

# World Journal of *Gastrointestinal Oncology*

*World J Gastrointest Oncol* 2018 January 15; 10(1): 1-61



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*World Journal of Gastrointestinal Oncology*  
Volume 10 Number 1 January 15, 2018

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Editorial Board Member of *World Journal of Gastrointestinal Oncology*, Anil Mishra, PhD, Doctor, Department of Allergy and Immunology, Children's Hospital Medical Center, Cincinnati, OH 45229, United States

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### INDEXING/ABSTRACTING

*World Journal of Gastrointestinal Oncology* is now indexed in Science Citation Index Expanded (also known as SciSearch®), PubMed, and PubMed Central.

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NAME OF JOURNAL  
*World Journal of Gastrointestinal Oncology*

ISSN  
ISSN 1948-5204 (online)

LAUNCH DATE  
February 15, 2009

FREQUENCY  
Monthly

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*World Journal of Gastrointestinal Oncology*  
Baishideng Publishing Group Inc  
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA  
Telephone: +1-925-2238242  
Fax: +1-925-2238243  
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PUBLISHER  
Baishideng Publishing Group Inc  
7901 Stoneridge Drive,  
Suite 501, Pleasanton, CA 94588, USA  
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PUBLICATION DATE  
January 15, 2018

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

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

Minoru Koi, Stephanie S Tseng-Rogenski, John M Carethers, Division of Gastroenterology, Department of Internal Medicine and Comprehensive Cancer Center, University of Michigan, Ann Arbor, MI 48109-5368, United States

ORCID number: Minoru Koi (0000-0002-1195-071X); Stephanie S Tseng-Rogenski (0000-0001-5195-3176); John M Carethers (0000-0003-2623-7332).

Author contributions: All three authors wrote and edited this manuscript.

Supported by United States Public Health Service, Nos. DK067287, CA162147 and CA206010; and the A. Alfred Taubman Medical Research Institute of the University of Michigan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

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Manuscript source: Invited manuscript

Correspondence to: John M Carethers, BSc, MD, Full Professor, Professor and Chair, Division of Gastroenterology, Department of Internal Medicine, and Comprehensive Cancer Center, University of Michigan, 3101 Taubman Center, 1500 East Medical Center Drive, Ann Arbor, MI 48109-5368, United States. [jcarethe@umich.edu](mailto:jcarethe@umich.edu)  
Telephone: +1-734-6151717  
Fax: +1-734-9367024

Received: October 26, 2017

Peer-review started: October 29, 2017

First decision: November 23, 2017

Revised: November 29, 2017

Accepted: December 6, 2017

Article in press: December 6, 2017

Published online: January 15, 2018

### 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 tetra-nucleotide 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.

Koi M, Tseng-Rogenski SS, Carethers JM. Inflammation-associated microsatellite alterations: Mechanisms and significance in the prognosis of patients with colorectal cancer. *World J Gastrointest Oncol* 2018; 10(1): 1-14 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v10/i1/1.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v10.i1.1>

## INTRODUCTION

The Cancer Genome Atlas (TCGA) for colorectal cancers (CRCs) clarified 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)<sup>[1]</sup>. Most MSI-H CRCs exhibit proximal location, mucinous, undifferentiated histology, abundant CD8<sup>+</sup>/Th1 T cell infiltrations, and less aggressive clinical behavior, and are susceptible for immune checkpoints blockade<sup>[2,3]</sup>. 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<sup>[4,5]</sup>. 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

