Inflammation-associated microsatellite alterations: Mechanisms and significance in the prognosis of patients with colorectal cancer

Minoru Koi, Stephanie S Tseng-Rogenski, John M Carethers

Abstract
Microsatellite alterations within genomic DNA frameshift as a result of defective DNA mismatch repair (MMR). About 15% of sporadic colorectal cancers (CRCs) manifest hypermethylation of the DNA MMR gene MLH1, resulting in mono- and di-nucleotide frameshifts to classify it as microsatellite instability-high (MSI-H) and hypermutated, and due to frameshifts at coding microsatellites generating neo-antigens, produce a robust protective immune response that can be enhanced with immune checkpoint blockade. More commonly, approximately 50% of sporadic non-MSI-H CRCs demonstrate frameshifts at di- and tetra-nucleotide microsatellites to classify it as MSI-low/elevated microsatellite alterations at selected tetranucleotide repeats (EMAST) as a result of functional somatic inactivation of the DNA MMR protein MSH3 via a nuclear-to-cytosolic displacement. The trigger for MSH3 displacement appears to be inflammation and/or oxidative stress, and unlike MSI-H CRC patients, patients with MSI-L/EMAST CRCs show poor prognosis. These inflammatory-associated microsatellite alterations are a consequence of the local tumor microenvironment, and in theory, if the microenvironment is manipulated to lower inflammation, the microsatellite alterations and MSH3 dysfunction should be corrected. Here we describe the mechanisms and significance of inflammatory-associated microsatellite alterations, and
propose three areas to deeply explore the consequences and prevention of inflammation’s effect upon the DNA MMR system.

**Key words:** Microsatellite instability; Microsatellite stable; Elevated microsatellite alterations at selected tetranucleotide repeats; Colorectal cancer; Mismatch repair; Inflammation; *MSH3*

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Core tip: Inflammation can trigger microsatellite stable colorectal cancers (CRCs) to acquire a nuclear-to-cytoplasm displacement of the DNA mismatch repair protein *MSH3*, rendering the CRC with di- and tetranucleotide microsatellite instability (MSI-low/elevated microsatellite alterations at selected tetranucleotide repeats) and modifying the biological behavior of the CRC towards metastasis and poor patient survival. We herein discuss the mechanisms and significance of these induced inflammatory-associated microsatellite alterations, and suggest three content areas to further examine interventions that may modify the observed behavior of these CRCs.

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INTRODUCTION

The Cancer Genome Atlas (TCGA) for colorectal cancers (CRCs) to acquire a nuclear-to-cytoplasm displacement of the DNA mismatch repair protein *MSH3*, rendering the CRC with di- and tetranucleotide microsatellite instability (MSI-low/elevated microsatellite alterations at selected tetranucleotide repeats) and modifying the biological behavior of the CRC towards metastasis and poor patient survival. We herein discuss the mechanisms and significance of these induced inflammatory-associated microsatellite alterations, and suggest three content areas to further examine interventions that may modify the observed behavior of these CRCs.

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INTRODUCTION

The Cancer Genome Atlas (TCGA) for colorectal cancers (CRCs) that there are two types of sporadic CRCs - hypermutated and non-hypermutated. Most hypermutated CRCs have a defect in their mismatch repair (MMR) system due to the loss of MLH1 function by promoter silencing of the *MLH1* locus, resulting in high levels of insertion/deletion (I/D) mutations at microsatellite loci (microsatellite instability high: MSI-H)[1]. Most MSI-H CRCs exhibit proximal location, mucinous, undifferentiated histology, abundant CDS7/Th1 T cell infiltrations, and less aggressive clinical behavior, and are susceptible for immune checkpoints blockade[2,3]. Among non-hypermutated CRCs, I/D mutations in microsatellite loci with larger repeat units (di- and tetra-nucleotide repeats) are frequent and have been shown to be caused by tumor cells’ exposure to inflammatory tumor-microenvironments[4,5]. In this review, we describe and discuss the penetrance and causes of inflammation-associated microsatellite alterations (IAMAs), and their significance to patients’ prognoses in CRC. We also raise “Provocative Questions” whose answers could contribute not only to understand the biology of IAMAs but also to treatment of CRC with IAMAs.

**MSI-H, MSI-L AND EMAST IN CRC**

Microsatellites or simple sequence repeats are composed of 1-6 nucleotide repeats, occupy 3% of the total human genome, and are located in both coding and non-coding regions[6]. MSI is defined as continuous length changes in simple DNA repeat sequences within microsatellite loci[7]. MSI in CRC was first reported by Aaltonen et al[8] and Thibodeau et al[9] followed by Ionov et al[10] in 1993. It was then shown that a subset of sporadic CRC tumors and tumors from hereditary nonpolyposis colon cancer (HNPCC) exhibit MSI and MMR-defects[11]. Subsequently, germline mutations in *MSH2, MLH1, PMS2 and MSH6* were found in different HNPCC families[12-18] and tumors from these families exhibited MSI[19,20]. A causal relationship between MMR-defect, MSI and cancer susceptibility was shown by knockout mouse studies[21-24]. Genetic complementation studies using tissue cultured MSI-positive CRC cells also confirmed that MSI is caused by MMR-deficiency in human cells[25-27]. It was also shown that MSI exhibited in 10%-15% of sporadic CRC cases was due to transcriptional down-regulation of *MLH1* expression through promoter hyper-methylation[28].

