

IN FOCUS

Colorectal Cancer: Looking for Answers in the Microbiota

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Summary: At a simplistic level, colorectal cancer arises from mutations in various proto-oncogenes and tumor suppressor genes. Aside from genetically inherited factors, environmental, lifestyle, and dietary habits have all been identified as risk agents promoting mutational events leading to the development of colorectal cancer. This “In Focus” presents evidence that the intestinal endogenous bacterial community represents a risk factor for the development of colorectal cancer. *Cancer Discov*; 3(4); 384–7. ©2013 AACR.

INTRODUCTION

Colorectal cancer is the third leading cause of cancer incidence, with an estimated 1,000,000 new cases per year worldwide and a significant mortality rate of nearly 33% in the developed world. Although integration of genomic, proteomic, and metabolomic approaches has provided significant advances in cancer prevention, detection, and therapy, translating into important improvements in patient survival and quality of life, colorectal cancer still represents an important health and socioeconomic burden in the United States. A low proportion of colorectal cancer cases are inherited (e.g., Lynch syndrome, familial adenomatous polyposis, and MUTYH-associated polyposis), but the vast majority of cases (~90%) are attributed to sporadic mutations in various genes controlling cellular proliferation, survival, differentiation, and migration. In addition to host genetics, environmental factors such as dietary habits and lifestyle influence colorectal cancer development. Understanding the contribution of these various environmental factors could provide new means to prevent the development of colorectal cancer or at least to help manage the pathology. It is interesting to note that the most ubiquitous environmental factor, the luminal microbiota, has been the subject of only sparse research. A bacterial community ranging from 10^{13} to 10^{14} organisms that contributes a collective genome evaluated at 3×10^6 genes resides in the colon. This microbial ecosystem lives in relatively close proximity to the intestinal epithelium and contributes essential functions involved in the maintenance of host homeostasis. Recent evidence based on metagenomics and experimental models has highlighted the potential contribution of the microbiome in modulating colorectal cancer development. Here, I discuss the interplay between microbes and colorectal cancer development and highlight key findings supporting the role of symbionts in this pathology. Because chronic inflammation represents a significant

risk factor for colorectal cancer, the literature is discussed predominantly in light of the experimental model of colitis-associated cancer (CAC).

MICROBIAL MOLECULAR PATTERNS AND PATTERN RECOGNITION RECEPTORS

The contribution of bacteria in the development of colorectal cancer could be investigated indirectly using a proxy approach focusing on the role of receptors responsible for sensing microorganisms and their associated ligands. In the Eukaria domain, a vast repertoire of receptors termed pattern recognition receptors (PRR) recognize specific conserved microbial patterns (bacterial cell walls, nucleic acids, motility apparatuses, etc.). The most studied PRRs in relation to colorectal cancer belong to the group of nucleotide-binding domain leucine-rich repeat proteins (NLR; also known as Nod-like receptors), and Toll-like receptors (TLR). Following microbial sensing, these PRRs engage a complex set of signaling proteins that shape the host immune and inflammatory response. The goal of this innate/immune response is to protect the host from any deleterious effects caused by microorganisms. Many of these sensors are expressed on the intestinal epithelial cells and on various mucosal immune cells, 2 important cellular compartments implicated in colorectal cancer development.

In a chemical model in which tumorigenesis is initiated with the colon-specific carcinogen azoxymethane (AOM) and colitis with dextran sodium sulfate (DSS), investigators have determined the impact of various PRRs in CAC. For instance, AOM/DSS-induced colonic tumors increased in *Tlr2*^{-/-} mice, whereas they decreased in *Tlr4*^{-/-} mice compared with control wild-type mice. The differential outcome on tumor development may be related to the different microbial pattern detected by these PRRs [lipopolysaccharide (LPS) vs. peptidoglycan]. The mechanism by which TLR4 favors colorectal cancer development is still unclear, but recent findings generated in *Apc*^{Min/+} (multiple intestinal neoplasia allele) mice suggest that defective barrier function favors LPS translocation and activation of mucosal immune cell-derived cytokines such as interleukin (IL)-23, IL-6, and IL-17A (1). This unique inflammatory milieu favors the progression of cancer-initiated cells and the development of adenocarcinomas.

With the exception of TLR3, most TLRs including TLR2 and TLR4 signal through the adaptor protein myeloid

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differentiation factor 88 (MyD88). Deletion of *Myd88* diminishes the development of spontaneous colorectal cancer in *Apc^{Min/+}* mice, reducing both intestinal tumor size and multiplicity. In contrast, AOM/DSS-induced colonic tumors increased in *Myd88^{-/-}* mice compared with wild-type mice, an effect linked to an impaired wound healing response due to defective IL-18/MyD88 signaling. These data suggest that host recognition of microbial entities through TLRs differentially affects colorectal cancer development. As functional polymorphisms in the human *TLR2* and *TLR4* genes have been shown to be associated with colorectal cancer risk (2), these findings may have important translational implications.