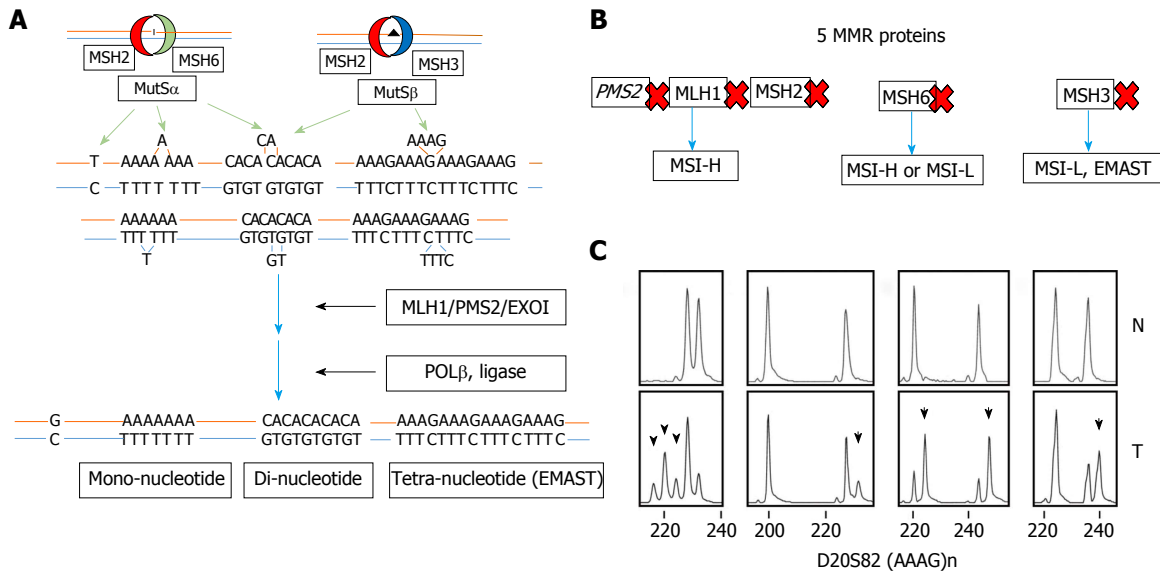
understand the biology of IAMAs but also to treatment of CRC with IAMAs.

## 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<sup>[6]</sup>. MSI is defined as continuous length changes in simple DNA repeat sequences within microsatellite loci<sup>[7]</sup>. MSI in CRC was first reported by Aaltonen *et al*<sup>[8]</sup> and Thibodeau *et al*<sup>[9]</sup> followed by Ionov *et al*<sup>[10]</sup> 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<sup>[11]</sup>. Subsequently, germline mutations in *MSH2*, *MLH1*, *PMS2* and *MSH6* were found in different HNPCC families<sup>[12-18]</sup> and tumors from these families exhibited MSI<sup>[19,20]</sup>. A causal relationship between MMR-defect, MSI and cancer susceptibility was shown by knockout mouse studies<sup>[21-24]</sup>. Genetic complementation studies using tissue cultured MSI-positive CRC cells also confirmed that MSI is caused by MMR-deficiency in human cells<sup>[25-27]</sup>. 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<sup>[28]</sup>.

MSI in CRCs was defined at an international workshop meeting sponsored by the National Cancer Institute in 1998<sup>[2]</sup>. 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 features compared to MSI-L/MSS sporadic CRCs<sup>[2]</sup>. 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 tetra-nucleotide 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<sup>[2]</sup>.

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



**Figure 1 Human DNA mismatch repair.** A: Two DNA recognition complexes MutS $\alpha$ , which recognizes insertion-deletion (I/D) loops of 1-2 repeated nucleotides for repair, and MutS $\beta$  which recognizes I/D loops of 2 or greater nucleotides for repair, are the key protein complexes of MMR. The MLH1 and PMS2 complex, also known as MutL $\alpha$ , then helps execute the repair with the exonuclease Exo1, polymerase $\beta$  and DNA ligase to fully effect repair; B: Specific efficiency in one of the five DNA MMR proteins yields differing microsatellite instability (MSI) results. Loss of MLH1, MSH2 or PMS2 will yield frameshifts at mono-, di- and tetra-nucleotide microsatellite markers. Loss of MSH6, inactivating MutS $\alpha$  only, will yield mononucleotide mostly but some dinucleotide microsatellite frameshifts, whereas loss of MSH3, inactivating MutS $\beta$ , will yield di- and tetranucleotide microsatellite frameshifts, but no mononucleotide microsatellite frameshifts; C: Examples of fragment analysis comparing normal colon tissue (upper panels) with tissue (lower panels) demonstrating frameshifts in the tetranucleotide marker D20S82. MMR: Mismatch repair; MSI: Microsatellite instability; CRC: Colorectal cancer.

that most of CRC is MSI-L, and that the NCI reference panel was inadequate for detection of MSI-L CRC<sup>[29,30]</sup>. 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<sup>[29,30]</sup>. The reason for the observed variation in instability and its pathological significance in patients' prognoses remained unclear.

I/D mutations in loci with selected tetra-nucleotide repeats (EMAST), such as (AAAG) $_n$  or (ATAG) $_n$ , have been reported in non-CRCs including non-small cell lung, bladder, ovary, head and neck, skin and kidney cancers<sup>[31]</sup>. Haugen *et al.*<sup>[32]</sup> 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<sup>[32]</sup>. 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<sup>[32]</sup>. Frequent incidence of EMAST in CRCs was confirmed by 2 other studies<sup>[33,34]</sup>.