MSI in CRCs was defined at an international workshop meeting sponsored by the National Cancer Institute in 1998[2]. A panel of five microsatellite markers - two markers with mononucleotide repeats and three markers with dinucleotide repeats - were validated to be classified as follows: High-frequency MSI (MSI-H: 2 or more of 5 markers show instability), low-frequency MSI (MSI-L: 1 of 5 markers shows instability), and microsatellite stable (MSS: none of 5 shows instability) CRCs. It was also confirmed that MSI-H in CRC is caused by defective MMR, mainly MSH2 and MLH1, and manifests as sporadic and hereditary forms of CRCs. Both sporadic and inherited MSI-H CRCs have unique clinical and pathological futures compared to MSI-L/MSS sporadic CRCs[2]. At this NIH meeting, the presence of CRCs with MSI-L was appreciated and discussed. However, the etiology of MSI-L and the distinction between MSI-L and MSS CRC remained unclear. Another type of microsatellite alteration, called elevated microsatellite alterations in selected tetranucleotide repeats (EMAST), where insertion/deletion mutations in the loci with tri- and/or tetra-nucleotide but not with mono- and/or dinucleotide repeats was recognized as a component of CRC but its etiology and clinic-pathological significance was not determined[3].

Although a consensus on the definition of MSI-L CRC was reached at the NCI meeting, two subsequent studies showed that approximately 80% of non-MSI-H CRC exhibited mutation at < 1 microsatellite locus when a large number of the loci with di-nucleotide repeats were tested for frame-shift mutations, indicating...
that most of CRC is MSI-L, and that the NCI reference panel was inadequate for detection of MSI-L CRC\cite{29,30}. These studies also showed that there were no genetic or clinic-pathological characteristics of tumors to separate MSI-L from MSS CRC. However, both studies observed that the incidence of MSI was non-randomly distributed among non-MSI-H CRC, suggesting that some tumors were more susceptible than others to slippage mutations at microsatellite loci, especially loci with dinucleotide repeats\cite{29,30}. The reason for the observed variation in instability and its pathological significance in patients’ prognoses remains unclear.

I/D mutations in loci with selected tetra-nucleotide repeats (EMAST), such as (AAAG)\textsubscript{n} or (ATAG)\textsubscript{n}, have been reported in non-CRCs including non-small cell lung, bladder, ovary, head and neck, skin and kidney cancers\cite{31}. Haugen \textit{et al} \cite{32} first described the frequency of EMAST in CRC, its relationship to MSI-L and its possible cause. They used the five NCI-endorsed MSI markers plus 2 additional markers with dinucleotide repeats to identify MSI-H, MSI-L and MSS CRC. They also used 7 EMAST markers and defined EMAST-positive if one or more of the 7 markers showed ID mutations\cite{32}. They found that EMAST is common in sporadic cases of non-MSI-H CRC (approximately 50%) and is associated with decreased nucleus MSH3 expression in tumor cells. Using MSH3-proficient and -deficient colon cancer cell lines, they also showed evidence that EMAST and low levels of instability at dinucleotide loci repeats - but not with mononucleotide repeats - in non-MSI-H CRC cells are caused by loss of MSH3\cite{32}. Frequent incidence of EMAST in CRCs was confirmed by 2 other studies\cite{33,34}.

The genetic cause of EMAST due to the loss of MSH3 was also proven by other studies using tissue cultured human cells\cite{35,36}.

**BIOCHEMICAL BASIS OF MICROSATELLITE ALTERATIONS**

Accumulated evidence supports that MSI-H, MSI-L and EMAST are caused by defects in some components of MMR\cite{37}. When DNA polymerase copies template DNA containing microsatellite loci, it mistakenly adds or deletes a repeat unit in the newly synthesized DNA strand (Figure 1A). The DNA polymerase slippage errors create loops between the two strands, which are recognized and repaired by MMR. In vitro experiments using cell extracts and/or purified proteins demonstrate that there are 5 MMR proteins involved in MMR reactions in human cells (Figure 1B)\cite{38}. MSH2 plays a major role in recognition of mismatched DNA. MLH1 and PMS2 are the main proteins responsible for down-stream MMR reactions. If MSH2, MLH1 or PMS2 lose their function, slippage errors at microsatellite loci with mono-, di- and tetra-nucleotide repeats are not fixed at all, resulting in MSI-H (Figure 1B). There are 2 pathways for mismatch recognition: (1) MSH2 and MSH6 form a dimer called MutS\textsubscript{b} that preferentially recognizes mismatched nucleotides and loops containing 1-2 nucleotides; (2) MSH2 and MSH3 form a dimer called MutS\textsubscript{a} that recognizes loops containing 2 or more nucleotides generated at di- and tetra-nucleotide repeats, including the EMAST loci (Figure 1A)\cite{39}. Defects in MSH6 result in increased...
missense mutations and in instability at mononucleotide repeats (Figure 1A)\cite{40}. When only MSH3 is disabled, increases in instability at di-, tri- and tetra-nucleotide repeats (EMAST) but not at mononucleotide repeats are observed (Figure 1)\cite{32}. Biochemical data indicates that loops containing 2 nucleotides are preferentially recognized by MutS\(\alpha\) over MutS\(\beta\)\cite{41}. Thus, when loss of MSH3 leaves many loops containing 2 or more nucleotides unrepaired, MutS\(\alpha\) may repair some but not all loops, resulting in low levels of mutation in di-nucleotide repeat loci and high levels of mutation in loci with tetra-nucleotide repeats (EMAST) loci (Figure 1B and C).

