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Impact of microbiota in colorectal carcinogenesis: lessons from experimental models

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A role of gut microbiota in colorectal cancer (CRC) growth was first suggested in germ-free rats almost 50 years ago, and the existence of disease-associated bacteria (termed pathobionts) had becoming increasingly evident from experimental data of fecal transplantation, and microbial gavage or monoassociation. Altered bacterial compositions in fecal and mucosal specimens were observed in CRC patients compared to healthy subjects. Microbial fluctuations were found at various cancer stages; an increase of bacterial diversity was noted in the adenoma specimens, while a reduction of bacterial richness was documented in CRC samples. The bacterial species enriched in the human cancerous tissues included *Escherichia coli*, *Fusobacterium nucleatum*, and enterotoxigenic *Bacteroides fragilis*. The causal relationship of gut bacteria in tumorigenesis was established by introducing particular bacterial strains in *in situ* mouse CRC models. Detailed experimental protocols of bacterial gavage and the advantages and caveats of different experimental models are summarized in this review. The microbial genotoxins, enterotoxins, and virulence factors implicated in the mechanisms of bacteria-driven tumorigenesis are described. In conclusion, intestinal microbiota is involved in colon tumorigenesis. Bacteria-targeting intervention would be the next challenge for CRC. **(Intest Res 2018;16:346-357)**

Key Words: Colorectal neoplasms; Microbiota dysbiosis; Mucosa-associated bacteria; Pathobiont; Virulence

INTRODUCTION

An ecosystem of microorganisms habituates the human intestinal tract which is defined as the gut microbiota, composed of bacteria, archaea, virus, and fungi.[1] The intestinal tract harbors around 10^{14} bacteria, with the highest amount in the colon.[2-4] Over 1,000 bacterial species belonging mainly to 4 phyla were identified in humans, with each individual possessing more than 160 species.[5] The collection of all genomes of the microbiota is termed microbiome, of which the number of intestinal microbial genes has been estimated to be ~100 times more than that of human genes.[1,4,5]

Accumulating evidence demonstrated that gut microbiota is involved in the maintenance of mucosal homeostasis, with an indispensable role in promotion of wound repair and epithelial barrier fortification.[6-9] While a symbiotic relationship is established between commensal bacteria and the healthy host, the presence of an altered gut microbial population or the emergence of opportunistic bacteria from commensals may contribute to the development of IBD and colorectal cancer (CRC).[10-13] Patients with IBD had higher risk of developing cancer later in life, termed colitis-associated CRC.[14,15] It is generally believed the genetic and dietary factors, as well as chronic inflammation and altered microbial population predispose to tumor development.[16-19]

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DETRIMENTAL AND BENEFICIAL ROLES OF BACTERIA IN EXPERIMENTAL MODELS OF CRC

A relationship between commensal bacteria and intestinal carcinogenesis was first suggested in rodent models

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by Reddy et al. in 1974.[20] Germ-free rats showed lower incidence of chemically induced duodenal and colonic tumors compared to those raised in conventional husbandry.[20] Reduction of colonic tumor burden was also reported in several chemically induced or genetic deficient mouse models when derived under germ-free conditions.[21-23] It should be kept in mind that the physiological relevance of germ-free models had been criticized since their mucosal immune systems were undeveloped due to the lack of microfloral establishment.[24-26] Additional evidence of bacterial involvement in carcinogenesis was supported by studies showing reduction of tumor load after antibiotic manipulation of gut microbiota.[22,27] The notion of disease-associated microbes originating from commensal-derived opportunistic bacteria (termed pathobionts) was shown by evidence of increased cancer growth in recipient mice after fecal transplantation from high tumor-bearing mouse.[28,29] These studies suggested the existence of pathobionts with a protumoral characteristic in the fecal bacteria population.

