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BASIC SCIENCE FOUNDATIONS IN COLORECTAL CANCER (S UMAR, SECTION EDITOR)

Deciphering the Colorectal Cancer Gut Microbiota: Association vs. Causality

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Abstract

Purpose of Review Studies have identified differences between the gut microbiota of colorectal cancer (CRC) patients versus healthy individuals. In this review, we assess the scientific literature to determine if gut microbes should be considered causal, co-varying, or a necessary but not sufficient agent in CRC development.

Recent Findings Oral bacteria may influence CRC susceptibility. Colonic biofilms in both sporadic and hereditary CRC suggest these bacteria are present in early neoplasia. Pathogenic drivers and opportunistic passenger bacteria may underlie direct effect of the gut microbiota on carcinogenesis.

Summary Members of multiple bacterial taxa have been implicated in CRC tumorigenesis and progression, with distinct mechanisms of action described for each. Individual bacterial organisms found in the colon are likely not enough to explain CRC development and progression. The entire colonic environment, including genetic factors, local tissue inflammatory state as well as dietary components may influence the way epithelial cells respond to the presence of certain bacteria. Longitudinal, human intervention studies are needed to completely clarify complex interactions in the colonic environment and specific causative pathways between the microbiota and CRC.

Keywords Gut microbiota · Microbiome · Colorectal Cancer · Inflammation · Diet · Colon

Introduction

Colorectal cancer (CRC) is the third most common cause of cancer death in both men and women in the USA. Epidemiology studies have identified many intersecting etiologic factors associated with CRC including, diet, tobacco and alcohol use, gut microbiota, inflammation, and host genetics [1–3]. The microbiota is hypothesized to play a role in both hereditary CRC (hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome) as well as sporadic CRC. HNPCC is characterized by a mutation in or inactivation of DNA mismatch repair genes (MMR). Mutations in the tumor-suppressor adenomatous polyposis coli (*APC* gene) cause familial adenomatous polyposis (FAP) and are also found in 70–80% of sporadic cases of CRC [4].

The complex nature of the interweaving factors associated with CRC makes causal determinations complex and has left the field with limited holistic models that capture the relative contributions of these factors to CRC tumorigenesis. A large and growing body of research has evaluated many aspects of each contributing factor. Unfortunately, uncertainty about the relationship between factors and eventual CRC development remains. For example, is the gut microbiota causing initiation or progression of CRC, is it the microbiota as a whole or individual phyla, genera, or species; is CRC only likely to develop when certain dietary or host genetic conditions are present; is the presence of harmful bacteria or the absence of beneficial bacteria required alongside host or behavioral

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factors; is a bacterial direct or indirect effect mediated through the production of metabolites or a downstream inflammatory host response?

The human gut microbiota is a complex and dynamic community of bacteria, viruses, fungi, and archaea. The bacterial component of the gut microbiota comprises a thousand different genera and species, totaling 100 trillion bacteria. In approximately 20% of human cancers, microbial organisms including pathobionts of the commensal microbiota have been implicated in inflammatory processes that promote tumor growth [5]. CRC has been linked to a dysbiotic gut microbiota [6–8]. A clear association between members of many bacterial taxa and the presence (versus the absence) of CRC has been demonstrated [9].

The microbiota has been described as having both a preventive effect and potentially causative influence on the initiation and progression of CRC. Preventive effects are mainly attributed to production of SCFAs, which promote DNA repair and provide a fuel source for epithelial cells. Essentially, microbes thought to be preventive tend to be high producers of SCFAs, which go on to provide benefit to the gut epithelium, countering deleterious effects of those bacteria thought to have damaging influence.

Composition of Gut Microbiota in Healthy vs. CRC Patients

It is unclear whether the initial cause of neoplasia in the colon is always or sometimes bacterial; however, there is a clear association between the microbiota and CRC. The association is so strong that many researchers have proposed using the gut microbiota as a biomarker for CRC. Repeated studies of patients with CRC have shown characteristic microbial patterns to be present. These patterns may be described as a "microbiota footprint." Researchers have used the CRC-associated microbiota footprint in stool to develop predictive models for development of CRC [9–15]. These models may one day be used to create stool-based, noninvasive screening tests for CRC. Others have proposed that blood-based verification of bacterial infection may be useful in predicting CRC development or risk. Recently, a retrospective analysis of 13,000 patients hospitalized with bacteremia showed increased risk of CRC for those who had bacteremia from intestinal microbes previously associated with CRC, and no increased risk in patients with bacteremia from bacteria not previously associated with CRC [16•]. Much attention is paid to the bacterial component of the gut microbiota, but a fungal dysbiosis has also been found to correlate with CRC in humans [17]. Further, the types of viruses present in the human gut, particularly those that infect bacteria (bacteriophages), have been found to vary significantly between CRC patients and healthy controls [18•].

