Fusobacterium nucleatum Infection in Colorectal Cancer: Linking Inflammation, DNA Mismatch Repair and Genetic and Epigenetic Alterations

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Abstract:
It has been recently reported that the population of Fusobacterium, particularly Fusobacterium nucleatum (Fn), is overrepresented in colorectal cancers (CRCs) and adenomas. The promoting effects of Fn infection on adenoma and/or carcinoma formation have been shown in ApcMin/+ mice. Characteristics of Fn-associated CRC were identified through studies using human CRC cohorts and include right-sided colon location, CpG island methylation phenotype (CIMP)-high, high level of microsatellite instability (MSI-H), and poor patient prognosis. A subset of Fn-associated CRC exhibits a low level of microsatellite instability (MSI-L) and elevated microsatellite alterations in selected tetra-nucleotide repeats (EMAST) induced by translocation of MSH3 from the nucleus to the cytoplasm in response to oxidative DNA damage or inflammatory signals. The association between CIMP/MSI-H and Fn infection can be explained by the role of the mismatch repair protein complex formed between MSH2 and MSH6 (MutSα) to repair aberrant bases generated by reactive oxygen species (ROS) to form 7,8-dihydro-8-oxo-guanine (8-oxoG). Clustered 8-oxoGs formed at CpG-rich regions including promoters by ROS is refractory to base excision repair. Under these conditions, MutSα initiates repair in cooperation with DNA methyltransferases (DNMTs) and the polycomb repressive complex 4. DNMTs at damaged sites methylate CpG islands to repress transcription of target genes and promote repair reactions. Thus, continuous generation of ROS through chronic Fn infection may initiate (1) CIMP-positive adenoma and carcinoma in an MSH2/MSH6-dependent manner and/or (2) MSI-L/EMAST CRC in an MSH3-dependent manner. The poor prognosis of Fn-associated CRC can be explained by Fn-induced immune-evasion and/or chemoresistance.

Keywords:
microsatellite instability-high (MSI-H), elevated microsatellite alterations at selected tetra-nucleotide repeats (EMAST), colorectal cancer (CRC), mismatch repair (MMR), Fusobacterium nucleatum, CpG island methylator phenotype (CIMP)

Introduction
Microbiota imbalances in the colon and rectum are associated with an inflammatory microenvironment and promotion of colorectal cancer (CRC)1,2. Several bacterial organisms including Fusobacterium nucleatum (Fn), Enterotoxigenic Bacteroides fragilis (ETBF), and colibactin-producing Escherichia col. (E. coli) are epidemiologically associated with CRC and have been found to be enriched in CRC1,2). Dejea et al showed that CRCs in the ascending colon and hepatic flexures but not on the left side of the colon are covered with invasive bacterial films3,4. The enrichment of Fn was found in colorectal adenomas relative to non-adenomas or surrounding normal tissues3,4. Recent studies further dem-
onstrated that the CRCs with a high load of Fn infection is associated with a high level of microsatellite instability (MSI-H) and a high level of CpG island methylation phenotype (CIMP-H) CRC and is associated with a right-side location and poor prognosis. There is also a subgroup of non-MSI-H CRC that exhibit microsatellite alterations in response to inflammatory tumor-microenvironment. For this review, we have compiled articles that deal specifically with Fn infection and CRC. On the basis of data in these articles, we discuss possible mechanisms for genetic and epigenetic change induced by Fn and the possible role of Fn in the prognosis of CRCs.