On the other hand, presence of beneficial bacteria (termed probionts or probiotics) with tumor-suppressive metabolites was also found in experimental models.[16,30] Short-chain fatty acids (SCFAs) such as butyrate are bacterial metabolites by fermentation of dietary fibers. Nutritional studies showed that high fiber diets or butyrate in the colon decreased the rate of aberrant crypt foci formation and reduced the tumor burden in animal models.[31,32] A recent study using a gnotobiotic mouse model also demonstrated that supplementation with high dietary fiber and butyrate-producing bacteria significantly decreased colon tumor growth.[30]

Taken together, intestinal bacteria are involved in tumorigenesis with both detrimental and beneficial roles. Potential tumorigenic bacteria and their underlying mechanisms to promote cancer growth will be highlighted in this review. For more information on probiotics and cancer, please refer to other review articles.[33,34]

MICROBIOTA DYSBIOSIS AT VARIOUS CANCER STAGES

Despite similar fecal bacterial counts between healthy subjects and CRC patients,[35] a clear difference in the composition of normal and tumor-associated microbiota was demonstrated. Numerous studies have shown reduction of microbial diversity in the stool samples and mucosal biopsies of patients with IBD[36-38] (including CD and UC) and in those with CRC.[39,40] In contrast, higher diversity and greater numbers of bacteria were noted in adenoma biopsies compared to healthy mucosa,[41,42] suggesting that there existed dynamic changes of microbial population throughout the different tumor stages. It should be noted these cross-sectional studies are based on specimens collected at single time points. Therefore, the changes in gut microbiota only indicated a correlation but did not prove a cause-and-effect relationship for tumor formation in CRC patients.

1. Microbiota in Stool Samples of CRC Patients

The Intestinal microbes are mixed in the outer loose mucus layer and in chyme and feces. Many clinical studies have utilized stool samples for analysis of bacterial composition as a surrogate for intestinal microflora. Although the fecal bacteria may not represent the complete population of gut microbiota, this noninvasive approach has been widely practiced and conveyed crucial information of the bacterial community residing in the intestinal lumen. However, comparison of fecal microbiota composition to that of mucosal specimens and tumor biopsies may provide a more comprehensive view of the changes in the gut ecosystem.

Four main phyla were identified in the fecal microbiota of healthy individuals, including Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria, with Bacteroidetes and Firmicutes being the 2 predominant phyla that constitutes more than 90% of the total bacterial population.[43,44] The percentages of Bacteroidetes and Firmicutes phyla in the fecal microbiota of healthy subjects were ~55% and ~40%, respectively.[43,44] In CRC patients, these 2 phyla still constitutes the largest proportion in the stool microbiota.[43,44] Being the 2 predominant phyla with higher total numbers in the individual, it is no doubt that alterations of the bacterial strains in the Bacteroidetes and Firmicutes phyla in the fecal population are easier to spot compared to other bacteria with lower counts. Nevertheless, accumulating evidence indicates that some bacteria belonging to the minor phyla in fecal microbiota may also play protumoral roles.

Under the Bacteroidetes phylum, enrichment of genera of *Porphyromonas* and *Prevotella* was reported in stool samples of CRC patients compared to healthy volunteers.[44,45] Conflicting results were shown for the *Bacteroides* genus; increased percentage were reported in CRC patients in some studies,[35,43] while others found a significant reduction.[45] Among the *Bacteroides* species, a significant increase of *Bacteroides fragilis* and a decrease of *Bacteroides vulgatus* and *Bacteroides uniformin* were found in fecal samples of CRC patients.[45,46]

Under the Firmicutes phylum, increase of *Enterococcus*

and *Streptococcus* genus and decrease of genera such as *Faecalibacterium*, *Roseburia* and *Eubacterium* and of *Lachnospiraceae* family were reported in stool samples of CRC patients compared to healthy volunteers.[45,47] At the species levels, decreases in *Ruminococcus obeum*, *Ruminococcus albus*, *Pseudobutyrivibrio ruminus*, *Lachnospira pectinoschiza*, *Lachnospira bovis* were seen in the fecal microbiota of CRC patients.[44] One of the common characteristics of the decreased bacterial genera and species in CRC patients belonging to the Firmicutes phylum is the ability of SCFA production.[43-45] The altered stool bacterial profiling corresponded to the relatively lower levels of fecal butyrate in CRC patients compared to control subjects.[44] In addition, no difference of *Lactobacillus* was shown in stool samples of cancer patients.[35,45,46,48]

Under the relatively minor phyla in fecal microbiota such as Proteobacteria, Actinobacteria and others, higher percentages of the genera of *Escherichia* (belonging to Proteobacteria phylum) and *Fusobacterium* (belonging to Fusobacteria phylum) were found in the stool samples of CRC patients compared to control subjects.[43,45,46] The *Bifidobacteria* genus (belonging to Actinobacteria phylum) in fecal samples were either increased[45] or showed no change[35,46] in CRC patients.