In the healthy colon, bacteria present in the lumen are separated from intestinal epithelial cells by a thick mucus layer. Together, the mucus layer and epithelial cells provide the gut barrier between the colonic microbiota and many immune cells. Disruption of barrier function leads to inflammation and has been implicated in CRC [19–22]. Biofilm formation on the mucus layer as well as invasion into epithelial cells has been demonstrated both in sporadic CRC and FAP [23]. Therapies to enhance barrier function, including bacterial-mediated therapies [24], can quell inflammation and boost the effect of CRC treatment [25].

The individual bacterial players present in the gut are likely not enough in isolation to explain CRC development and progression. There is a lack of consistency in the bacteria associated with cancerous colon tissue, precancerous (adenomatous) colon tissue, and the stool of patients with CRC or adenomatous polyps [26]. The "driver-passenger" model for the involvement of bacteria in CRC takes into account these inconsistencies [27]. The driver-passenger model proposes that members of several different bacterial taxa are capable of causing DNA-damage and/or initiating a prolonged inflammatory response, resulting in cell proliferation and loss of mucus barrier function. The "driver" bacteria that trigger this protumorigenic environment are then replaced by "passenger" bacteria that are better adapted to the conditions in and around cancerous and precancerous cells, and therefore outcompete the driver bacteria. The passenger bacteria may or may not contribute to cancer progression. The presence of passenger bacteria and their ability to efficiently supersede previously dominating driver bacteria adds additional complexity to models of how the microbiota influences or relates to tumorigenesis.

Enterotoxigenic *Bacteroides fragilis* (ETBF) exemplifies a CRC-associated "driver" bacterium. ETBF secretes a toxin, *B. fragilis* toxin (Bft), which is genotoxic to colon cells, cleaves E-cadherin resulting in cell proliferation and loss of mucus barrier function, and triggers a proinflammatory microenvironment in colonic epithelial cells through increased IL-17, IL-6, and IL-8 secretion [23, 27, 28, 29••]. Recent evidence for an early role in CRC came from a study demonstrating increased incidence of colonization and abundance of ETBF on early-stage carcinogenic lesions (low-grade dysplasia, tubular adenomas, and serrated polyps) rather than later-stage tumors [29••]. In addition, colonizing strains of *Escherichia coli* and ETBF harboring genes for secreted toxins were found to dominate mucosal biofilms from FAP patients [23].

Fusobacterium nucleatum is one of the most common CRC- associated bacterial species [30]. *F. nucleatum* is found in cancerous colon tissue and has been shown to be abundant in chemo-resistant colon tumors [31, 32]. *F. nucleatum* is considered a "passenger" bacterium as it is more commonly associated with established tumors rather than early neoplasia

[27]. It is likely drawn to existing colorectal tumors via binding to the polysaccharide, Gal-GalNAc, overexpressed in CRC cells [33]. In recent years, many studies have demonstrated multiple mechanisms of action for *F. nucleatum* pathogenesis in CRC [34]. Broadly, these mechanisms include (1) invasion of colonic epithelial and endothelial cells, resulting in an increase of many inflammatory cytokines, and production of a proinflammatory microenvironment; (2) promotion of tumor cell proliferation via β-catenin, nuclear factor kappaB (NF-κB), and Toll-like receptor (TLR); and (3) creation of a tumor immunosuppressive environment by either lowering T cell activation or inducing lymphocyte cell death [34, 35].

In mice carrying xenografts of *F. nucleatum*-positive CRC from humans, *F. nucleatum* was found in remote metastases, suggesting an intracellular localization and transport of viable bacteria to different organs via metastasis. Further, antibiotic treatment-reduced tumor growth, indicating a role for *F. nucleatum* in cancer progression [36••].

Bifidobacterium has been described as having both anti-tumor effects and, specifically within the tumor microenvironment, tumor-promoting effects [37]. The anti-tumor effects of *Bifidobacterium* have been linked with suppression of inflammation-associated colon carcinogenesis in *Ffar2*$^{-/-}$ mice. In mice lacking the SCFA binding, G protein-coupled receptor, free fatty acid receptor 2 (FFAR2), there is higher susceptibility to intestinal carcinogenesis. Researchers were able to mitigate carcinogenesis in these mice by treating with *Bifidobacterium*, suggesting that FFAR's tumor suppressive function depends upon modulating the gut bacteria [38].