**Discovery of Fusobacterium in Colorectal Neoplasms**

Fn is one of 14 species belonging to the genus *Fusobacterium*. It is Gram-negative and anaerobic and is associated with various infections including periodontitis, otitis media, Lemierre’s disease, anamnestic fluid infections and inflammatory bowel disease. Fn is a common resident in the human gut mucosa and is one bacteria that colonizes CRC tumors more frequently than adjacent normal mucosa. Clear evidence for the abundance of Fn in CRC tumor tissues compared with nearby normal tissues was first reported by two studies in 2012. Kostic et al used a whole genome sequence approach where they identified microbial DNA sequences by subtracting human DNA sequences from total RNA reads. They found an enrichment of genus *Fusobacterium* in most CRC tissues (8/9 cases). They further amplified microbial 16S ribosomal-DNA and sequenced from 95 tumor/normal CRC pairs and also performed fluorescence in situ hybridization to show that *Fusobacterium* is enriched in tumor tissues. They identified Fn as the most enriched species among *Fusobacterium* species in CRC. Castellarin et al used an RNA-seq approach where human transcript sequences were subtracted from total RNA reads in 11 paired CRC tumor/normal tissues. They found enrichment of Fn in tumors (more than twofold) as compared with matching normal tissues (9/11 cases). They developed a quantitative PCR assay to determine Fn-specific copy numbers using the *nusG* gene of Fn and measured Fn copies in genomic DNA from 99 CRC tumor/normal tissues. Again, Fn was enriched in tumors compared with normal tissues. They also showed that Fn isolated from one CRC patient was invasive to cultured colon cancer cells. Finally, they showed that a high level of Fn in CRC was associated with lymph node metastasis. Later, an enrichment of Fn in CRC tumor tissues was found in cohorts from Japan, Europe, and China, indicating that this phenomenon is universal among human populations.

McCoy et al first described the association between *Fusobacterium* and colorectal adenoma. They compared the abundance of *Fusobacterium* in normal rectal mucosa isolated from subjects with adenoma (48 cases) and from subjects without adenoma (67 cases) and found that rectal mucosa from adenoma patients were significantly enriched in *Fusobacterium* compared with mucosa from subjects without adenoma. In contrast to McCoy’s study, Kostic et al directly quantified *Fusobacterium* spp. loads in adenomas and matched adjacent normal tissues from the same patient. They found significant enrichment of the *Fusobacterium* species in adenoma tissues (*P* < 0.004). Flanagan et al quantified Fn loads in 52 colorectal adenomas and nearby normal mucosa and found that there was a trend but no significant association between the Fn level and adenoma state. However, they found a significantly higher Fn load in high-grade dysplasia and in CRC, thus suggesting that the Fn level was associated with progression of adenoma to carcinoma. Although a higher level of Fn load was detected in adenoma and carcinoma tissues compared with adjacent normal tissues, carcinoma tissues contained more Fn than adenoma tissues. Similarly, Yu et al observed a gradual increase in the Fn load during the transition from adenoma to carcinoma. Taken together, these results suggest that Fn infection may be involved in progression from adenoma to carcinoma in some CRC cases.

**Mouse Model of Fn-associated Intestinal Tumorigenesis**

To evaluate Fn infection in intestinal tumorigenesis, Kostic et al used C57BL/6 Apc<sup>Min/+</sup> mice, which develop intestinal tumors because of an inactive mutation in one copy of the Apc gene. Oral introduction of Fn (13 of 15 mice) but not of control Streptococcus spp. (two of 12 mice) or tryptic soy broth (one of 20 mice) significantly increased the number of colonic tumors in Apc<sup>Min/+</sup> mice. Kostic et al also replicated observations found in human tissues that of enrichment of Fn in tumor tissues compared with surrounding normal mucosa and invasiveness of Fn to tumor tissues. Yu et al used both C57BL/6 and C57BL/6 Apc<sup>Min/+</sup> mice models. In the C57BL/6 model, mice were treated with a chemical carcinogen, 1,2-dimethylhydrazine (DMH), followed by introduction of Fn or control bacteria (*E. coli*). Introduction of Fn increased the number of DMH-induced aberrant crypt foci and tumors compared with introduction by *E. coli*. Similar to Kostic’s results, introduction of Fn increased the number of tumors in C57BL/6 Apc<sup>Min/+</sup> mice. Similarly, Yang et al showed that introduction of Fn into C57BL/6 Apc<sup>Min/+</sup> mice increased the number and size of tumors and shortened overall survival compared with the non-treated controls. Although these three studies suggest that Fn infection may promote loss of a wild-type copy of Apc in Apc<sup>Min/+</sup> colon cells to form adenoma, they do not give evidence that Fn infection may be involved in adenoma-to-
carcinoma transition in the colon and rectum. It would be interesting to see the genetic and epigenetic landscapes of the Fn-induced tumors.

