranucleotide repeats, or EMAST [3•, 10•, 12–15].

### Generation of Microsatellite Instability: Defective DNA Mismatch Repair

Human MMR is an evolutionarily conserved DNA repair system that is maximally operative after the replication of DNA in cells and functions to accurately and faithfully repair single nucleotide base mispairs and slippage mistakes at microsatellite sequences [1, 2, 3•, 6•]. Thus, when MMR is not functional, single base mutations and MSI result [1, 2]. MMR proteins are encoded for by MMR genes, and the proteins form heterodimers to properly function. MSH2 is a key protein that partners with MSH6 as well as MSH3, and MLH1 pairs with PMS2. The fidelity for repair of DNA lies with the two MSH2 complexes: MSH2-MSH6 recognizes single base mispairs as well as slippages at mono- and dinucleotide sequences, and MSH2-MSH3 recognizes slippages at dinucleotide or longer repeats. The MLH1-PMS2 complex binds to the MSH2-MSH6 and MSH2-MSH3 complexes and signals the cell to target repair (triggering use of an exonuclease that

removes the affected bases from the newly synthesized DNA strand, followed by re-synthesis via DNA polymerase) or demise (if the DNA damage is overwhelming, thus maintaining the fidelity of DNA by preventing an inaccurate and changed genome for daughter cells) [1, 2, 3•]. Loss of MMR function through mutation or inactivation of any of the MMR genes or proteins can be detected by MSI assays and depends on which MMR gene/protein is affected. Figure 1 demonstrates the type of MSI that results with each dysfunctional MMR complex. For instance, MSH2, MLH1, and PMS2 defects show total absence of MMR at any of the microsatellite sequences, whereas MSH6 or MSH3 defects are more specific, with MSH6 dysfunction at mono- and dinucleotide sequences (as well as at single base pairs) and MSH3 defective at dinucleotide or longer repeats [1, 2, 3•, 10•, 13].

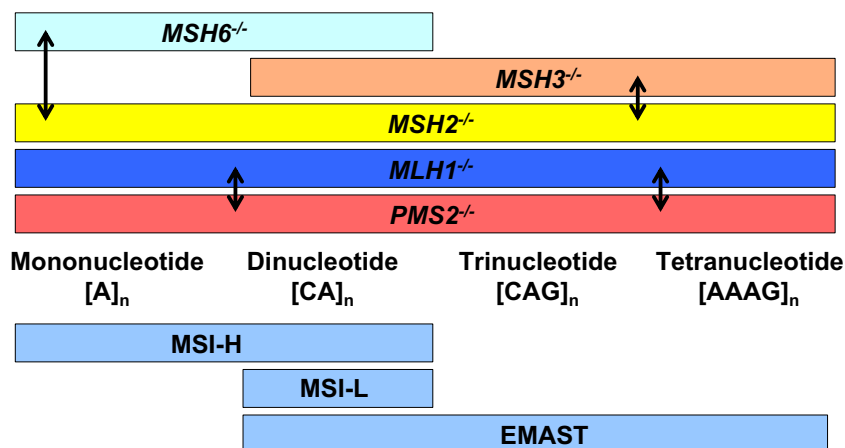
With the understanding of the fidelity of the two MSH2 recognition complexes and the function of MMR, one can infer which MMR protein complex is dysfunctional based on the type of MSI detected biochemically. MSI-low, detected by generally 1 dinucleotide frameshift out of five mono- and dinucleotide markers, is consistent with MSH3 dysfunction in the absence of any mononucleotide marker frameshifts; this hypothesis has been experimentally confirmed [3•, 10•, 11, 13, 14]. EMAST detection using tetranucleotide markers is consistent with isolated MSH3 dysfunction [3•, 6•, 11, 13, 14, 16–18]. The presence of both mono- and dinucleotide frameshifts can be attributed to MSH2, MLH1, PMS2, or MSH6 dysfunction [10•]. Dysfunction of MSH2, MLH1, and PMS2 not only causes tri- and tetranucleotide frameshifts, like those seen with MSH3 dysfunction, but also shows mononucleotide frameshifts (Fig. 1). The best way to detect which MMR protein complex is not functioning is to perform MSI analysis using mono-, di-, and tetranucleotide markers; however, most commercial entities only assay mononucleotide or mono- and dinucleotide markers.