The genetic cause of EMAST due to the loss of MSH3 was also proven by other studies using tissue cultured human cells<sup>[35,36]</sup>.

## BIOCHEMICAL BASIS OF MICROSATELLITE ALTERATIONS

Accumulated evidence supports that MSI-H, MSI-L and EMAST are caused by defects in some components of MMR<sup>[37]</sup>. 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 1)<sup>[38]</sup>. 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 $\alpha$  that preferentially recognizes mismatched nucleotides and loops containing 1-2 nucleotides; (2) MSH2 and MSH3 form a dimer called MutS $\beta$  that recognizes loops containing 2 or more nucleotides generated at di- and tetra-nucleotide repeats, including the EMAST loci (Figure 1A)<sup>[39]</sup>. Defects in MSH6 result in increased

**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 <i>MSH3</i> germline mutation	Bi-allelic <i>MSH3</i> germline mutation
Normal colonic mucosa	MSH3 expressed	MSH3 absent
Colon adenoma	Not obtained	MSH3 absent

Extracted from Adam *et al.*<sup>[42]</sup>.

missense mutations and in instability at mononucleotide repeats (Figure 1A)<sup>[40]</sup>. 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)<sup>[32]</sup>. Biochemical data indicates that loops containing 2 nucleotides are preferentially recognized by MutS $\beta$  over MutS $\alpha$ <sup>[41]</sup>. Thus, when loss of MSH3 leaves many loops containing 2 or more nucleotides unrepaired, MutS $\alpha$  may repair some but not all such 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.*<sup>[32]</sup> 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<sup>[25]</sup>. 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.*<sup>[42]</sup>. 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 MSI at different loci, masking each alteration that occurred in individual colon cells with the exception of 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)<sup>[1]</sup>.

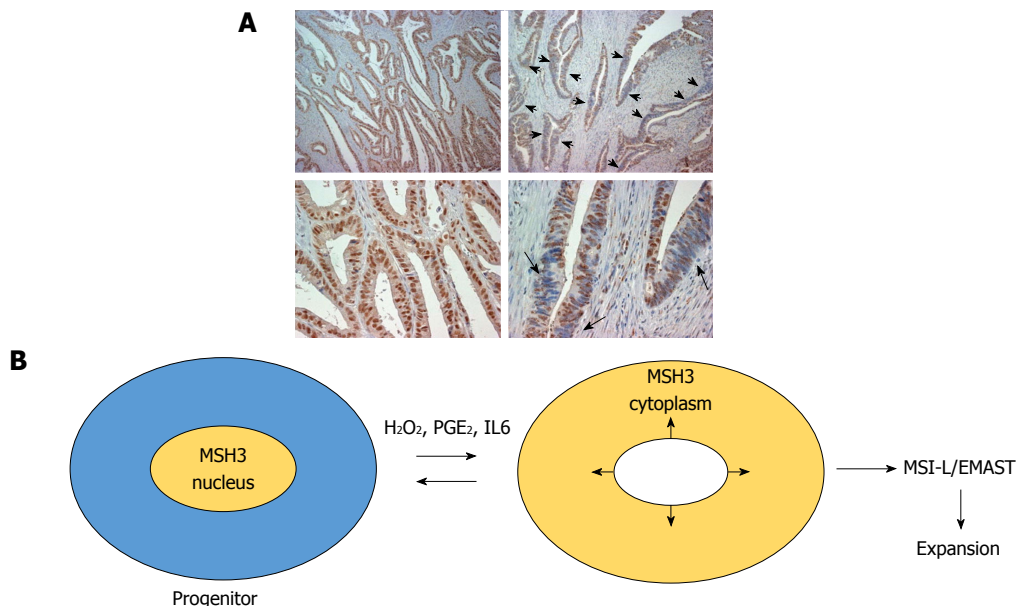
Lee *et al.*<sup>[34]</sup> found that EMAST CRC is enriched in in the tumor microenvironment of CD8<sup>+</sup> T cells compared to non-EMAST CRC, suggesting that some immunological and inflammatory responses are active in EMAST CRC. They also found that EMAST is significantly high in ulcerated tumors. Devaraj *et al.*<sup>[43]</sup> 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.*<sup>[4,36]</sup> 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