The microbiota of stool and colon tissue samples are typically assessed for CRC-associated or predictive bacterial species. However, several recent studies have demonstrated that assessment of the oral microbiota may be a useful tool for identifying CRC risk. *Fusobacteria* and *Streptococcus*, both oral bacteria are associated with CRC [15, 39]. Combining oral and fecal microbiota data was shown to increase the sensitivity of a CRC predictive model [15].

Diet, Gut Microbiota, and CRC

Microorganisms are influenced by and dependent on their environment for survival. The environment of the colon is shaped in large part by the food consumed by the host. Depending on the upstream dietary input present, bacteria are stimulated to produce downstream metabolites with CRC protective or promoting effects [40]. Diets rich in fruits and vegetables create an environment abundant in fiber, polysaccharides, oligosaccharides, lignin, and associated plant substances, indigestible to humans, but easily fermentable by bacteria. Many of these substances are thought to promote proliferation of those bacteria which offer protective epithelial effects. The effect of diet on the microbial community is so profound that changes in diet can significantly alter what bacteria are present and a severe lack of fiber can cause the gut to be uninhabitable to many bacterial species [41].

Many fiber compounds have been analyzed as prebiotics, dietary substances capable of promoting the growth of beneficial bacteria. In human and animal studies, prebiotic fiber compounds have been shown to slow the progression of CRC [42]. Conversely, diets high in fat lead to a build-up of the bile acid deoxycholic acid (DCA) in the lumen of the colon, which reduces tumor-suppressor activation and apoptosis pathways in colonocytes, promoting tumor growth [43]. Researchers have been able to demonstrate a decrease in colonic mucosal inflammation and proliferation of cancer suppression associated biomarkers in human subjects when introducing a high fiber, low-fat diet. The reverse has been documented when study participants are given a low-fiber, high-fat diet [44]. However, other studies suggest particular prebiotic compounds may have more of a preventive role against CRC than a total increase in dietary fiber [45, 46].

The anti-CRC effects of pre- and probiotics have been largely attributed to the downstream production of short-chain fatty acids (SCFAs), the end-product of bacterial fermentation of fiber in the colon. SCFAs, particularly propionate, acetate, and butyrate have been shown to be anti-inflammatory in the colon [47] and inhibit the growth of CRC cells [48]. In a mouse model of colon polyposis, treatment with a high-fiber diet increased SCFA-producing bacteria, increased SCFAs, and reduced polyp development [49]. Butyrate inhibits tumor cell proliferation, repairs DNA in epithelial cells [50], and induces tumor cell apoptosis through inhibition of histone deacetylase and through regulation of microRNA expression [48, 51]. However, these anti-tumor effects may be most effective in the early stages of CRC, as exposure of cancer cells to butyrate was shown to cause butyrate resistance in vitro [52]. Butyrate also supports the growth of a healthy colonic epithelium as a preferred energy source for normal colonocytes but not cancer cells, which prefer glucose [51]. Studies suggest that SCFAs also exert their anti-tumor effects via G protein-coupled receptors such as FFAR2 and that these receptors are significantly downregulated in human colon cancers [38, 53, 54].

Immune Function, Microbiota, and CRC

Commensal and pathogenic bacteria have been shown to interact with the host immune response to cancer. In a mouse model of melanoma, *Bifidobacterium* was shown to increase a T cell-mediated anti-tumor response; further, this could be transferred to mice lacking *Bifidobacterium* either through co-housing, fecal transfer, or oral *Bifidobacterium* supplement [55]. Mutations in the innate immune receptor, AIM2, are often detected in CRC patients and increase the rate of

mortality. Aim2-deficient mice were shown to be more susceptible to tumorigenesis, which was exacerbated by dysbiotic colonic microbiota and reduced after fecal transplantation of healthy colonic microbiota [56].

Gram-negative gut bacteria are covered in the immune-stimulatory molecule, lipopolysaccharide (LPS). LPS triggers inflammation through activation of the Toll-like receptor 4 (TLR4) and NF-κB pathways [57]. Increased LPS levels in blood and colon tissue are associated with CRC and LPS has been shown to play a role in CRC metastasis [58, 59].