The MMR complexes are heterodimers of two MMR proteins that keep each individual protein stable from decay [1, 2, 3•, 19]. With *MSH2* mutated, for instance, its partners MSH6 and MSH3 become unstable since its binding partner is not present. However, if *MSH6* alone is mutated, for instance, MSH2 can still partner with MSH3 and thus MSH2 remains stable. If *MLH1* is not transcribed as a full protein, PMS2 becomes unstable. MLH1 can also bind to one or more other MMR proteins that are not thought to function in pre-mitotic MMR; thus, *PMS2* mutation may not cause loss of MLH1. These MMR protein patterns can be seen by immunohistochemistry of CRCs to help determine which MMR protein is not expressed.

### MSI Presence in Human Colorectal Cancer

Microsatellite instability (MSI) is generated when MMR function is compromised. There are five human conditions

**Fig. 1** Spectrum of MMR gene and protein defects and category of frameshift microsatellite mutation to define MSI-H, MSI-L, and EMAST



described to date in which MMR is dysfunctional: two germline causes and three somatic causes (Table 1) [6•, 7•, 8, 20•]. Among germline causes, autosomal dominant transmission of a monoallelic germline mutation in a MMR gene causes Lynch syndrome, the most common inherited form of CRC, and found in 3% of all CRC patients based on population studies [6•, 7•, 20•]. Lynch families may demonstrate CRC and other gastrointestinal tract cancers such as gastric tumors, biliary tract cancers, cancers of the female reproductive tract (particularly endometrial and ovarian cancers), cancers of the urinary tract, specific skin tumors, and glioblastomas. These patients often present 10–30 years younger than sporadic CRC patients. Lynch families with *MSH6* or *PMS2* mutations tend to present at later ages (50s to 60s) as compared to families with *MSH2* or *MLH1* mutations that are more common (40s) [7•, 20•]. Germline mutation of *MSH3* as a cause of Lynch syndrome has yet to be described; such patients likely have a presentation phenotype indistinguishable from sporadic CRC patients [10•]. Another germline cause for MSI is the autosomal recessive inheritance of the constitutional mismatch repair deficiency (CMMRD) syndrome (Table 1). These very rare patients inherit biallelic germline MMR gene mutations, one from each parent, to cause a virulent and early presentation of CRC associated with café au lait spots, typically <10 years of age [6•, 21, 22]. Most commonly, patients carry biallelic *PMS2* or *MSH6* mutations, as their Lynch syndrome parents may have childbearing prior to knowledge of carrying a mutation. Biallelic *MSH3* mutations have been described; consistent with the mild (or null) presentation of patients with sporadic monoallelic *MSH3* mutation, patients with biallelic *MSH3* germline mutation present after age 30 years—much later in age than CMMRD caused by *PMS2* or *MSH6* biallelic mutations—and demonstrate colonic adenomatous polyposis [23•]. Lynch CRCs and some adenomas demonstrate MSI, whereas in CMMRD both tumors and normal tissue demonstrate MSI

[6•, 21, 22]. Those CMMRD patients with biallelic *MSH3* germline mutations show EMAST and lack mononucleotide frameshifts [23•].