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

<i>MLH1</i> promoter hypermethylation	22/35 (63%) of hypermutated CRCs	8/22 (36%) with MSH3 frameshift mutation 1/22 (4.5%) with MSH3 missense/nonsense mutation 0/22 (0%) with MSH2 mutation 5/22 (23%) with MSH6 frameshift mutation 4/22 (18%) with MSH6 missense/nonsense mutation
<i>POLE</i> mutation	13/35 (37%) of hypermutated CRCs	3/13 (23%) with MSH3 frameshift mutation 2/13 (15%) with MSH3 missense/nonsense mutation 5/13 (38%) with MSH2 missense/nonsense mutation 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; EMAS: Elevated microsatellite alterations at selected tetranucleotide repeats.

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 EMAS loci. However, other inflammatory cytokines including *TNF $\alpha$* , *IFN $\alpha$* , *IFN $\beta$* , and *IL1 $\beta$*  did not have such an effect. Tseng-Rogenski *et al.*<sup>[41]</sup> 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 EMAS 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.*<sup>[44]</sup>, 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.*<sup>[45]</sup>, 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.*<sup>[45]</sup> 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/EMAS<sup>[46]</sup>.



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 EFAST genotypes on patient prognoses in CRC. There have been 4 studies evaluating the prognosis values of MSI-L<sup>[47-50]</sup>. Kohonen-Corish *et al.*<sup>[47]</sup> 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.*<sup>[48]</sup>. 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<sup>[48]</sup>. They also observed that most MSI-L CRC exhibited MSI at one di- or tetra-nucleotide but not at mono-nucleotide repeat markers<sup>[48]</sup>. Lee *et al.*<sup>[49]</sup> 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<sup>[49]</sup>. Although the previous 2 studies suggested that MSI-L may have a significant prognostic value for stage C CRC patients, Lee *et al.*<sup>[49]</sup> 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.*<sup>[50]</sup> 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.*<sup>[51]</sup> did not find any association between MSI-L and disease-free survival (DFS) or OS in stage II and III CRC cohorts.

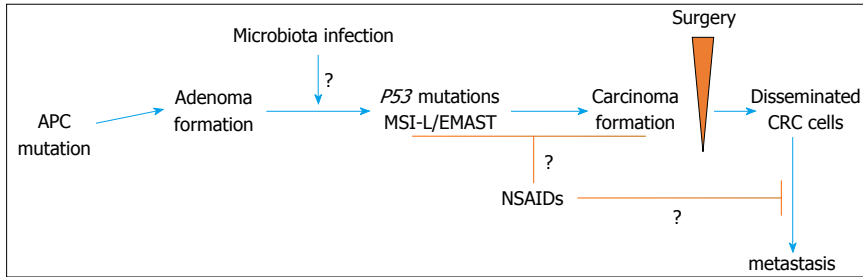
There are 2 studies examining the relationship between EFAST and OS in CRC; they found no association between the two<sup>[33,51]</sup>. However, when both MSI-L and EFAST cases were combined, Garcia *et al.*<sup>[51]</sup> found that MSI-L/EFAST was associated with shorter DFS but not OS compared with non-MSI-L/EFAST CRC. In their cohort, MSI-H CRC patients exhibited the highest survival. Thus, the MSI-L/EFAST genotype in CRC may be associated with recurrence and/or metastasis after surgery. There appears to be heterogeneity even among MSI-L/EFAST CRC patients<sup>[52,53]</sup>. One group of MSI-L/EFAST 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<sup>[53]</sup>. The results also showed that MSI-L/EFAST/9p24.2 LOH is an independent factor that predicts improved OS in stage II/III CRC. Thus, MSI-L/EFAST may be associated with recurrence, but additional genetic or epigenetic changes may modify the behavior of recurrent tumors<sup>[53]</sup>. Overall, the data presented so far suggest that MSI-L and/or EFAST 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 EFAST.

One concern regarding EFAST is that various studies have not reached a full consensus on the definition of EFAST. As described above, current evidence supports the idea that MSI-L and EFAST 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<sup>[4]</sup>. Based on these observations, we propose that EFAST cancer is a non-MSI-H cancer, and MSI at EFAST markers is not caused by loss of other MMR proteins including MLH1, MSH2, PMS2<sup>[51,53]</sup>. The next question should be whether or not non-EFAST CRC really exists. Similar to MSI-L in CRC<sup>[29,30]</sup>, almost all CRC could be EFAST-positive if a large number of EFAST markers are used. A recent study by Cortes-Ciriano *et al.*<sup>[54]</sup> 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 EFAST<sup>[46]</sup>. Thus, while the purpose of the MSI assay is primarily to detect MMR-deficient CRC, the purpose of an EFAST 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/EFAST 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 EFAST markers and cut-off levels for determining EFAST-positive/negative used so far are adequate to distinguish CRCs with different prognoses<sup>[31]</sup>.