In contrast to the three studies discussed above, Tomkovich et al did not see any tumor-enhancing effects of Fn on ApcMin/+ mice. They used germ-free (GF) or GF-derived, specific-pathogen-free (SPF) 129/SvEv ApcMin/+ mice, whereas other studies used SPF C57BL/6 ApcMin/+ mice. Thus, the mouse genetic background and/or different microbial exposures before and/or after Fn infection may change the susceptibility of ApcMin/+ mice to Fn. There was also a difference in the strain of Fn used by Tomkovich’s study as compared with others. These discrepancies highlight the possibility that (1) there may be individual genetic and/or epigenetic backgrounds that are susceptible for Fn-associated tumorigenesis and/or (2) there may be a select group of Fn that are a component of diverse microbiota associated with colorectal tumorigenesis.

Characteristics of Fusobacterium-associated CRCs

Following the discovery of the association between Fusobacterium infection and CRC, it was recognized that the infection is significantly associated with two subgroups of CRCs. The first group of CRCs exhibit a high level of CpG island methylation phenotype (CIMP-H) and/or a high level of microsatellite instability (MSI-H) and are located on the right side of the colon. The second group of CRCs exhibit a low level of microsatellite instability (MSI-L) and/or elevated microsatellite alterations in selected tetra-nucleotide repeats (EMAST) and are also located on the right side of the colon (unpublished data).

CIMP and MSI-H CRCs

Tahara et al found that 74% of tumor tissues from 149 CRC cases were tested positive for infection by the Fusobacterium species, including Fn (52.3%;78/149). Among infected CRCs, 14 (9.4%) and 8 (5.4%) cases were heavily infected with Fusobacterium spp. or Fn, respectively. They further showed that the Fusobacterium-high CRCs were enriched in MSI-H (P = 0.018), CIMP-positive (P = 0.001), hMLH1 methylation-positive (P = 0.0028), p53 wild-type (P = 0.015), and CDH7/8 mutant CRCs (P = 0.002) and located on the right side. Ito et al also showed that 56% of tumor tissues from 511 cases of CRC were positive for Fn infection. They divided Fn-infected groups into high and low on the basis of the median number of Fn bacteria present and found that Fn-high was associated with tumor tissues rather than premalignant tissues (P = 0.0001), larger tumor size (P = 0.0005), and CIMP-high (P = 0.0013). Mima et al also showed that a high load of Fn in CRC tissues was associated with MSI-H, CIMP, and BRAF mutation by univariate analysis but not by multivariate analysis adjusted for MSI-H.

The human cancer genome exhibits aberrant DNA methylation. Increased methylation of promoter region CpG islands is prominent in tumor DNA and is associated with transcriptional inactivation of tumor-suppressor genes. Toyota et al first systematically examined CpG islands that were differentially methylated between tumor and normal genomic DNA from CRC patients. They found a group of the CpG sites specifically associated with the cancer state in a subset of CRC and gave this phenotype the name CpG island methylation phenotype (CIMP). Thus, CRC can be divided into CIMP-positive (CIMP+) and CIMP-negative (CIMP). They further demonstrated that (1) CIMP is seen in a subgroup of adenomas; (2) CRC with MSI-H due to promoter hypermethylation of hMLH1 is a subgroup of CIMP+ CRCs; (3) CDKN2A and THBS1 are frequently methylated in CIMP+ CRCs; and (4) CIMP– CRC is associated with proximal site. Later, it was recognized that CIMP is not a dichotomous trait but rather continuous and can be divided into CIMP+, CIMP-intermediate, and CIMP–.

Sánchez-Vega et al analyzed methylation data from The Cancer Genome Atlas (TCGA) CRC cohort (274 cases: reference 27) using CIMP+, CIMP-intermediate, and CIMP categories and confirmed that a large portion of CIMP+ exhibit hypermethylation of hMLH1 (MSI-H) and are associated with the right side of the colon. They also showed that hypermethylation of MGMT is frequent in CIMP+ CRC and is associated with FBXW7, APC, and KRAS mutations.

Microsatellites, or simple sequence repeats, are composed of 1-6 repeated nucleotides. Microsatellite instability (MSI) is defined as continuous length changes in simple DNA repeats within microsatellite loci. MSI-H is caused by deficiencies in mismatch repair (MMR) genes including MSH2, MLH1, MSH6, and PMS2. As mentioned above, the MSI-H exhibited in 10-15% of sporadic CRC cases is due to transcriptional down-regulation of MLH1 expression through promoter hypermethylation.