PRRs of the NLR family including NOD1, NOD2, NLRP3, NLRP6, and NLRP12 have also been investigated for their involvement in colorectal cancer. Importantly, polymorphisms in some of these PRR genes such as *NLRP3* and *NOD2* have been linked to the development of human inflammatory bowel diseases (IBD) or colorectal cancer, respectively. NOD2, an intracellular NLR detecting both gram-positive and gram-negative bacteria, seems essential for a proper healthy host/bacterial interaction. Indeed, *Nod2*-deficient mice have a dysbiotic microbiota, a microbial state characterized by an unbalanced ratio of bacteria compared with eubiosis, the default normal microbial state found in a healthy host. Interestingly, *Nod2*-deficient mice are more susceptible to AOM/DSS-induced colorectal cancer (3). The intracellular NLR NOD1 that detects peptidoglycan has also been shown to play a protective role against the development of colorectal cancer. In AOM/DSS or spontaneous colorectal cancer (*Apc^{Min/+}*) models, tumor development was augmented in *Nod1^{-/-}* mice compared with wild-type mice, a phenomenon attenuated with broad-spectrum antibiotic treatment. Although both NOD1 and NOD2 are protective against the development of experimental colorectal cancer, it is not clear whether these NLRs operate through unifying mechanisms. The intracellular sensors NLRP3 and NLRP6 also seem to be protective against colitis-associated colorectal cancer, a role attributed to the production of IL-18. A recent report showed that NLRP3/6-induced IL-18 expression was essential in regulating the expression of IL-22-binding protein (IL-22BP), a critical negative regulator of the pro-proliferative cytokine IL-22 (4). Finally, NLRP12 signaling from the hematopoietic compartment protects against the development of colitis-associated colorectal cancer. From these findings, it appears that PRRs are important modulators of colorectal cancer development, with intracellular NLRs being mostly protective, whereas TLRs, with the exception of TLR2, may be procarcinogenic. The mechanism responsible for the protective function is not entirely clear, but production of IL-18, a protein-controlling cellular proliferative response, seems to be a central component of the PRR-mediated effect.

Although these findings have greatly contributed to our understanding of the role of innate sensors in the development of colorectal cancer, they provide minimal insight into the impact of microorganisms. Indeed, some of the PRRs discussed above have no identified ligands (NLRP6, NLRP12) or alternatively detect a wide range of patterns that make an association with specific microorganism a daunting task. For example, TLR2 and TLR4 respond to nonmicrobial ligands such as HSPs and histones, endogenous components often

released in the environment in response to stress and cellular injury, as seen in inflammatory conditions. Therefore, assessing the contribution of microorganisms to colorectal cancer development using mice with various PRR gene deletions is fraught with limitation.

THE MICROBIOME

For decades, the field of host/bacterial interaction in the gastrointestinal tract has mostly focused on the study of enteropathogenic bacteria. Indeed, tremendous efforts have been directed at understanding the molecular mechanisms by which certain pathogens cause gastrointestinal illnesses. Although of prime public health importance, enteropathogenic bacteria represent a small proportion (3%) of the total microbial community (~7,000 strains). Moreover, most of these microorganisms are not natural residents of the gastrointestinal tract, and the transient nature of their passage in the intestine rarely affects long-term health status once eliminated. With more than 100 trillion microorganisms in the gastrointestinal tract, most of them found in the colon, researchers have begun to question the potential implication of these symbionts in health and disease. This field of research is relatively new and stems from a worldwide effort to catalog the microbiome at different human biologic sites, including the gastrointestinal tract. Using next-generation sequencing and taxonomic studies based on ribosomal 16S bacterial genes, a clearer picture has emerged regarding the identity of the microorganisms inhabiting our intestine. At the phylum level, 95% of the gastrointestinal tract is dominated by *Firmicutes* (~75%) and *Bacteroidetes* (~20%), followed by *Proteobacteria* and *Actinobacteria*. Although a healthy core microbiome has not been clearly identified, studies using cases and controls have provided new insights into changes in microbial composition, a phenomenon referred to as dysbiosis. Applying microbiome analysis of tissues and fecal materials, researchers have identified various microbial groups associated with colorectal cancer. Although these studies have not identified a consensus group of bacteria associated with colorectal cancer, these investigations have systemically shown differences between the microbiome of patients and the one present in healthy subjects. For example, the stool of patients with colorectal cancer harbors a larger population of bacteria belonging to the group *Bacteroides-Prevotella* compared with normal controls (5). Another study showed that the genera *Enterococcus*, *Escherichia/Shigella*, *Klebsiella*, *Streptococcus*, and *Peptostreptococcus* were significantly more prevalent in the luminal compartment of patients with colorectal cancer than in controls, whereas the family *Lachnospiraceae*, which contains butyrate-producing bacteria thought to exert intestinal protection function, was less abundant (6). At the intestinal mucosal surface, an increased abundance of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* was observed in patients with adenoma compared with nonadenoma subjects (7). Using resected tissues from patients with adenocarcinoma and adjacent nonmalignant sites, another group showed expansion of the phyla *Bacteroidetes* and reduction of *Firmicutes* in patients compared with controls (8). At the genera level, increases in *Coriobacteridae*, *Roseburia*, *Fusobacterium*, and *Faecalibacterium* were observed