**MSI-L AND EMAST ARE CAUSED BY MSH3 FUNCTIONAL LOSS IN CRC**

The first evidence that loss of MSH3 may result in MSI-L and/or EMAST in CRC was reported by Haugen et al\cite{32} in 2008. They used the colon cancer cell line HCT116 that is deficient in MLH1 due to a hemizygous inactivating mutation in exon 9, and is also deficient in MSH3 due to a homozygous frameshift inactivating mutation in exon 7. Thus, this cell line showed the MSI-H phenotype. Introduction of a normal human chromosome 3 carrying a wild-type MLH1 to HCT116 complemented MLH1-deficiency\cite{23}. The resulting HCT116 with chromosome 3 exhibited stability in loci with mononucleotide repeats but showed low levels of instability at loci with dinucleotide repeats: MSI-L, and high degree of instability at EMAST loci. They further introduced a normal human chromosome 5 carrying wild-type MSH3 into HCT116 + 3 + cells. The resulting HCT116 + 3 + 5 cells exhibited complete stability at loci with mono-, dinucleotide repeats and EMAST loci. Finally, they introduced MSH3-shRNA to HCT116 + 3 + 5 cells to knock-down MSH3 and showed that specific knock-down of MSH3 resulted in an MSI-L/EMAST phenotype.

The second evidence that loss of MSH3 results in MSI in loci with di- and tetra-but not mono-nucleotide repeats is from a discovery of two families with bi-allelic MSH3 germ-line mutations, reported by Adam et al\cite{42}. Patients with bi-allelic inactivation mutations of the MSH3 locus suffered from a colorectal adenoma polyposis syndrome and early occurrence of multiple adenoma polyps and tumors in other organs. As expected, the expression of MSH3 was null in normal colon and adenoma polyps from these patients (Table 1). MSI assays showed that instability at di-nucleotide repeat loci and EMAST loci, but not loci with mononucleotide repeats, was detected in adenoma polyps but not in normal colon cells from the same patient. This is because adenoma is monoclonal while the normal colon of these patients consists of mixture of cells with MSH3 at normal colon cells from the same patient. This is because adenoma is containing germline alleles. However, there is likely dinucleotide and tetranucleotide instability within normal tissues if they were compared to heterozygous MSH3 germline relatives, or relatives that are homozygous normal for MSH3 mutation. These results support that MSI-L/EMAST in sporadic CRC is caused by loss of MSH3 function.

**EVIDENCE THAT MSI-L/EMAST IN SPORADIC CRC IS INDUCED BY INFLAMMATION THROUGH DISPLACEMENT OF MSH3 FROM NUCLEUS TO CYTOPLASM**

While homogeneous loss of nuclear MSH3 can be detected in adenoma polyp with bi-allelic germline MSH3 mutations, heterogeneous loss of nuclear MSH3 is frequently detected in sporadic CRC exhibiting MSI-L/EMAST (Figure 2A). These results suggest that local loss of MSH3 expression in sporadic MSI-L/EMAST CRC may be not due to genetic loss of MSH3. TCGA data shows that the frequency of MSH3 somatic mutations in CRC is about 6.6%. This does not explain the high incidence of MSI-L/EMAST (approximately 50%) in CRC. Furthermore, most MSH3 mutations are frameshift mutations in exon 7 that are a resulting target from MLH1 inactivation in sporadic CRC (Table 2)\cite{41}.

Lee et al\cite{43} found that EMAST CRC is enriched in in the tumor microenvironment of CD8⁺ T cells compared to non-EMAST CRC, suggesting that some immunological and inflammatory responses are active in EMAST CRC. They also found that EMAST is significantly high in ulcerated tumors. Devaraj et al\cite{43} further showed that EMAST-positive rectal tumors are associated with the presence of chronic inflammation. These observations led them to hypothesize that inflammation may somehow affect MSH3 function that induces MSI-L/EMAST.

Tseng-Rogenski et al\cite{4,36} demonstrated that several main inflammatory factors, including oxidative stress (hydrogen peroxide), interleukin 6 (IL6) and prostaglandin E2 (PGE2) induce displacement of MSH3 from the nucleus to the cytoplasm in several

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**Table 1** Expression of MSH3 protein within the epithelium of normal colonic mucosa and adenoma of patients with mono- or bi-allelic germline mutation in MSH3

| Tissue (epithelium) | Monoallelic MSH3 germline mutation | Bi-allelic MSH3 germline mutation |
|---------------------|-----------------------------------|----------------------------------|
| Normal colonic mucosa | MSH3 expressed | Not obtained |
| Colon adenoma | Not obtained | MSH3 absent |

Extracted from Adam et al\cite{32}.