CRCs have been divided into several subgroups on the basis of their genetic and epigenetic landscapes, their transcriptional or proteomic similarities or differences, and their immunological landscapes. In 2012, genetic and epigenetic landscapes of sporadic CRC identified through massive DNA/RNA sequencing were reported by the TCGA Consortium. One of the major findings was that there are two types of CRC that differ in respect to the frequency of somatic gene mutations. Sixteen percent of CRC showed a hypermutated genotype where three-quarters exhibited MSI-H because of MLH1 silencing by promoter-methylation, and one-quarter had somatic mutations in the MMR gene and polymerase ε (POLE).

The transcriptional landscapes in CRC can be divided into four distinctive subgroups: (1) the consensus molecular sub-
type 1 (CMS1) (14%) that is enriched in hypermutated and MSI-H CRC and characterized by gene expression with strong immune activation; (2) CMS2 (37%), marked with WNT and MYC signaling activation; (3) CMS3 (13%) with metabolic dysregulation; and (4) CMS4 (23%), marked with prominent transforming growth factor-β activation, stromal invasion, and angiogenesis. CRCs with mixed features (13%) also exist and may represent a transitional phenotype. Although most of MSI-H CRC is grouped into CMS1, some of them are grouped into CMS3 and CMS4. In proteomic subgrouping, there are five distinctive groups within the TCGA CRC cohort. Proteomic subtype B and C are enriched in MSI-H/CIMP and hypermutated CRC. These observations indicate that MSI-H/CIMP CRCs are heterogeneous in terms of m-RNA and protein expression. Angelova et al analyzed TCGA CRC data to identify a dominant immune cell subpopulation associated with a particular molecular subtype of CRCs. They found that tumor-infiltrating lymphocytes (TILs) from MSI-H CRC tumors are enriched by lymphocytes with antitumor functions including central and effector memory CD4+CD8+ cells and natural killer T cells, compared with TILs from MSS CRC.

**MSI-L/EMAST CRCs**

MSI in CRS was defined in 1998 at an international workshop meeting sponsored by the National Cancer Institute. In this meeting, MSI-L, low-frequency of MSI (MSI-L), and microsatellite stable (MSS) CRCs were defined using a specific panel of microsatellite markers. Although MSI-H is caused by the functional loss of MMR proteins, the etiology of MSI-L and the distinction between MSI-L and MSS CRC remained unclear until recently. Another type of microsatellite alteration, termed EMAST, where insertion/deletion mutations in the loci with tetra-nucleotide but not with mononucleotide and/or dinucleotide repeats, was recognized as a component of CRC. MSI-L and EMAST have been observed in many human cancers. Haugen et al examined the frequency of EMAST in CRC, its relationship to MSI-L, and its possible causes. They found that EMAST is frequent in sporadic cases of non-MSI-H CRC (~50%) and is associated with decreased nuclear MSH3 expression in tumor cells. Using isogenic MSH3-proficient and MSH3-deficient cell lines, they also showed that EMAST and MSI-L in non-MSI-H CRC cells are caused by loss of MSH3. The frequent incidence of EMAST in CRCs was confirmed by two other studies. The fact that EMAST is due to the loss of MSH3 was also proven by other studies using tissue cultured human cells. Later, Adam et al found patients with bi-allelic inactivation mutations at the MSH3 locus who suffered from colorectal adenoma polyposis syndrome. The adenoma polyps from these patients exhibited MSI at loci with dinucleotide repeats and EMAST loci but not at loci with mononucleotide repeats. These results strongly suggest that MSI-L/EMAST is caused by loss of the MSH3 function. The biochemical basis for MSI-L/EMAST formation through loss of MSH3 has been described elsewhere.

Immunohistochemical staining using anti-MSH3 showed that nuclear MSH3 is completely lost in adenoma polyps with bi-allelic germline MSH3 mutations, whereas localized loss of nuclear MSH3 is detected in sporadic CRCs exhibiting MSI-L/EMAST. These results suggest that loss of MSH3 expression in sporadic MSI-L/EMAST CRC may be due to a somatic epigenetic event. The frequency of MSH3 somatic mutations in CRC is about 6.6%, which does not explain the high incidence of MSI-L/EMAST (~50%) in CRC.