2. Microbiota in Tumor Biopsies of CRC Patients

Recent studies have utilized tumor/mucosal biopsies and lavage samples instead of fecal samples to validate and clarify the dysbiosis of gut microbiome. Data obtained from tumor biopsies indicated the alteration in mucosa-associated bacteria. Accumulating evidence showed distinct profiles between mucosal bacteria and fecal microbiota,[47,49-51] whereas microfloral results obtained from mucosal biopsy and similar to those of lavage samples.[52] For the evaluation of mucosal microbiota, tumor tissues are usually compared to normal mucosa of healthy individuals or to patient-matched non-cancerous tissues. One report had shown that *Proteobacteria* was the most predominant phylum (~60%) in the gut mucosa of healthy individuals.[49] Another study showed the predominance of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, of which the 3 phyla accounts for 80% of the microbiota in the control subjects.[50] The findings showed that fecal microbiota as a surrogate only partially reflect the composition of the mucosa-associated bacteria.

By using mucosal specimens, the genera of *Escherichia* and *Fusobacterium* were found significantly increased in CRC patients compared to healthy controls.[49-51] It is noteworthy that *Escherichia* and *Fusobacterium* constitute a minority of the healthy stool microbiome,[43,44] and are also of low percentages (0.22% and 0.01%, respectively) of the bacterial population in healthy gut mucosa.[49] However, a 15- and 1,000-fold increase of the percentage of *Escherichia* and *Fusobacterium* were noted in the microflora of biopsy tissues of CRC patients compared to that of healthy controls.[49] When comparing patient-matched cancerous samples and adjacent non-cancerous tissues, abundance of *Fusobacterium* was also observed in tumors.[49-51] The enrichment of *Escherichia* and *Fusobacteria* in tumor biopsies is in keeping with the findings in stool samples of CRC patients.[43,45,46]

Higher levels of *Bacteroides* genus was found in the mucosal microbiota of CRC patients than healthy controls.[47] When comparing microbial composition in tumor biopsies to adjacent non-cancerous tissues, the percentage of *Bacteroides* were decreased in one report[50] and increased in another study.[49] The inconsistent result of Bacteroides in tumor tissues seems to reflect the fecal microbial composition which also shows conflicting data in the literatures.[35,43,45]

Decreased mucosal levels of *Firmicutes* and *Actinobacteria* phyla were observed in cancerous tissues of CRC patients compared to normal mucosa of healthy subjects or to adjacent non-cancerous tissues.[49,51] A downregulation of *Lachnospiraceae* and *Ruminococcaceae* was observed in cancerous tissues compared to adjacent non-cancerous tissues, which also correlated with the finding of decreased bacterial genus in fecal samples of CRC patients.[45,50] The data of mucosa-associated *Lactobacillus* and *Bifidobacterium* were inconsistent and a few reports showed no difference of these bacteria between tumor and control samples.[35,48]

Overall, the aforementioned studies all pointed to microbiota dysbiosis in fecal and mucosal samples of CRC patients despite inconsistency of the wax or wane of particular bacterial taxa. The fecal bacterial profiling only partially reflected mucosal microbiota in healthy controls and in CRC. Moreover, a higher ratio of the Proteobacteria and Fusobacteria phyla was observed in CRC patients when the analysis was based on mucosal specimens.