## PROVOCATIVE QUESTIONS

Here, we have raised three questions whose answers can be important for not only clinical but also basic



**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; EMAS: Elevated microsatellite alterations at selected tetranucleotide repeats.

aspects of MSI-L/EMAST in CRC (Figure 3).

**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<sup>[55]</sup> and CRC formations<sup>[56]</sup>, but also prevent recurrence and metastasis of CRC following surgery<sup>[57]</sup>. 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<sup>[58-60]</sup>. But it was also found that COX-2 inhibitors suppressed colorectal tumor growth and metastasis in mouse models<sup>[61,62]</sup>. Furthermore, Chan *et al.*<sup>[63]</sup> 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<sup>[64]</sup>. Also, CRCs with *PIK3CA* mutations responded better to aspirin treatment after diagnosis than did CRCs with wild-type *PIK3CA*<sup>[65]</sup>. However, a recent study by Gray *et al.*<sup>[66]</sup> 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.*<sup>[67]</sup> 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<sup>[57]</sup>.

Ma *et al.*<sup>[68]</sup> 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 (*Ptges*)-knockout mice bore a significantly reduced number of colon tumors.

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- $\kappa$ B-COX-2 cycles, and produce TNF- $\alpha$  and IL6<sup>[68]</sup>. 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<sup>[69,70]</sup>. Interestingly, Zelenay *et al.*<sup>[71]</sup> 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 in cancer cells and/or in tumor-microenvironments. This could be the reason why patients with MSI-L/EMAST CRCs exhibit a shorter RFS<sup>[51,53]</sup>. Thus, reduction of PGE2 by NSAIDs may reduce the incidence and recurrence/metastasis of MSI-EMAST. If this is the case, MSI-L/EMAST could be a biomarker for susceptibility to the NSAIDs treatment.

**Question 2: Do microbiota play a role in MSI-L/EMAST formation, adenoma/carcinoma transition and recurrence/metastasis?**

Lee *et al.*<sup>[34]</sup> discovered that EMAS is less frequent in colorectal adenomas and well-differentiated adenocarcinomas than in moderately differentiated and poorly differentiated adenocarcinomas, suggesting that EMAS is progressively acquired during the histological adenoma-carcinoma sequence, from adenoma to well-differentiated carcinomas to moderately and poorly differentiated carcinomas. Because a key gene alteration responsible for adenoma-carcinoma sequence

in CRC is *p53* mutation<sup>[72]</sup>, MSI-L/EMAST formation may be associated with *p53* mutation. In fact, Ahrendt *et al.*<sup>[73]</sup> reported that EMAST is associated with *p53* mutations in non-small cell lung cancer. Li *et al.*<sup>[74]</sup> 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%)<sup>[75,76]</sup>. 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<sup>[75]</sup>. 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<sup>[77]</sup>. 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<sup>[77,78]</sup>. The enrichment of *F. nucleatum* was also found in colorectal adenoma relative to non-adenoma or surrounding tissues<sup>[79-81]</sup>. McCoy *et al.*<sup>[79]</sup> 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.*<sup>[80]</sup> investigated the effect of *F. nucleatum* infection on the development of intestinal tumors in *APC*<sup>Min/+</sup>, IL10<sup>-/-</sup> and T-bet<sup>-/-</sup> X Rag2<sup>-/-</sup> mice. There was an increase in the number of tumors in *APC*<sup>Min/+</sup> mice. Importantly, infection with *F. nucleatum* accelerated adenocarcinoma formation in the small intestines of *APC*<sup>Min/+</sup> mice compared to sham-treated control mice. In contrast, infection with *F. nucleatum* did not induce any tumor formation in IL10<sup>-/-</sup> and T-bet<sup>-/-</sup> X Rag2<sup>-/-</sup> 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<sup>[80]</sup>. It would be interesting to determine whether *F. nucleatum* -induced adenocarcinomas in *APC*<sup>Min/+</sup> mice gain *p53* mutations. Kostic *et al.*<sup>[80]</sup> 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 micro-environment that promote tumor progression. In