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in this study, whereas the family *Enterobacteriaceae* decreased. Interestingly, an expansion of *Fusobacterium* in rectal swab samples from patients with colorectal cancer compared with healthy controls was also reported by another group (9). In addition, using whole-genome sequencing and RNA-sequencing approaches, 2 independent groups have shown that *Fusobacterium* is highly prevalent in colonic tissues of patients with colorectal cancer compared with normal controls (10, 11). As the presence of *Fusobacterium nucleatum* correlates with the development of IBD, this bacterium could provide a potential link between the development of IBD and colorectal cancer. Unfortunately, despite the extensive amount of effort dedicated to surveying the microbiomes of colorectal cancer patients and normal subjects, these findings remain essentially correlative and caution is warranted about interpreting these data. In the absence of longitudinal analysis and functional experiments using experimental models, microbiome changes in patients with colorectal cancer could simply represent a consequence of environmental changes caused by neoplastic lesions. In addition, it is not clear which environmental factors contribute to the expansion or contraction of these various microbial entities.

To circumvent the limitations of human microbiome surveys, novel studies using germ-free mice and gnotobiotic technology have been initiated, and the functional impact of certain bacteria on the development of colorectal cancer has been reported. The *Apc^{Min/+}* mouse represents a popular model of human familial adenomatous polyposis and develops tens to hundreds of intestinal adenomas when maintained in regular housing conditions. Interestingly, *Apc^{Min/+}* mice raised in germ-free conditions show a reduction in tumor load in both the small intestine and the colon compared with mice housed in specific pathogen-free conditions (12). It would be interesting to investigate the functional impact of bacteria associated with human colorectal cancer (e.g., *Fusobacterium*, *Escherichia coli*, and others) using germ-free *Apc^{Min/+}* mice. The enterotoxigenic *Bacteroides fragilis* (ETBF) promotes the development of colorectal cancer in *Apc^{Min/+}* mice, an effect caused by the production of *B. fragilis* toxin. The prevalence of ETBF appears higher in the stool of patients with colorectal cancer compared with controls, suggesting a potential role of this bacterium in colorectal cancer pathology. Patients with IBD or colorectal cancer have an increased number of adherent-invasive *E. coli* on their mucosal surface compared with normal controls. Interestingly, a recent study showed that luminal proteobacteria, especially *E. coli*, expand during the development of experimental colitis in *Il10^{-/-}* mice (13). Using a murine adherent-invasive *E. coli* strain (NC101), the authors showed that this bacterium promotes the development of colorectal cancer through the action of a genotoxic island named polyketide synthases. Another important observation from this study is that although intestinal inflammation developed in monocolonized *Enterococcus faecalis* *Il10^{-/-}* mice, these mice failed to develop tumors as observed in *E. coli*-associated *Il10^{-/-}* mice. This finding showed in an experimental model that (i) chronic intestinal inflammation alone, an established risk factor for colorectal cancer, is not sufficient to promote tumors, and (ii) microbial composition is an important environmental factor in the pathology of colorectal cancer.