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cancer cell lines. Importantly, other MMR proteins including MLH1, MSH2 and MSH6 do not move from the nucleus to the cytoplasm in response to these stimuli. Repeated treatment of several microsatellite stable colon cancer cell lines with IL6 induced microsatellite instability at EMAST loci. However, other inflammatory cytokines including TNFα, IFNα, IFNβ, and IL1β did not have such an effect. Tseng-Rogenski et al. also showed that phosphorylation of STAT3 may be required for displacement of MSH3 when induced by IL6. These studies convincingly show that not all, but some, inflammatory factors induce EMAST through loss of MSH3 from the nucleus (Figure 2B).

Evidence that an inflammatory micro-environment induces MSI-L (low levels of MSI at the loci with dinucleotide repeats) has been shown in regenerated colon tissues from ulcerated colitis (UC) patients. The first study, reported by Brentnall et al., showed for the first time the presence of MSI-L but not MSI-H in colon tissues from UC patients. The second study, by Ozaki et al., isolated crypts from UC-derived CRC, UC-derived hyperplasia and UC-regenerated colons through laser micro-capture and tested for the presence of microsatellite instability in DNA. Ozaki et al. detected MSI-L but not MSI-H in some crypts but not in others, regardless of whether they were from cancer or non-cancer tissues. They also showed that MSI was not detected from stroma cells from these UC patients. Each crypt showed a different MSI-profile, indicating that MSI-L occurs independently at the crypt level. Our recent study showed that regenerated colon cells and CRCs from UC patients have a high frequency of MSH3 displacement from the nucleus to the cytoplasm, and demonstrate MSI-L/EMAST.

Table 2 Comparison of type of mismatch repair gene mutations between sporadic hypermethylated MLH1 colorectal cancers and POLE mutation colorectal cancers from TCGA

| Gene    | Type of Mutation | MLH1 promoter hypermethylation | POLE mutation |
|---------|------------------|--------------------------------|---------------|
| MLH1    | frameshift       | 8/22 (36%) with MSH3 frameshift mutation |
|         | missense/nonsense| 1/22 (4.5%) with MSH3 missense/nonsense mutation |
|         |                  | 0/22 (0%) with MSH2 mutation |
|         | frameshift       | 5/22 (23%) with MSH6 frameshift mutation |
| POLE    | frameshift       | 4/22 (18%) with MSH6 frameshift mutation |
|         | missense/nonsense| 3/13 (23%) with MSH3 frameshift mutation |
|         |                  | 2/13 (15%) with MSH3 missense/nonsense mutation |
|         | frameshift       | 5/13 (38%) with MSH2 frameshift mutation |
|         | missense/nonsense| 0/13 (0%) with MSH6 frameshift mutation |
|         |                  | 7/13 (54%) with MSH6 missense/nonsense mutation |

Both types of CRCs are hypermutated, containing hundreds of somatic mutations in genomic DNA. Note that the MLH1 hypermethylated CRCs demonstrate higher frequency and consistent frameshift mutations in MSH3 and MSH6 as compared to POLE mutated CRCs, which contain some frameshifts but higher frequency of missense/nonsense mutations in MSH3, MSH2 and MSH6. Extracted from: Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012; 487: 333-337. CRCs: Colorectal cancers.

Figure 2 MSH3 expression in sporadic colorectal cancer. A: Immunohistochemistry for MSH3 in sporadic CRC. Arrows show heterogeneous expression of MSH3 in cells and within nuclei in the epithelium; B: Model of MSH3 displacement from the nucleus to the cytosol with inflammatory stimuli to allow accumulation of tetranucleotide frameshift mutations. Progenitor cells could be affected earlier such that subsequent daughter cells amplify the accumulated frameshift mutations. MSI: Microsatellite instability; CRC: Colorectal cancer; EMAST: Elevated microsatellite alterations at selected tetranucleotide repeats.

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These results further support the role of inflammation in displacement of MSH3-induced MSI-L/EMAST in human tissues including cancers.

**PROGNOSTIC VALUE OF MSI-L/EMAST IN CRC**

Several studies have examined the impact of MSI-L and/or EMAST genotypes on patient prognoses in CRC. There have been 4 studies evaluating the prognosis values of MSI-L[47-50]. Kohonen-Corish et al[47] showed that patients with stage C colon cancers defined as MSI-L by the NCI panel plus one tetra-nucleotide marker (MYCL1) showed poor overall survival (OS) compared to patients with MSI-H and/or MSS colon cancers. Similar results were obtained by Wright et al[48]. They showed that stage C CRC patients that are positive for MSI-L as defined by the NCI panel, plus an additional 2 markers with mono-nucleotide repeats, 3 with di-nucleotide repeats and the tetra-nucleotide MYCL1 marker, exhibited poor cancer-specific survival compared to MSS CRC patients[48]. They also observed that most MSI-L CRC exhibited MSI at one di- or tetra-nucleotide but not at mono-nucleotide repeat markers[48]. Lee et al[49] examined 3019 CRC cases for MSI using an NCI microsatellite marker panel and evaluated prognoses of those patients. Similar to other studies, they showed that most MSI-L CRC exhibited MSI at dinucleotide repeats, and patients with MSI-L CRCs was associated with poor OS by Cox regression analysis[49]. Although the previous 2 studies suggested that MSI-L may have a significant prognostic value for stage C CRC patients, Lee et al[49] did not examine the prognostic significance of MSI-L for cancer-specific survival in their large cohort. In contrast to the above three studies, Azzoni et al[50] reported that MSI-L is associated with improved patient survival as compared to MSS CRC. However, the percentage of MSI-H cases in their cohort was unusually high (37%; 68 of 184 cases) compared to other studies (10%-15%), suggesting the presence of some bias in the studied cohort. Lastly, a study reported by Garcia et al[51] did not find any association between MSI-L and disease-free survival (DFS) or OS in stage II and III CRC cohorts.