Inflammation of the colon is highly associated with CRC. Patients with inflammatory bowel disease (IBD) have increased rates of CRC [60]. The current literature shows that several bacterial taxa are capable of modulating the host immune system and triggering a cancer promoting, proinflammatory state within the colonic epithelium [28, 61, 62]. This proinflammatory state seems to act as a feed-back loop in which more inflammation leads to more dysbiosis and more dysbiosis leads to more inflammation. Does the microbiota exacerbate or initiate this process? It remains unclear whether microbial dysbiosis precedes development of inflammation or whether inflammation arises independently of the microbiota and leads to dysbiosis? To answer this question, studies focused on the mechanism of colonization and enrichment of early CRC-associated bacteria ("driver" bacteria) are essential.

One recent study demonstrated that *Streptococcus gallolyticus subsp. gallolyticus* (SGG), an early CRC-associated bacterium, colonized tumor-bearing mice significantly more than mice without tumors and that *APC* mutation and Wnt pathway activation promote SGG colonization [63••]. SGG were shown to outcompete commensal enterococci by secretion of an enterococci-killing bacteriocin. Activity of the SGG bacteriocin was found to be enhanced by the secondary bile acid, DCA. Further, researchers linked activation of the Wnt pathway in colonic epithelial cells to downregulation of a major bile acid transporter, resulting in build-up of DCA [63••], thereby enhancing the capacity for SGG colonization. Taken together, these data seem to suggest that Wnt pathway activation, DCA build-up, and tumor formation precede and enable SGG colonization.

Dietary supplementation of DCA in mice has also been shown to select for microbial dysbiosis. Further, fecal transplant of these dysbiotic bacteria from DCA-treated mice resulted in activation of the Wnt/β-catenin signaling pathway, impaired mucus layer integrity, increased inflammation, and tumor progression [43].

In mouse models, bacteria have been shown to promote cancer cell proliferation through alterations in epigenetic signaling [55]. Recently, the stool of CRC patients was shown to promote tumorigenesis and increase markers of inflammation in mice compared to stool from healthy individuals [64]. *Citrobacter rodentium* (*C. rodentium*) is an attaching and effacing murine pathogen that serves as a great template to study the human pathogen Enteropathogenic *Escherichia coli* (EPEC). In response to *C. rodentium* infection, there is restructuring of the microbiota concomitant with the upregulation of β-catenin, NF-κB, and Notch signaling pathways that promote cellular proliferation and increase susceptibility to colorectal cancer in mouse models as a component of driver bacteria implicated in CRC [65–68].

There is some evidence that mutations in the *APC* gene, implicated in both hereditary and sporadic CRC, result in changes in the microbiota, particularly an increase in abundance of *Bacteroidetes*, before the development of precancerous polyps [4].

Colon cancer pathogenesis is driven by the relationship between the microbiome (including all bacterial, fungal, and viral components), the immune system, and the colonic epithelium where neoplasia arises. The interactions between the three are governed by host genetics, inflammatory state, and environmental factors, such as diet, alcohol, and tobacco use (Fig. 1).

Intestinal bacteria influence the intestinal epithelial differentiation via transcription factors Hes1, Hath1, and KLF4 in addition to Muc1 and HBD2, in vitro and in vivo. The induction of Muc1 and HBD2 seems to be triggered directly by bacteria and not by Notch [69]. Moreover, propionate has been associated with enhancement of gut epithelial KLF4 expression via a PPAR-γ-dependent mechanism, independent of its HDAC-inhibitory activity [70].

Microbiota-Modulating Therapies for CRC

Modulation of the gut microbiota has been shown to alter colon tumorigenesis in mice. In an inflammation-based model of CRC, mice treated with antibiotics developed significantly fewer tumors [14]. Antibiotic treatment early in the inflammatory process blocked subsequent tumor formation [71]. Researchers were also able to predict the number of subsequent tumors based on initial bacterial composition of the mouse gut [71]. Taken together, these results suggest a causal role for bacteria in inflammatory models of CRC and a potential role for antibiotic treatment. Further studies are needed to determine whether this model applies to human CRC.

In addition to antibiotic treatment to alter CRC outcome, the use of prebiotics has been studied. In a mouse model of CRC, treatment with extracellular polysaccharide from the fungus, *Rhizopus nigricans*, modified the composition of the gut microbiota to that resembling healthy control mice, increased SCFA-producing bacteria and overall SCFA abundance, and improved intestinal barrier function [72].