Somatic MMR dysfunction is seen in (a) sporadic MSI CRCs; (b) double somatic mutation of MMR genes, sometime referred to Lynch-like syndrome; and (c) EMAST CRCs. Sporadic MSI CRCs, about 12–15% of all CRCs, demonstrate biallelic hypermethylation of the *MLH1* gene promoter, preventing the transcription of *MLH1* [1, 2, 3•, 24–28]. This completely inactivates MMR function to manifest MSI. Sporadic MSI CRCs may show mutations in the oncogene *BRAF* in ~25% of cases, distinguishing themselves from Lynch CRCs which lack *BRAF* mutations (Table 1) [3•, 7•, 8, 9]. Sporadic MSI CRC patients more often, but not exclusively, present past 70 years of age and trend towards female gender [2, 3•, 29]. Lynch-like patients present on average in their 50s, younger than sporadic MSI CRC patients but older than Lynch syndrome patients. These patients lack a germline MMR mutation, but the CRCs show two somatic hits to any of the MMR genes, typically by mutation in one allele and loss of heterozygosity of the non-mutated MMR gene allele, and may be seen in ~1% of all CRCs [3•, 8, 30•, 31]. EMAST CRCs are driven by any pathogenic pathway (MSI, chromosomal instability, or CpG island methylator phenotype) [3•, 6•, 9, 10•, 12, 17, 18]. Best studied in MSS CRC patients (eliminating the effects of total complete loss of MMR function) [6•, 10•, 16, 32•, 33•], EMAST CRCs demonstrate isolated loss of *MSH3* function, not by mutation or epimutation, but by change in *MSH3* cellular location shifting from the nucleus (where MMR acts to survey and repair DNA) to the cytosol [13, 34•]. Loss of *MSH3* from the nucleus allows genomic frameshifts at tetranucleotide sequences to be detected [13, 34•]. EMAST CRCs are the most common demonstration of loss of MMR among all CRCs, occurring in ~50% of all tumors (Table 1). The trigger for the nuclear-to-cytosol shift appears to be inflammation and oxidative stress and, in particular, interleukin-6 signaling [3•, 13, 34•].

**Table 1** Human hereditary and sporadic conditions that manifest MSI

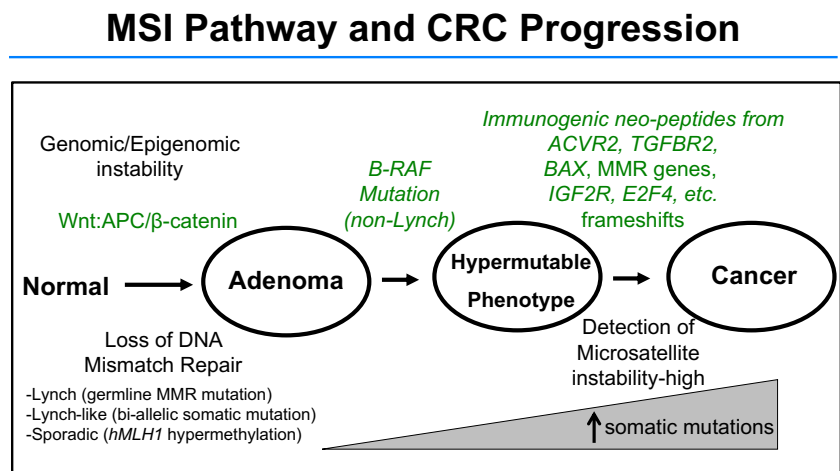
	Hereditary conditions		Sporadic conditions		
	Lynch syndrome	Constitutional mismatch repair deficiency (CMMRD)	Lynch-like syndrome	Sporadic MSI CRC and sessile serrated polyps	EMAST
Approximate percent in the CRC population	3%	Extremely rare	1%	12–15%	50%
Germline mutation	One allele of a MMR gene: <i>MSH2</i> , <i>MLH1</i> , <i>MSH6</i> , <i>PMS2</i> , <i>EPCAM</i>	Both alleles of a MMR gene: <i>MSH2</i> , <i>MLH1</i> , <i>MSH6</i> , <i>PMS2</i> , <i>MSH3</i>	None	None	None
Somatic mutation	2nd allele of MMR gene	None	Both alleles of a MMR gene	<i>BRAF</i>	None
Tumor MMR phenotype	MMR deficient (MSI)	MMR deficient (MSI)	MMR deficient (MSI)	MMR deficient (MSI)	MMR deficient (EMAST)
Epigenetic	Germline deletion in 3' end of <i>EPCAM</i> leads to somatic allele-specific <i>MSH2</i> methylation in tissues	None reported	None reported	Somatic biallelic promoter methylation for <i>MLH1</i>	None
MMR protein subcellular localization	Nucleus	Nucleus	Nucleus	Nucleus	Cytosol ( <i>MSH3</i> only)