POTENTIAL TUMORIGENIC PATHOBIONTS

Clinical studies indicated a positive correlation between mucosal bacteria and disease pathogenesis. The gut commensals mostly reside in the intestinal lumen in physiological conditions, separated by the inner mucus layer and are rarely in direct contact with the epithelial cells.[53-56] The concept of bacterial adhesion to host epithelial cells was first reported in uropathogenic *Escherichia coli* in 1908.[57] In

the past two decades, high numbers of mucosa-associated bacteria were reported in clinical studies of IBD and CRC. Enrichment of mucosa-associated *E. coli* was found in tissue biopsy of patients with CD and CRC.[38,58-62] Abundance of mucosa-associated *Fusobacterium* was noted in CRC patients compared to healthy individuals.[49,50] Invasive strains of *Fusobacterium nucleatum* were also isolated from biopsy specimens of CD or acute appendicitis.[63-65] Moreover, higher levels of enterotoxigenic *B. fragilis* were found in colonic tumor samples obtained from patients.[66,67] Evidence of the protumorigenic potentials of *E. coli*, *F. nucleatum*, and *B. fragilis* by using experimental models are discussed. The detailed experimental protocols of bacterial gavage in *in situ* mouse CRC models are listed in Table 1,[58,68-79] and the advantages and caveats of different experimental models are summarized as followed.

1. *Escherichia coli*

Adherent-invasive *E. coli* (AIEC) was first isolated from ileal lesions in CD patients.[80-84] Although AIEC was unable to colonize the intestine of wild type mice, the colitogenic activity of AIEC was shown in transgenic mice expressing human carcinoembryonic antigen-related cell adhesions molecules (CEACAMs; a receptor for type-1 pili or fimbriae [fim]).[85] The data suggested that mucosal colonization of AIEC through fimbriae-mediated adhesion was a crucial step for its colitogenic ability as a pathobiont.[85] However, there remains no direct evidence for the involvement of AIEC in colon cancer development, except the general link between inflammation and tumorigenesis. It is noteworthy that the fimbrial adhesin is historically known as a common feature for host colonization by various strains of gram-negative bacteria.[57] Although fimbriae-mediated adhesion also affected bacterial uptake or promote proinflammatory response in epithelial cells,[57] it has not been traditionally regarded as a strain-specific pathogenic virulence.[86,87] Previous studies demonstrated increased tumor burden in human CEACAM-transgenic mice after azoxymethane (AOM) injections,[88] but did not specify the bacterial strains responsible for the fimbriae-dependent tumor growth. It remains undetermined whether the adherent or invasive virulence of *E. coli* is involved in colorectal tumorigenesis.

A clear evidence of *E. coli* involvement in CRC development was reported with the use of 3 bacterial strains (i.e., NC101, CCR20, and 11G5) which harbors genotoxin-encoding polyketide synthase (pks) pathogenicity islands (Table 1).[68,69,71,72] The colibactin produced by pks$^+$ *E. coli* induced DNA damage and cellular senescence in intestinal epithelial cells.[68,69,71,72] The pks$^+$ NC101 strain was a mouse adherent-invasive *E. coli*, and the CCR20 and 11G5 strains were clinical isolates from tumor biopsies of CRC patients. Increased colonic tumor load was observed by monoassociation of *E. coli* NC101 in gnotobiotic interleukin 10 (IL-10)$^{-/-}$ mice given injections of AOM[68,69] and in adenomatous polyposis coli (APC)$^{Min/+}$-IL-10$^{-/-}$ mice.[70] Moreover, higher tumor susceptibility was found after oral gavage of pks$^+$ *E. coli* CCR20 to wild type mice administered AOM/dextran sulfate sodium (DSS).[71,72] Increased tumor numbers and volume were documented in APC$^{Min/+}$ mice following oral inoculation of pks$^+$ *E. coli* 11G5 compared to the K12 strain.[58] Details of the experimental design are described as followed and also summarized in Table 1. The germ-free IL-10$^{-/-}$ and APC$^{Min/+}$-IL-10$^{-/-}$ mice were orally gavaged with 10^8 colony-forming units (CFU) of *E. coli* NC101 strain in one bolus for bacterial monoassociation.[68-70] For oral gavage of *E. coli* CCR20 strain, the wild type mice were raised on specific pathogen free (SPF) conditions and given intraperitoneal injection of AOM, followed by pretreatment of streptomycin (a bactericidal antibiotic to G(–) bacteria to disturb microflora) in drinking water for 2 days prior to gavage of 10^9 CFU of *E. coli* CCR20 to facilitate bacterial colonization, and then subjected to 2 cycles of DSS in drinking water to induce colitis.[71,72] In addition, APC$^{Min/+}$ raised in SPF conditions were gavaged with 10^8 CFU of *E. coli* 11G5 strain 3 days after streptomycin treatment to facilitate bacterial colonization, and tumors were inspected at 7 weeks after infection.[58] Due to the nature of these intestinal isolated *E. coli* which are not pathogens *per se*, microbiota disturbance by antibiotics prior to bacterial gavage or monoassociation in gnotobiotic mice are necessary to facilitate bacterial colonization to understand their effects on tumor development. Overall, higher tumor loads were found in the mouse groups given pks$^+$ *E. coli* compared to the pks-deleted bacteria or pks-negative strains, indicating a crucial role of *pks* operon in promoting carcinogenesis.[68-71]