Determining protective populations, rather than focusing on cancer promoting bacteria may lead to useful CRC therapies. However, host genetics must be considered, as some

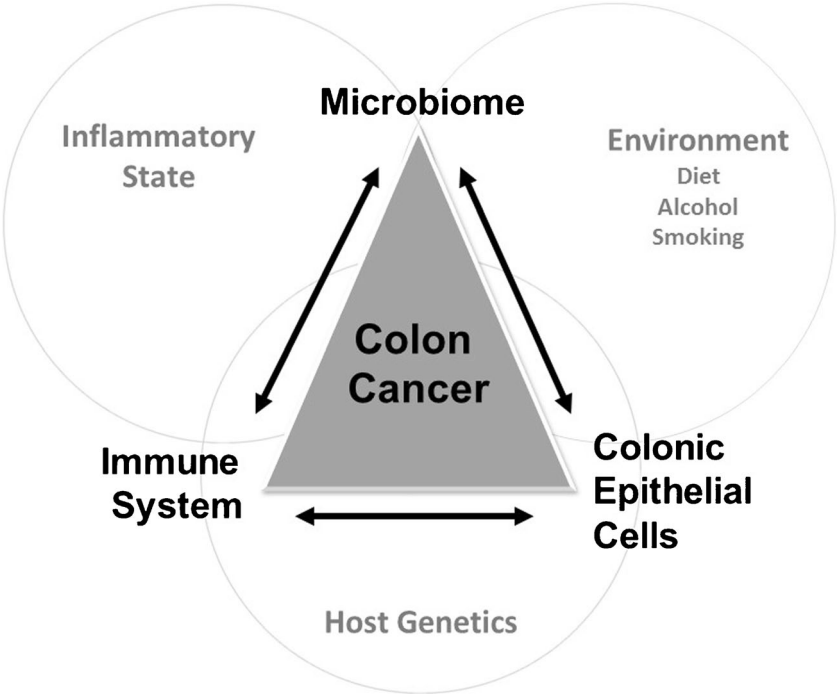

Fig. 1 Tripartite relationship contributing to colon cancer pathogenesis within the multivariate context of inflammatory state, environmental factors, and host genetics adapted from Sears CL, Pardoll DM, "Perspective: Alpha-Bugs, Their Microbial Partners, and the Link to Colon Cancer," *Journal of Infectious Diseases*, 2011, 203(3), 306–311, by permission of the Infectious Diseases Society of America

protective species may act in a CRC-promoting manner in different genetic environments [73]. In a particular genetic context, certain bacteria, or functional isotypes, may exacerbate the effects of genetic mutations. For example, butyrate-producing bacteria are associated with a decreased risk of CRC; however, in a mouse model mimicking common genetic mutations in hereditary forms of CRC, the presence of butyrate-producing bacteria in the colon or a high-fiber diet (representing a substrate for butyrate production) caused $APC^{Min/+}$ $MSH2^{-/-}$ epithelial cells to hyper proliferate [73]. This lack of consensus regarding butyrate's role seems multifactorial with variations in the amounts of experimental butyrate used in various studies, differences in the type of fiber tested, and the strategies for butyrate measurement, contributing towards the paradox.

Conclusions

The individual bacterial players present are likely not enough to explain CRC development and progress. Multiple bacterial taxa have been implicated in tumorigenesis and CRC progression, with distinct mechanisms of action described for each. Therefore, it is unlikely that one bacterial species, or even one microbial-dependent pathway, is responsible for all CRC. The entire colonic environment, including inflammatory state and carbohydrates and other dietary components present may influence the way epithelial cells respond to the presence of certain bacteria. Furthermore, host genetics will influence the outcome; epithelial cells with mutations in APC or MMR will respond differently to the presence of particular bacteria. The functional classification (virulence factors, LPS producer, SCFA metabolizers) rather than taxonomic classification may prove to be a better identifier of pro- or anti-carcinogenic bacterial species.

Longitudinal, human intervention studies are needed to verify a causative link between the microbiota and CRC, particularly in a cohort that precedes the onset of disease. However, larger studies must be rooted in trends or correlations between the microbiome and human health discovered in small, well-defined human populations. Currently, the critical role of microbiota in CRC is based on analysis of patients for whom there is limited data on medication use, prior diagnoses, recent illnesses, dietary intake, and environmental factors. The correlations discovered in smaller cohorts, with a depth of clinical and social data, can then be modeled in an easily controlled system, like tissue, cell culture, or animals. Animal models, where diet, environment, genetics, and other possible confounding variables can be controlled, permit cause and effect relationships to be determined.

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Compliance with Ethical Standards

Conflict of Interest Kristina M. Bridges, K. Allen Greiner, and Shahid Umar declare they have no conflict of interest.

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

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- • Of importance
- •• Of major importance

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