## MSI the Biomarker

MSI-H CRCs are a result of loss of function of MMR (particularly *MSH2*, *MLH1*, *MSH6*, and *PMS2* proteins). The presence of MSI-H in a CRC is associated with certain histological and patient characteristics. MSI-H CRCs tend to be poorly differentiated and demonstrate mucin production and subepithelial lymphoid aggregates (sometimes referred to as a “Crohn’s-like reaction” as it simulates the non-caseating granuloma formation in this disease) [1, 2, 3•, 35]. Approximately 70% of all MSI-H CRCs are located in the right colon (transverse, ascending, and cecum portions) [1, 2, 3•, 6•, 7•, 35]. Overall survival is higher in patients with MSI-H CRCs as compared to patients with MSS CRCs [1, 2, 3•, 36, 37, 38•].

MSI-H CRCs are hypermutated, meaning that the cancer has accumulated hundreds to a thousand somatic mutations in its genome, as compared to MSS CRCs, which accumulate only tens of mutations [3•, 4]. As expected from a defect in MMR, there are single base mutations as well as frameshift mutations in the cancer genome [3•, 4]. Whether a patient inherits a germline mutation in a MMR gene, acquires methylation of the *MLH1* promoter, or acquires two somatic MMR gene hits in the CRC, a colonic crypt stem cell must be affected to propagate the defect, coupled with disruption of Wnt signaling to generate an adenoma (Fig. 2) [1, 2, 3•]. With defective MMR, the ability to mutate is accelerated greatly, and it is believed that this rapid mutation ability shortens the typical time frame for adenoma-to-carcinoma formation from 1–2 decades to 1–2 years [1, 2, 3•, 5, 8, 39]. Sporadic MSI-H CRCs, but not Lynch CRCs, may further acquire activating mutations in the oncogene *BRAF* [3•, 7•, 8, 9]. Following inactivation of MMR genes, mutations continually accumulate in the cancer genome, as there is no ability to repair post-DNA replicative base substitutions or slippage mistakes at microsatellite sequences (Fig. 2) [1, 2, 3•, 6•]. Although most frameshift mutations that accumulate are in non-coding regions, as many as 300 microsatellites are in coding regions [1, 2, 3•]. This fact is believed to be the driver of improved outcome for patients with MSI-H CRCs. The frameshifted genes are transcribed and translated as shorter novel peptides due to the frameshift and new stop codon (Fig. 2) [1, 2, 3•, 40–45]. These neopeptides are immunogenic and are recognized by the immune system, which causes the development of subepithelial lymphoid aggregates. Lymphocytes that respond to the neopeptides then contain and/or limit the spread of the tumor [1, 3•, 46]. Essentially, MSI-H CRC patients immunize themselves with the neopeptides, which is likely the principal mechanism that extends patient survival [3•, 46–48]. It is probable that some of the frameshifted genes are driver genes for the pathogenesis of MSI-H CRCs (e.g., *ACVR2*, *TGFBR2*) [1, 2, 3•, 40, 41], while others are passenger genes, with both driver and