There are 2 studies examining the relationship between EMAST and OS in CRC; they found no association between the two[33,51]. However, when both MSI-L and EMAST cases were combined, Garcia et al[51] found that MSI-L/EMAST was associated with shorter DFS but not OS compared with non-MSI-L/EMAST CRC. In their cohort, MSI-H CRC patients exhibited the highest survival. Thus, the MSI-L/EMAST genotype in CRC may be associated with recurrence and/or metastasis after surgery. There appears to be heterogeneity even among MSI-L/EMAST CRC patients[52,53]. One group of MSI-L/EMAST CRC exhibited loss of heterozygosity (LOH) at chromosome 9p24.2. and the other did not exhibit 9p24.2 LOH. When the prognoses of these two groups were compared, the one with 9p24.2 LOH at stage III showed improved survival after surgery and OS in Kaplan-Meier analysis and in multi variate analysis over the one without 9p24.2. LOH at stage III[53]. The results also showed that MSI-L/EMAST/9p24.2 LOH is an independent factor that predicts improved OS in stage II/III CRC. Thus, MSI-L/EMAST may be associated with recurrence, but additional genetic or epigenetic changes may modify the behavior of recurrent tumors[53]. Overall, the data presented so far suggest that MSI-L and/or EMAST could be a biomarker for DFS and/or OS of stage II and/or III CRC. However, additional studies using a population-based large cohort are needed to confirm the prognostic value of MSI-L and EMAST.

One concern regarding EMAST is that various studies have not reached a full consensus on the definition of EMAST. As described above, current evidence supports the idea that MSI-L and EMAST in sporadic CRC share the same etiology: both are induced by the absence of nuclear MSH3 in response to exogenous inflammatory factors such as IL6, and oxidative stress[4]. Based on these observations, we propose that EMASCT cancer is a non-MSI-H cancer, and MSI at EMAST markers is not caused by loss of other MMR proteins including MLH1, MSH2, PMS2[51,53]. The next question should be whether or not non-EMAST CRC really exits. Similar to MSI-L in CRC[29,30], almost all CRC could be EMAST-positive if a large number of EMAST markers are used. A recent study by Cortes-Ciriano et al[54] showed that all non-MSI-H cancers contain various levels of frame-shift mutations in microsatellite loci with mono-, di-, tri- and tetra-nucleotide repeats. Considering that all tumor tissues contain some degree of inflammatory elements, many of those mutations could be induced by the loss of MSH3 triggered by inflammation in the tumor-microenvironment. Furthermore, a study for UC suggested that frequent exposure to inflammation increased the incidence of MSI-L and EMAST[55]. Thus, while the purpose of the MSI assay is primarily to detect MMR-deficient CRC, the purpose of an EMAST assay could be to distinguish CRCs whose precursors were exposed to high levels of inflammation to CRCs whose precursors were exposed to lower levels of inflammation. Therefore, the results of the studies by Kohonen-Corish, Wright, Lee and Garcia could be re-interpreted according to the idea that high levels of inflammatory tumor-microenvironments not only induce MSI-L/EMAST in cancer cells at the primary site but also include some property that promotes recurrence and/or metastasis when they disseminate. Additional studies will be required to determine whether the numbers and kinds of EMAST markers and cut-off levels for determining EMAST-positive/negative used so far are adequate to distinguish CRCs with different prognoses[51].

**PROVOCATIVE QUESTIONS**

Here, we have raised three questions whose answers can be important for not only clinical but also basic
They identified neutrophil, probably myeloid-derived suppressor cells (MDSC), and cancer-associated fibroblast (CAF) as the main cell components recruited in tumor-microenvironments, expressing EP2, responding to PEG2, and contributing to tumor formation. These cells form a positive-feedback loop of COX-2-PGE2-EP2-NF-kB-COX-2 cycles, and produce TNF-α and IL6[68]. The presence of MDSC and CAF in the tumor-microenvironment are also significantly associated with stage progression and a poor prognosis for CRC, while activation of Th1 helper and cytotoxic memory T cells play a key role in anti-tumor activities preventing recurrence and/or metastasis in CRC[69,70]. Interestingly, Zelenay et al[71] showed that, depending on the level of COX-activity in cancer, the immunological landscape of tumor-microenvironments can be switched between anti-tumor and inflammatory pro-tumor. Therefore, the level of PEG2 and of COX-2 may be major factors in controlling immunological responses to cancer cells, and thereby a patient’s prognosis. Regarding the relationship between MSI-L/EMAST and PEG2, we have observed that the exposure of colon cancer cells in tissue cultures to PGE2 triggers movement of MSH3 from the nucleus to the cytoplasm, which may induce MSI-L/EMAST. Therefore, it is reasonable to speculate that MSI-L/EMAST in CRC may be associated with high levels of COX-2 expression and/or with mutational status of PIK3CA in CRCs. Ng et al[67] showed that aspirin and COX-2 inhibitors improved recurrence-free survival, DFS and OS of stage III CRC patients who either received fluorouracil (FU) plus leucovorin (LV) or FU plus LV with irinotecan. These studies support the idea that NSAIDs can be used as part of adjuvant therapy for stage I-III CRC, however, the efficacy of NSAIDs on the recurrence/metastasis of CRC are still under investigation through several randomized controlled trials[53].