Lee et al first showed evidence that inflammation may be linked to MSI-L/EMAST in CRC. They found that EMAST CRC is enriched in CD8+ T cells in the tumor microenvironment compared with non-EMAST CRC. They also found that EMAST is significantly high in ulcerated tumors. These results suggest that some immunological and inflammatory responses are active in EMAST CRC. Later, Tseng-Rogenski et al demonstrated that in several cancer cell lines, inflammatory factors including oxidative stress (hydrogen peroxide), interleukin-6 (IL6), and prostaglandin E2 (PGE2) induce displacement of MSH3 from the nucleus to the cytoplasm, whereas the other MMR proteins do not displace. Repeated treatment of microsatellite stable colon cancer cell lines with IL6 induced EMAST. These studies convincingly showed that some inflammatory factors induce EMAST through loss of MSH3 in the nucleus.

Evidence that the inflammatory microenvironment induces MSI-L had been found in regenerated colon tissues from ulcerated colitis (UC) patients. Brentnall et al showed for the first time the presence of MSI-L but not MSI-H in colon tissues from UC patients. Ozaki et al also examined crypts from UC-derived CRC, UC-derived hyperplasia, and UC-regenerated colons and tested them for the presence of microsatellite instability. They again detected MSI-L but not MSI-H in some crypts but not in stroma cells regardless of whether they were from cancer or non-cancer tissues. Recently, we have shown that regenerated colon epithelial cells and tumors from UC patients show a high frequency of MSH3 displacement from the nucleus to the cytoplasm and of MSI-L /EMAST. These results support the role of inflammation in displacement of MSH3, which induces MSI-L/EMAST in human tissues including cancers.

Our recent investigations not only confirmed that Fn is associated with MSI-H but also showed that Fn is associated with MSI-L/EMAST when compared with non-MSI-H, non-MSI-L/EMAST CRC (unpublished data). These findings raise the possibility that inflammatory factors generated in tumor-microenvironments in response to Fn infection and/or
Fn itself may induce translocation of MSH3 from the nucleus to the cytoplasm in colon tumor cells, resulting in MSI-L/EMAST.

**Poor Prognosis for patients with Fn-associated CRCs**

Increased loads of Fn are associated with advanced CRC stages, and with poor prognoses. Mima et al showed that colon cancer-specific death, but not overall survival, is associated with high levels of tissue Fn. Yu et al looked at the relationship between CRC recurrence and microbiota alterations in the gut. They found that Fn is the most enriched bacteria species in tumors from recurrent CRC compared with tumors from non-recurrent CRC. Using additional cohorts, they showed that the higher load of Fn is an independent determinant and predictor of recurrence-free survival (RFS). Yan et al also showed that a high level of Fn in stages III and IV CRC is associated with shorter cancer-specific survival and shorter RFS. They also observed that adjuvant chemotherapy is more effective in preventing recurrence in stage III patients with a low level of Fn compared with those with a high level of Fn. Bullman et al showed that patients from TCGA cohorts who suffered from right-side colon cancer with a high load of Fn infection had poor overall survival compared with patients with a low load of Fn infection. Interestingly, they detected Fn infection not only in primary CRC but also in matching liver metastasis. They also demonstrated that antibiotic treatment not only reduced the *Fusobacterium* load but also inhibited growth of xenograft tumors from patients, suggesting that infection by *Fusobacterium* accelerates tumor growth in vivo.