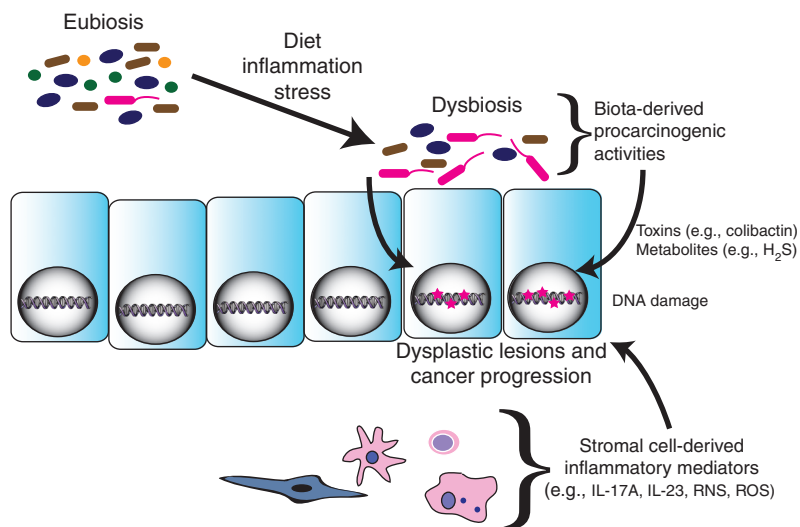
FUTURE DIRECTIONS

Although infectious microorganisms such as *Streptococcus bovis*, *Chlamydia trachomatis*, and *Helicobacter pylori* have been associated with certain types of cancer, the potential implication of symbionts or nonpathogenic bacteria in the development of tumorigenesis is a relatively new concept. Human microbiome studies conducted in patients with colorectal cancer at different stages have provided novel insights into microbial communities living at various ecological sites. These studies have offered potential links between microbial entities and the development of cancer. In addition, proof-of-principle studies in preclinical models have shown that bacteria impact the development of colorectal cancer. Although the findings are sparse, novel mechanisms have been proposed to explain the procarcinogenic effect of some of these microbes.

Although it is still in its infancy, cancer microbiome investigation opens a new era for colorectal cancer research and a new set of questions has been generated from these investigations. For example, is there a specific group of microorganisms responsible for cancer initiation, progression, and even metastasis? How do microorganisms influence cancer development? Could microbial-derived metabolites or proteins affect cancer susceptibility and progression? How do environmental factors such as stress, diet, and lifestyle modulate microbial activities to influence colorectal cancer development? Could biomarkers be generated from microbiome research? Is efficacy of current therapeutic modalities (radiation, immunotherapy, surgery) influenced by the microbiome? Could the microbiome be manipulated (e.g., by pre- and probiotics or bacteriophages) for therapeutic purposes? Addressing these questions would undeniably contribute to our understanding of the interplay between the microbiome and cancer development. Nevertheless, from the current state of knowledge, one could hypothesize that the microbiota is subjected to internal/external pressure (e.g., diet, stress, inflammation, and others) affecting cancer development/progression through production of microbial-derived carcinogenic products (e.g., Colibactin, H₂S, and others; Fig. 1). This bacterial contribution occurs in the background of host responses to these environmental cues, resulting in the production of different immune and stromal cell-mediated procarcinogenic mediators such as cytokines (IL-17A, IL-23, IL-6, and others), radical oxygen species, and radical nitrogen species.

Although still a work in progress, existing data linking microbes to colorectal cancer provide a strong rationale to pursue investigation into the role of the microbiota in cancer development. However, the microbiome encompasses numerous microscopic entities besides bacteria, including viruses and fungi. It is reasonable to speculate that components of this complex ecosystem interact with each other, their environment, and the host to impact intestinal homeostasis, which poses an enormous challenge to researchers. Metagenomic studies on viruses and fungi have already shown their potential effect on gastrointestinal health (14, 15). Nevertheless, it has become clear that taxonomy-based investigations using a ribosomal 16S approach will not be sufficient to provide a clear understanding of the bacterial contribution to cancer.

Figure 1. Environmental factors such as diet, inflammation, and stress influence microbial composition and cause microbial dysbiosis. Expansion of cancer-promoting bacteria with procarcinogenic activities (toxins, metabolites) promotes epithelial cell DNA damage, which, in conjunction with host responses (cytokines, DNA damaging products) from the underlying tissues, fosters dysplastic events and cancer progression. H₂S, hydrogen sulfide; RNS, radical nitrogen species; ROS, radical oxygen species.



Microorganisms are closely attuned to their environment and respond to various changes (diet, stress, inflammation, etc.) by inducing complex transcriptional responses that lead to the production of various molecules (e.g., toxin, metabolites, and enzymes), and future studies would need to incorporate various approaches in which the bacterial metatranscriptome, metagenome, and metabolome are investigated in conjunction with the host response. This comprehensive and integrative approach would capture the complex interaction between the microbiota and the host and could reveal novel pathways that contribute to the development of colorectal cancer. Mechanistic studies using experimental models and microbial genetic manipulation would be required to prove functional relevance. Together, such studies have the potential to reveal new paradigms that could have a significant impact on our understanding of colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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