addition, they found the up-regulation of genes that are down-stream of NF- $\kappa$ B including *PTGS2* (*COX-2*), *IL6*, *IL1 $\beta$* , and *TNF* in both human and mouse CRC infected with *F. nucleatum*<sup>[80]</sup>. 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<sup>[81-84]</sup>. Yu *et al.*<sup>[85]</sup> 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<sup>[85]</sup>. One of the reasons why 5-FU-based adjuvant therapy does not have benefit for a sub-group of MSI-H CRC<sup>[86,87]</sup> could be partly explained by the infection of *F. nucleatum*<sup>[85]</sup>. 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<sup>[82]</sup>. 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<sup>[51,53]</sup>.

Another bacterium, *ETBF*, is also associated with CRC<sup>[88-90]</sup> and can target colorectal cells to promote an adenoma and/or adenoma-carcinoma transition in *APC*<sup>Min/+</sup> mice<sup>[91]</sup>. *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<sup>[91,92]</sup>. Th-17/IL-17 plays a major role in *ETBF* tumorigenesis because the depletion of CD4<sup>+</sup> 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<sup>[92]</sup>. Colibactin-producing *E. coli* is also associated with CRC<sup>[93,94]</sup> and initiates inflammation and promotes adenoma formation in *APC*<sup>Min/+</sup> mice<sup>[94]</sup> and in *APC*<sup>Min/+</sup>, IL10<sup>-/-</sup> mice<sup>[95]</sup>. 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.*<sup>[96]</sup> 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)<sup>[97-100]</sup>. 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 II $\beta$ -binding protein 1 (TOPBP1), which is recruited to the DSB site, interacts with ATRIP and activates ATR. Activated ATR phosphorylates CHEK2 that regulate cell cycle progression. TOPBP1 also interacts with polo-like kinase (PLK) that phosphorylate RAD51 for its loading on resected ssDNA<sup>[101]</sup>. Burdova *et al.*<sup>[99]</sup> showed that recruitment of ATR/ATRIP to RPA-coated ssDNA is mediated by MutS $\beta$  which binds to the loop structure formed within the ssDNA. Therefore, MutS $\beta$  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<sup>[98,100]</sup>. Thus, when oxidative stress causes DSBs, it may induce elimination of MSH3 from the nucleus, resulting in activation of Ataxia-telangiectasia mutated (ATM)<sup>[102]</sup> 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)<sup>[4,36]</sup>. H<sub>2</sub>O<sub>2</sub> and oxidative stress causes DSBs, resulting in activation of NF- $\kappa$ B<sup>[103,104]</sup>. IL6 and PGE2 are mediators that possibly form a loop associated with activation of NF- $\kappa$ B through STAT3<sup>[105-107]</sup>. IL6 activates STAT3, which directly interacts with the NF- $\kappa$ B family member RELA, contributing to constitutive NF- $\kappa$ B activation<sup>[105]</sup>, and COX2/PGE2 also activates STAT3, leading to NF- $\kappa$ B activation<sup>[106]</sup>. 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 main 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- $\kappa$ B Essential Modulator (NEMO), when used as a bait, can pull down MSH3, suggesting physical interaction between these two proteins<sup>[108]</sup>. As one of the three components of the IKK complex, NEMO's role in regulating the NF- $\kappa$ B pathway is well documented<sup>[104]</sup>. It is possible that simultaneous or sequential movement of MSH3, NEMO and ATM in

the cell may transmit a DNA damage signal to NF- $\kappa$ B, 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<sup>[109]</sup>, cervical<sup>[110]</sup>, pancreatic<sup>[111]</sup>, ovarian<sup>[112]</sup>, skin<sup>[113]</sup>, nerve<sup>[114]</sup>, breast, endometrial<sup>[115]</sup>, liver<sup>[116]</sup>, esophageal<sup>[117]</sup>, eye<sup>[118]</sup>, soft tissue<sup>[119]</sup>, gallbladder<sup>[120]</sup>, head and neck<sup>[121]</sup>, prostate<sup>[122]</sup>, lung<sup>[123]</sup> and cancers of the urinary tract<sup>[124]</sup>. EMAST has also been widely detected in other various human cancers<sup>[31]</sup>. A recent study by Cortes-Ciriano *et al.*<sup>[54]</sup> 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<sup>[54,125,126]</sup>. 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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