**Possible Explanation of Why *Fusobacterium* Infection is Associated with CIMP CRC**

CIMP has been detected in many human cancers, but its causes are not clear. Several observations suggest that infection by microorganisms and/or inflammation associated with microorganism infection may induce CIMP in tumor genomes. For instance, in gastric cancers, there are two types of CIMP tumors: one is associated with Epstein-Barr virus (EBV) infection, and another is associated with MSi-H (hMLH1 promoter hypermethylation). Infection of Helicobacter pylori in gastric cells also induces inflammation that causes aberrant DNA methylation. In fact, when human cell lines were co-cultured with Fn *in vitro*, expression of pro-inflammatory genes including TNF, IL8, and IL1β as well as ROS was induced. Kostic et al analyzed transcriptome sequences from 133 cases of CRC in the TCGA data set to identify the genes associated with *Fusobacterium species* infections. They found that the expression of inflammatory response genes including IL1β, IL24, PTGS2 (COX-2), IL8, IL6, and TNF were enriched in *Fusobacterium*-infected CRCs. Importantly, these gene signatures were specific to *Fusobacterium* but not to other bacteria detected in CRC tissues including *Bacteroides*, *Escherichia*, *Streptococcus*, and *Propionobacterium*. They further demonstrated that Fn-induced tumors in Ape mice express elevated levels of mouse homologs of PTGS2, IL8, IL6, and TNF genes compared with tumors not induced by Fn. Finally, they showed that NF-xB, which drives the pro-inflammatory response, was activated in Fn-enriched CRC tissues. Similarly, Yang et al reported that Fn-infection activates Toll-like receptor 4 (TLR4)/NF-xB axis in cultured cells, and NF-xB is highly activated in Fn-enriched CRC tissues. These observations strongly suggest that the tumor-microenvironment of Fn-infected CRC is highly inflammatory.

One of the factors that would be constantly generated by chronic *Fusobacterium* infection and could be responsible for an aberrant DNA methylation is ROS. Although there are many cases where ROS is associated with DNA hypermethylation, until recently, there has been no direct evidence that oxidative DNA damage causes a genome-wide hypermethylation of promoter CpG islands and hypomethylation of CG sites at other parts of the genome. The aberrant base, 7,8-dihydro-8-oxo-guanine (8-oxoG), is the most abundant DNA modification generated by ROS. Therefore, 8-oxoG would be enriched at promoter CpG islands within the whole genome after ROS exposure. Base excision repair (BER) is primarily responsible for removing 8-oxoG; however, clustered 8-oxoG lesions are known to be refractory to BER. Zlatanou et al first reported that MutSα, the heterodimer formed between the MMR proteins MSH2 and MSH6, recognizes and initiates removal of the clustered 8-oxoG in cooperation with mono-ubiquitinated PCNA and DNA polymerase eta. Independently, O’Hagan et al discovered that a large protein complex consisting of DNA methyltransferase I (DNMT1), DNMT3B, and a member of the polycomb repressive complex 4 (PRC4), linked to transcriptional silencing, tightly binds to a promoter CpG island of the expressed gene when the island is damaged by ROS. Binding of this complex leads to a reduction of gene expression and induces DNA methylation at the CpG islands of the target gene. Ding et al successively demonstrated when MutSα recognizes and repairs 8-oxoG, it also recruits DNMT1 to the damaged site. Taken together, these results support the idea that enriched 8-oxoG at the promoter CpG islands attracts MutSα, and at the same time, DNMTs and PRC4 are recruited to reduce the transcriptional activity of the target gene through promoter methylation during MutSα-directed repair. This imbalance in DNMTs/PRC4 localization between promoter and non-promoter CpG sites explains how genome-wide hypermethylation of promoter CpG islands, CIMP, and hypomethylation of CG sites in other parts of the genome develop in response to ROS dam-
age in the cancer genome. Finally, this mechanism has been proven by Maiuri et al using a mouse model of colitis-associated CRC\(^{36}\). They showed that inflammation-induced tumors gained DNA hypermethylation at CpG islands in an MSH2-dependent manner\(^{36}\). They used Min\(^{APC^{Δ716-721}}\) and MSH2\(^{VC/Min}\) mice and inoculated mice with Bacteroides fragilis (ETBF) to generate colitis-associated CRC\(^{37}\). They found that ETBF-induced tumors had more hypermethylated regions and fewer hypomethylated regions in their genome compared with ETBF-mock tumors. They observed a transient increase in the amount of 8-oxoG in colon epithelium cells from ETBF-infected mice and a synchronous reduction of gene expression from the loci that were hypermethylated in ETBF-induced tumors. They detected MSH2 interacting with DNMTs and components of PRC4 in ETBF-inflamed colon cells. Lastly, they demonstrated that loss of MSH2 reduces CpG island hypermethylation in ETBF-induced tumors\(^{36}\).  

Although the ETBF-induced mouse CRC model highlights a casual role of ROS in the CIMP phenotype, whether Fn-infection also activates MSH2/MSH6-dependent hypermethylation of promoter CpG islands in CRC remains to be determined (Figure 1).  