Ma et al[69] showed that PGE2 and its receptor, the prostaglandin E receptor 2 (EP2), are necessary for colon cancer formation in inflammatory tissue environments. Compared to wild-type mice treated with azoxymethan (AOM) followed by dextran sodium sulfate (DSS), AOM/DSS-treated EP2-knockout and prostaglandin E synthase (Ptgese)-knockout mice bore a significantly reduced number of colon tumors. They presented microsatellite instability; CRC: Colorectal cancer; EMAST: Elevated microsatellite alterations at selected tetranucleotide repeats.

Figure 3 Model of adenoma-to-carcinoma formation in the human colon, with actual and potential sites of interventions to improve survival. MSI: Microsatellite instability; CRC: Colorectal cancer; EMAST: Elevated microsatellite alterations at selected tetranucleotide repeats.

Question 1: Does treatment of CRC with non-steroidal anti-inflammatory drugs reduce not only recurrence/metastasis but also the incidence of MSI-L/EMAST?

The idea that inflammation is associated with recurrence and/or metastasis is indirectly supported by observations that an intake of the anti-inflammatory drug, aspirin, may not only prevent adenomas[53] and CRC formations[56], but also prevent recurrence and metastasis of CRC following surgery[57]. Other non-steroidal anti-inflammatory drugs (NSAID) including celecoxib and rofecoxib, specific inhibitors of cyclooxygenase 2 (COX-2), have been shown to reduce the incidence of adenomas[58-60]. But it was also found that COX-2 inhibitors suppressed colorectal tumor growth and metastasis in mouse models[61,62]. Furthermore, Chan et al[63] reported that the regular intake of aspirin after curative surgery reduced cancer-specific mortality in a sub-group of CRC cells expressing a high level of COX-2 protein. In addition, CRCs expressing HLA class I compared to those not expressing HLA class I are susceptible for aspirin treatment after diagnosis[64]. Also, CRCs with PIK3CA mutations responded better to aspirin treatment after diagnosis than did CRCs with wild-type PIK3CA[65]. However, a recent study by Gray et al[66] showed that the efficacy of aspirin on cancer-specific survival, and OS was associated with levels of COX-2 expression but not with mutational status of PIK3CA in CRCs. Ng et al[67] showed that aspirin and COX-2 inhibitors improved recurrence-free survival, DFS and OS of stage III CRC patients who either received fluorouracil (FU) plus leucovorin (LV) or FU plus LV with irinotecan. These studies support the idea that NSAIDs can be used as part of adjuvant therapy for stage I-III CRC, however, the efficacy of NSAIDs on the recurrence/metastasis of CRC are still under investigation through several randomized controlled trials[53].

Ma et al[69] showed that PGE2 and its receptor, the prostaglandin E receptor 2 (EP2), are necessary for colon cancer formation in inflammatory tissue environments. Compared to wild-type mice treated with azoxymethan (AOM) followed by dextran sodium sulfate (DSS), AOM/DSS-treated EP2-knockout and prostaglandin E synthase (Ptgese)-knockout mice bore a significantly reduced number of colon tumors.
in CRC is p53 mutation\[^{72}\], MSI-L/EMAST formation may be associated with p53 mutation. In fact, Ahrendt et al\[^{73}\] reported that EMAST is associated with p53 mutations in non-small cell lung cancer. Li et al\[^{74}\] observed an association between LOH at TP53 and EMAST in CRC. Interestingly, p53 mutations are the most frequently found in inflammatory bowel disease (IBD)-associated CRC among other gene mutations (60%-90%)\[^{75,76}\]. One half of the p53 mutations are C>G>T:G transitions, thought to be caused by nitric oxide exposure due to increased inducible nitric oxide synthase expression in IBD\[^{75}\]. Our preliminary data showed that IBD-associated CRC exhibit a higher frequency of MSI-L/EMAST than do sporadic CRC (unpublished data). Taken together, these results suggest that the inflammatory tissue environment may enhance p53 mutations and MSI-L/EMAST formation in sporadic adenomas, leading to carcinoma transition. As mentioned earlier, MSI-L/EMAST in stage II CRC patients is associated with shorter RFS, suggesting that the inflammatory tumor-environment in primary tumor tissues somehow promotes recurrence or metastasis. These observations lead to the next question: What establishes an inflammatory environment in colorectal adenoma and carcinoma?