**Fig. 2** Genetic path of colonic neoplasia with loss of MMR function



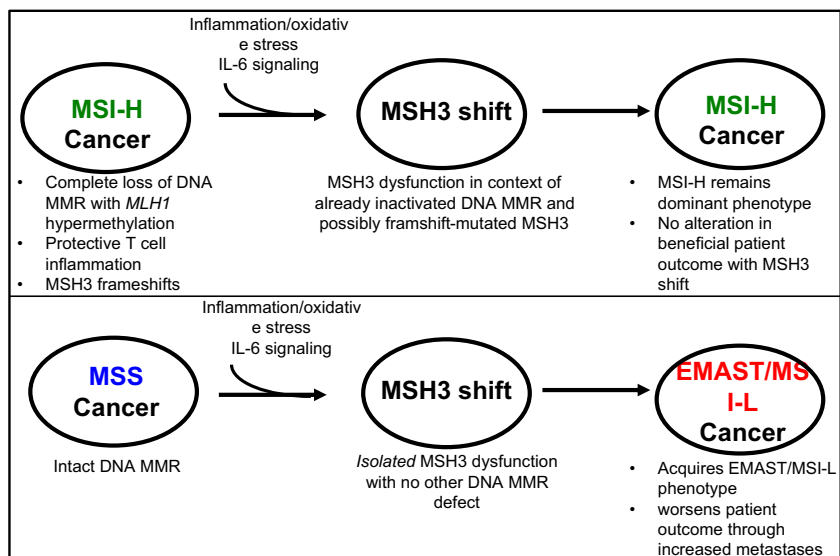
passenger frameshifted genes contributing to the immune induction [46–48].

EMAST CRCs with isolated MSH3 protein dysfunction show a markedly different histological and patient characteristic pattern than MSI-H CRCs. EMAST CRCs demonstrate a high prevalence of intraepithelial lymphocytes, showing an intimate association with the epithelial cancer cells, which supports the notion of localized interleukin-6 ligand generation and interaction [3•, 6•, 10•, 11, 16–18]. EMAST CRCs also show a decreased heterogeneous expression of MSH3 from within the nuclei of CRC cells [10•, 13, 15, 17, 18]. Patients with EMAST CRCs are associated with a higher prevalence of metastases and decreased patient survival [3•, 6•, 12, 16, 33•, 39]. The prevalence of EMAST is higher among African American CRC patients than in Caucasian patients [3•, 6•, 10•, 16, 29, 39, 49]. Available evidence suggests that once a CRC is formed, inflammation may occur to generate interleukin-6, triggering nuclear-to-cytosol shift of MSH3 within the cancer cell (accounting for the observed decreased nuclear expression), causing a loss-of-function of

MSH3 (generating EMAST and DNA double strand breaks), which ultimately causes more aggressive cell behavior and generation of metastasis and contributes to poor patient survival [10•, 12–16, 18, 33•, 34•, 50, 51].

It should be noted that the biochemical detection of tetranucleotide frameshifts (EMAST) can be observed with complete loss of MMR (i.e., *MLH1*, *MSH2*, *PMS2* inactivation) in addition to the isolated loss-of-function of MSH3 (Fig. 1). Patient outcome is determined by which of these scenarios is present within the tumor. As depicted in Fig. 3, an MSI-H CRC generates neopeptides that immunize the patient against the tumor, and even if inflammation occurs to shift MSH3 protein out of the nucleus, the MSI-H phenotype is more important for patient outcome, with improved survival over patients with MSS CRCs and no apparent effect from any MSH3 nuclear shift. However, a MSS CRC does not generate immunogenic neopeptides like the MSI-H CRC, and if inflammation occurs that can cause MSH3 to shift out of the nucleus, the CRC acquires the EMAST phenotype that

**Fig. 3** Schema showing the effect of inflammation on MMR-defective and MMR-intact cancers



increases metastatic behavior and worsens patient outcome (Fig. 3) [6•, 10•].

Clinically, immunohistochemistry is commonly used in most pathology labs to determine the presence of the MMR proteins MSH2, MLH1, MSH6, and PMS2 [1, 2, 3•, 7•, 9, 20•]. The absence of one or more of these proteins may be caused by either a sporadic or germline mutation. MSI can be detected in certain specialty laboratories commercially, and typically tests for mono- and dinucleotide repeat instability only [1, 2, 3•, 7•, 9, 20•]. MSH3 protein is not routinely assayed for in pathology labs, and tetranucleotide microsatellites are not typically tested for in MSI assays. Thus, EMAST is not assayed for outside of the research setting at present.