**Possible Explanation of Why Fusobacterium Infection is Associated with MSI-H CRC**

Although the CpG island of the MLH1 promoter would be one of many sites damaged by ROS and methylated by DNMTs/PRC4, resulting in MSI-H (Figure 1), it is not clear why inactivation of this locus has an advantage for CRC initiation triggered by inflammation. One possible explanation is that silencing MLH1 may aid the damaged cells in escaping apoptosis induced by oxidative stress. Hardman et al showed that MLH1-deficient cells are more resistant to the cytotoxic effects of hydrogen peroxide or tert-butyl hydroperoxide than are MLH1-proficient cells. They observed a lack of apoptotic events including increased mitochondrial
permeability, release of cytochrome c, and caspase 3 activation in MLH1-deficient cells after exposure to hydrogen peroxide. Yanamadala et al confirmed Harman’s results and showed that apoptosis induced by hydrogen peroxide is MLH1-dependent and associated with global inhibition of m-RNA. Chen et al found that MLH1 is cleaved by caspase-3 during apoptosis activated by DNA damage and that this cleaved MLH1 product plays a role in the execution of apoptosis. Thus, for colonic epithelial cells continuously exposed to ROS by chronic infection of Fn, silencing MLH1 would be one way to avoid apoptosis.

**Fusobacterium Infection is Associated with MSI-L/EMAST**

As mentioned above, cumulative evidence supports the idea that the etiology of MSI-L/EMAST formation in cancer genome is the inflammation-induced loss of nuclear MSH3 in dividing cells. Therefore, it is reasonable to speculate that a subgroup of MSI-L/EMAST CRC may be associated with pathogenic infections of microbiota including *Fusobacterium*. We have examined a CRC cohort for the MSI-H and MSI-L/EMAST genotypes and for the level of Fn in each CRC case. We have confirmed that Fn-positivity is associated with MSI-H compared with non-MSI-H. We have also found that Fn-positivity is significantly associated with MSI-L/EMAST as opposed to non-MSI-H and non-MSI-L/EMAST CRC (unpublished data). We are currently determining whether direct infection by Fn induces MSI-L/EMAST in tissue cultured cells and/or in mouse models (Figure 1).

Furthermore, it is possible that MSI-L/EMAST CRC also exhibits CIMP. In support of this assumption, a subset of CIMP CRC exhibits hypermethylation of MGMT, which is associated with MSI-L CRC.

**Possible Explanation of Why Fusobacterium Infection is Associated with Poor Prognosis of CRC**

**Enhancement of Tumor Cell Growth and Survival**

Yang et al demonstrated that Fn directly promotes CRC cell growth and survival in vitro and in vivo. Fn activates NF-κB through TLR4 signaling, resulting in up-regulation of microRNA-21 that could be a marker for poor clinical outcomes for CRC patients. Fn secretes adhesion and FadA through which attachment and invasion of Fn to a host cell occurs. Rubinstein et al showed that FadA binds to E-cadherin on the host cell and activates β-catenin, resulting in oncogenic and inflammatory responses. They observed that (1) Fn and purified FadA stimulate cell growth in several CRC cell lines in an E-cadherin-dependent manner; (2) FadA binds to E-cadherin on CRC cells and promotes Fn attachment and invasion via E-cadherin; (3) the binding of FadA to E-cadherin initiates β-catenin nuclear translocation and increases transcription of the downstream oncogenic pathway including T cell factors and Myc and cyclin D genes. Further internalization of the FadA/E-cadherin complex by endocytosis also activates inflammatory NF-κB signaling. Thus, FadA on the surface of Fn stimulates tumor growth through bacterial attachment but also triggers the inflammatory response through bacterial invasion. There are many other uncharacterized Fn proteins that may control host-pathogen interactions. Further studies are required to identify and characterize these proteins in relation to CRC carcinogenesis.