Microbiota in the colon and rectum create an inflammatory microenvironment and promote CRC formation\[^{77}\]. Several bacterial organisms including Fusobacterium nucleatum (F. nucleatum), Enterotoxigenic Bacteroides fragilis (ETBF), and colibactin-producing Escherichia coli (E. coli) are epidemiologically associated with CRC, and have been found to be enriched in CRC\[^{77,78}\]. The enrichment of F. nucleatum was also found in colorectal adenoma relative to non-adenoma or surrounding tissues\[^{79-81}\]. McCoy et al\[^{79}\] showed that F. nucleatum abundance in colorectal adenoma is associated with local inflammatory cytokine gene expression including IL-10 and TNF-\alpha. Kostic et al\[^{80}\] investigated the effect of F. nucleatum infection on the development of intestinal tumors in APC\[^{Min/+}\], IL10\(^{-/-}\) and T-bet\(^{-/-}\) X Rag2\(^{-/-}\) mice. There was an increase in the number of tumors in APC\[^{Min/+}\] mice. Importantly, infection with F. nucleatum accelerated adenocarcinoma formation in the small intestines of APC\[^{Min/+}\] mice compared to sham-treated control mice. In contrast, infection with F. nucleatum did not induce any tumor formation in IL10\(^{-/-}\) and T-bet\(^{-/-}\) X Rag2\(^{-/-}\) mice. These results suggest that the effects of F. nucleatum may manifest on existing adenomas, and may stimulate adenoma-carcinoma transition by creating an oxidative stress-rich, carcinogenic environment\[^{80}\]. It would be interesting to determine whether F. nucleatum-induced adenocarcinomas in APC\[^{Min/+}\] mice gain p53 mutations. Kostic et al\[^{80}\] further showed that infection of tumor tissues with F. nucleatum results in recruitment of MDSCs, tumor-associated macrophages, and dendritic cells in tumor tissues, and modulate the tumor immune microenvironment that promote tumor progression. In addition, they found the up-regulation of genes that are down-stream of NF-kB including PTGS2 (COX-2), IL6, IL13, and TNF in both human and mouse CRC infected with F. nucleatum\[^{80}\]. It is tempting to speculate that F. nucleatum-induced adenocarcinoma may gain MSI-L/EMAST in response to oxidative stress, PEG2 and/or IL6 that cause displacement of MSH3 from the nucleus to the cytoplasm. Recently, a heavy load of F. nucleatum has been associated with MSI-H CRC, proximal colon cancer and a poor prognosis\[^{81-84}\]. Yu et al\[^{85}\] showed that F. nucleatum infection in primary CRC is associated with recurrence after surgery followed by adjuvant chemotherapy. They showed that F. nucleatum induces chemoresistance in infected cells through autophagy\[^{85}\]. One of the reasons why 5-FU-based adjuvant therapy does not have benefit for a sub-group of MSI-H CRC\[^{86,87}\] could be partly explained by the infection of F. nucleatum\[^{85}\]. It is also possible that the CpG Island Methylator Phenotype (CIMP) including promoter methylation of the MLH1 locus could be induced by chronic inflammation due to a heavy load of F. nucleatum infection\[^{82}\]. Considering that infection of F. nucleatum is associated with recurrence of CRC after surgery, a group of such CRCs may exhibit MSI-L/EMAST CRC\[^{51,53}\].

Another bacterium, ETBF, is also associated with CRC\[^{88-90}\] and can target colorectal cells to promote an adenoma and/or adenoma-carcinoma transition in APC\[^{Min/+}\] mice\[^{91}\]. ETBF produces a metalloprotease toxin called BFT. BFT binds to the surface of colorectal epithelial cells and induces E-cadherin cleavage, resulting in an increase in barrier permeability and inducing an inflammatory micro-environment with Th-17/IL-17 predominance\[^{91,92}\]. Th-17/IL-17 plays a major role in ETBF tumorigenesis because the depletion of CD4\(^+\) T cells and blockade of IL-17 inhibited it. IL-17 attracts neutrophils, MDSCs and macrophages, and induces carcinogenic and immunosuppressive factors including nitric oxide, ROS, and Arg1 in mouse models\[^{92}\]. Colibactin-producing E. coli is also associated with CRC\[^{93,94}\] and initiates inflammation and promotes adenoma formation in APC\[^{Min/+}\] mice\[^{94}\] and in APC\[^{Min/+}\], IL10\(^{-/-}\) mice\[^{95}\]. Taken together, infection with all three bacterial organisms, that are found to be associated with CRC, induces an inflammatory environment in adenoma tissue and promotes adenoma and/or a transition from adenoma to carcinoma in mouse models. It would be interesting to determine whether MSI-L/EMAST and p53 mutations coincide with bacterial-induced transitions to adenoma/carcinoma. Recently, Scott et al\[^{96}\] showed that the efficacy of 5-FU treatment maybe largely influenced by microbiota in the gut.