### MSI and Modification of Treatment Approaches for Patients With CRC

In addition to MSI being a prognostic marker, MSI can be used to direct patient therapy. The MMR recognition complexes recognize and bind to ingested or parenteral 5-fluorouracil (5FU) and are then converted and incorporated into DNA. Both MSH2-MSH6 and MSH2-MSH3 complexes can bind 5FU within DNA and trigger cell death [52–59, 60•]. When the MMR recognition complexes are not present, cell death does not occur and cells may proceed with mitosis with 5FU-induced and other novel mutations contained within DNA. The ability of MMR to trigger cell death after 5FU recognition is 30-fold higher than when MMR is absent [52]. Retrospective and prospective studies on stage II and III MSI-H CRC patients as well as Lynch syndrome CRC patients confirm there is no increased survival time with adjuvant 5FU therapy, as compared to increased survival among MSS CRC patients [53, 54]. Current clinical practice refrains from using 5FU monotherapy for stage II MSI-H CRC patients [55, 56]. At present, stage III MSI-H CRC patients are offered adjuvant 5FU in combination with oxliplatin and/or irinotecan (both of which show activity against MSI-H CRC cells in culture), but generally not 5FU alone or solely with leucovorin. Interestingly, the nature of an MMR-deficient, MSI-H tumor may modify the resistance to 5FU in some cases. MSI-H CRC cells in which the DNA glycosylase *MBD4* is frameshift mutated (which removes its glycosylase domain while keeping its DNA-binding domain and occurs in 20–35% of MSI-H CRCs) re-acquires some sensitivity to 5FU through a yet-to-be-determined mechanism [57, 58].

Patients with stage II/III EMAST CRCs (i.e., those with MSS tumors and isolated MSH3 dysfunction) respond to adjuvant 5FU therapy to the same degree as non-EMAST MSS CRC patients. This is likely because MSH2-MSH6 is fully present and functional to bind 5FU within DNA to trigger cell death despite the MSH2-MSH3 dysfunction [32•].

The immunogenic nature of MSI-H CRCs goes beyond the generation of neopeptides from frameshifted coding microsatellite sequences as a consequence of defective MMR and activation of T lymphocytes. The majority of MSI-H CRCs acquire expression of the immune checkpoint molecule PD-1, which prevents immune destruction of the cancer cell by cytotoxic lymphocytes when engaged with PD-L1 [47•]. This makes MSI-H CRCs more susceptible to immune checkpoint blockade with PD-1 antibodies [48, 59, 60•]. Indeed, patients with deficient MMR tumors showed 78% progression-free survival as compared to 11% for patients with proficient MMR tumors and anti-PD-1 more than doubled the average survival of MSI-H patients [59, 60•]. Thus, immune checkpoint blockade could further improve the overall good survival of MSI-H CRC patients, in contrast to the limited efficacy of adjuvant 5FU.

### Conclusions

Since its discovery as a part of human disease, MSI is proving to be a valuable biomarker for the diagnosis, prognosis, and determination of treatment approaches in CRC patients. Defective MMR generates MSI and hypermutated cancers, and detection via immunohistochemistry and mono-, di-, tri-, and tetranucleotide microsatellite frameshifts helps determine which germline or somatic MMR-defective condition is present (although only mono- and dinucleotide microsatellites are assayed currently commercially). Immunorecognition of neopeptides improves survival of MSI-H CRC patients; in contrast, isolated MSH3 dysfunction that causes EMAST worsens outcome for MSS patients. This outcome difference depends on if the MMR defect drives an immune response, or if acquired inflammation causes disruption of MMR via MSH3 cellular mislocation. Loss of MMR function not only prevents repair at single nucleotides and microsatellite sequences but also prevents recognition of altered nucleotides such as 5FU that gets incorporated into DNA. MSI-H CRCs often acquire immune checkpoint molecule expression which may be exploited therapeutically to prolong patient survival.

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### Compliance with Ethical Standards

**Conflict of Interest** The author declares that he has no conflict of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by the author.

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