**Immune evasion**

CRC infected with a high load of Fn is associated with MSI-H and poor prognoses. However, it is widely accepted that the prognosis of MSI-H CRC is better than for non-MSI-H CRC. Popat et al showed that MSI-H CRC exhibits better overall survival than does non-MSI-H CRC through meta-analysis of 32 studies examining 7642 cases including 1277 MSI-H tumors. As mentioned above, the tumor-microenvironment of MSI-H CRC is enriched in lymphocytes with antitumor functions including CD4+CD8+ T cells and natural killer (NK) cells. A high immunoscore based on the abundance of CD3+ and CD8+ T cells at the central and invasion fronts of tumor tissues is associated with improved prognosis, and the majority of MSI-H cases have this high immunoscore. The reason for the improved prognosis of MSI-H CRC compared with non-MSI-H CRC is the presence of an antitumor T cell population in the tumor-microenvironment, elicited by continuous generations of frameshift neoantigens in hypermutated MSI-H CRC. However, Mlecnik et al showed that MSI-H CRC is heterogeneous and consists of at least two subgroups; the major group exhibiting a positive prognosis with high T cell activity and the minor group exhibiting poor prognosis with reduced T cell activity. Therefore, it could be that CRC with a high Fn load is a subgroup of MSI-H CRC with reduced T cell activity. Compatible with this assumption, Mima et al showed that CRCs with high levels of Fn exhibited a reduced density of CD3+ T cells in their tumor-microenvironments.

Reduced T cell activity in the tumor-microenvironment can be achieved by cancer cells through loss of HLA expression and/or disabling the antigen-processing machinery (APM). In fact, alterations in the HLA complex containing B2M and APM occur more frequently in MSI-H CRCs. Thus, this immune evasion mechanism may partially explain the immunological heterogeneity seen in MSI-H CRC.

MSI-H tumors that have lost HLA or APM function are still targets of NK cells. Fn secretes several proteins in-
tions

TIGIT (T cell immunoglobulin and ITIM domain) interaction, cells from cytotoxic attacks of NK and/or T cells via Fap2-TIGIT (T cell immunoglobulin and ITIM domain) interactions. The NK cell activity is controlled by inhibitory and activating NK receptors. TIGIT is one of the inhibitory NK receptors, expressed in all NK cells. When its ligands (for instance, Fap2) bind to TIGIT, NK cells stop killing their target cancer cells. Copenhagen-Glazer et al demonstrated that Fap2 co-aggregates other microorganisms and attaches to the mammalian cell surface by binding to galactose- and N-acetyl-d-galactosamine (Gal/GalNAc). Abed et al showed evidence that enrichment of Fn in CRC tumor tissues, as opposed to normal tissues surrounding these tissues, may be due to selective binding of Fap2 to Gal/GalNAc, which is overexpressed on the surface of tumor cells. Thus, cancer cells coated by Fap2 would be protected from an NK attack.

Chemoresistance

Yu et al convincingly demonstrated that a higher load of Fn in CRC is associated with cancer recurrence after surgery. They reasoned that chemoresistance is a major factor for recurrence and proved that Fn induces cancer cells’ chemoresistance through activation of autophagy. We previously showed that stage II/III MSI-L/EMAST CRC exhibit a shorter RFS compared with MSI-H or non-MSI-H, non-MSI-L/EMAST CRCs. Because MSI-L/EMAST CRCs are infected by Fn, it is tempting to speculate that a majority of Fn-associated CRCs that exhibit poor prognoses, especially shorter RFS, could be MSI-L/EMAST CRCs but not MSI-H CRCs. Considering that only 4% of stage IV CRCs are MSI-H, a majority of Fn-infected CRCs with poor prognoses could be MSI-L/EMAST. These possibilities are under intensive investigation.

Conclusion

It is still questionable whether Fn and/or other bacterial infections cause CRC. However, cumulative evidence shows that Fn infection affects the course of CRC carcinogenesis linked to DNA repair and genetic/epigenetic alterations. In conclusion, Fn-associated CRCs are located on the right side of the colon, are inflamed by ROS, and are associated with CIMP/MSI-H and MSI-L/EMAST. Fn-associated CRCs exhibit shorter RFS. Mechanistically, DNA damage caused by ROS triggers MSH2/MSH6-dependent repair that results in CIMP and in MSI-H when MLH1 is silenced, while it also induces translocation of MSH3 from the nucleus to the cytoplasm, resulting in MSI-L/EMAST. Poor prognoses for patients with Fn-associated CRC could be explained by Fn-induced chemoresistance and/or immune evasion and/or immune suppression. It would be worthwhile to conduct further studies, especially into the detection, prevention, and treatment of Fusobacterium.

Acknowledgements

This work was supported by the United States Public Health Service (DK067287 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.

Conflicts of Interest

There are no conflicts of interest.

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Acknowledgements

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