**Question 3: Is MSH3 a component of DNA damage signaling?**

The MutS\(^{\beta}\) hetero-duplex between MSH3 and MSH2 not only functions in MMR but may also play a role
in double strand break (DSB) repair via homologous recombination (HR)\textsuperscript{[97-100]}, DNA double strand breaks (DSB) induce cell death if not repaired. Cells have evolved two pathways to re-connect the broken DNA ends: Non-homologous end joining (NHEJ) and homologous recombination (HR). If one of these pathways is disabled when DSB is created, cells use the other pathway for survival. The HR reaction starts with a nuclease-mediated resection of broken DNA ends to be coated by the single stranded (ss) DNA-binding protein, replication protein A (RPA). Then, Ataxia telangiectasia and Rad3-related (ATR) kinase is recruited to the RPA-coated ssDNA via an ATR-interacting partner (ATRIP). The topoisomerase IIβ-binding protein 1 (TOBP1), which is recruited to the DSB site, interacts with ATRIP and activates ATR. Activated ATR phosphorylates CHEK2 that regulate cell cycle progression. TOBP1 also interacts with polo-like kinase (PLK) that phosphorylate RAD51 for its loading on resected ssDNA\textsuperscript{[101]}. Burdova et al\textsuperscript{[102]} showed that recruitment of ATR/ATRIP to RPA-coated ssDNA is mediated by MutS\textsubscript{b} which binds to the loop structure formed within the ssDNA. Therefore, MutS\textsubscript{b} is required in the early stage of HR-DSB repair and its loss due to an MSH2 or MSH3 defect forces a cell to use NHEJ for survival under the presence of DSBs\textsuperscript{[98,100]}. Thus, when oxidative stress causes DSBs, it may induce elimination of MSH3 from the nucleus, resulting in activation of Ataxia-telangiectasia mutated (ATM)\textsuperscript{[102]} but not ATR, and dependence of NHEJ for survival.

An intriguing question is why and how nuclear MSH3 proteins translocate in response to oxidative stress or exposure to IL6 or PGE2 (Figure 2B)\textsuperscript{[103,104]}. H\textsubscript{2}O\textsubscript{2} and oxidative stress causes DSBs, resulting in activation of NF-κB\textsuperscript{[105-107]}. IL6 and PGE2 are mediators that possibly form a loop associated with activation of NF-κB through STAT3\textsuperscript{[105-107]}. IL6 activates STAT3, which directly interacts with the NF-κB family member RELA, contributing to constitutive NF-κB activation\textsuperscript{[105]}, and COX2/PGE2 also activates STAT3, leading to NF-κB activation\textsuperscript{[106]}. We found that MSH3 itself is a shuttling protein. It contains a bona fide bipartite nuclear localization signal (NLS) that directs its nuclear import to perform DNA repair (unpublished data). It also contains two functional nuclear export signals (NESs) that allow it to exit the nucleus upon the treatment of a pro-inflammatory cytokine, IL-6 (unpublished data).

Among the other MMR proteins including MSH2, MSH3 is the only MMR protein that shifts into the cytoplasm upon oxidative stress or IL6 treatment, suggesting that MSH3 moves alone or does so with other unknown partner proteins. Recent data indicate that the NF-κB Essential Modulator (NEMO), when used as a bait, can pull down MSH3, suggesting physical interaction between these two proteins\textsuperscript{[108]}. As one of the three components of the IKK complex, NEMO’s role in regulating the NF-κB pathway is well documented\textsuperscript{[104]}. It is possible that simultaneous or sequential movement of MSH3, NEMO and ATM in the cell may transmit a DNA damage signal to NF-κB, depending on the degree of DNA damage. Further studies are necessary for clarify these possibilities.

**MSI-L/EMAST IS COMMON IN HUMAN CANCERS**

Since the discovery of MSI in CRC, MSI-L and EMAST have been examined in cancers from other organs and tissues. MSI-L has been found in stomach\textsuperscript{[109]}, cervical\textsuperscript{[110]}, pancreatic\textsuperscript{[111]}, ovarian\textsuperscript{[112]}, skin\textsuperscript{[113]}, nerve\textsuperscript{[114]}, breast, endometrial\textsuperscript{[115]}, liver\textsuperscript{[116]}, esophageal\textsuperscript{[117]}, eye\textsuperscript{[118]}, soft tissue\textsuperscript{[119]}, gallbladder\textsuperscript{[120]}, head and neck\textsuperscript{[121]}, prostate\textsuperscript{[122]}, lung\textsuperscript{[123]} and cancers of the urinary tract\textsuperscript{[124]}. EMAST has also been widely detected in other various human cancers\textsuperscript{[211]}. A recent study by Cortes-Ciriano et al\textsuperscript{[154]} showed that there are MSI-H prone cancers including colorectal, esophageal, stomach and endometrial cancers, and non-MSI-H prone cancers that include ovarian, kidney, liver, breast, head and neck, cervical, lung, pancreatic, bladder, prostate, skin, adrenal, cortical and thyroid cancers. They also showed that most non-MSI-H cancers exhibit different degrees of MSI at not only loci with mono- but also loci with di-, tri- and tetra-nucleotide repeats, suggesting that inflammation-induced MSH3 replacement from the nucleus to the cytoplasm is probably common in human cancers\textsuperscript{[104,125,126]}. Thus, the answers to the provocative questions raised above may also apply to many human cancers.

**CONCLUSION**

MSI-L/EMAST is common in human cancers. MSI-L/EMAST is caused by displacement of MSH3 from the nucleus to the cytoplasm in replicating cells triggered by inflammatory stimuli, and can be termed Inflammatory-Associated Microsatellite Alterations (IAMAS). MSI-L/EMAST is associated with recurrence and/or metastasis in CRC patients. MSI-L/EMAST CRC is a heterogeneous group and consists of sub-groups with different genetic changes and prognoses.

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