Other reports have demonstrated that the human CRC-associated *E. coli* (11G5 strain) induced colitis and increased crypt cell proliferation in transgenic mice with epithelial overexpression of human CEACAM.[73] The *E. coli* 11G5 strain was gavaged at 2×10^8 CFU twice a week for 3 weeks after streptomycin and 0.25% DSS treatment in the human CEACAM-transgenic mice.[73] Moreover, the human CRC-associated *E. coli* 11G5 triggered the production of cyclooxygenase (COX)-2 in macrophages after phagocytosis *in vitro*, in a *pks*-independent manner.[74] Furthermore, human CRC-associated *E. coli* obtained from tumor biopsies were

Table 1. Bacteria Promoted Tumorigenesis in *In Situ* Mouse CRC Models

Bacterial strain	Animal models	Housing and pretreatment	Dose and time for bacterial gavage	Outcome	Proposed mechanisms	Reference
Escherichia coli						
NC101 (a mouse adherent-invasive *E. coli*)	IL-10$^{-/-}$ mice administered AOM	GF	Monoassociation by gavage at 10^8 CFU, followed by 6 wk AOM injections starting at 1–4 wk post-monoassociation	Mice showed more severe colitis and invasive mucinous adenocarcinoma	Colibactin encoded by pks operon caused DNA damage	68-70
	APC$^{Min/+}$-IL-10$^{-/-}$ mice	GF	Monoassociation by gavage at 10^8 CFU, and tumor inspection after 16 wk	Mice showed more severe colitis and higher colon tumor loads	Colibactin encoded by pks operon caused DNA damage	70
CCR20 (a clinical isolate of *E. coli* from CRC samples)	Wild type mice administered AOM/DSS	SPF; streptomycin for 2 day starting on the first day of AOM injection	Single gavage at 10^9 CFU 1 day after the end of antibiotic pretreatment	Mice developed higher tumor numbers	Colibactin encoded by pks operon induced cellular senescence	71, 72
11G5 (a clinical isolate of *E. coli* from CRC samples)	APC$^{Min/+}$ mice	SPF; streptomycin	Single gavage at 10^8 CFU after antibiotic pretreatment, and tumor inspection at 7 wk after infection	Mice showed increase tumor numbers and volume	Colibactin encoded by pks operon caused DNA damage	58
	Transgenic mice expressing epithelial-specific human CEACAM	SPF; streptomycin and 0.25% DSS	Gavage twice wk at 2×10^8 CFU for 3 wk, after antibiotic and DSS pretreatment	Mice displayed more severe colitis, epithelial damage and proliferation	*E. coli* induced epithelial proliferation, partly via induction of macrophage COX-2 synthesis in a pks-independent manner	73, 74
Fusobacterium nucleatum						
ATCC25586 (a clinical isolate of cervical-facial lesion)	APC$^{Min/+}$ mice; wild type mice administered AOM/DSS AOM/DSS	SPF; streptomycin for 3 day	Daily gavage at 10^9 CFU for 20 wk	Mice displayed higher tumor burden	Activation of TLR4/NF-κB signaling and upregulation of miR-21	75
EAVG_002; 7/1 (a clinical isolate from sigmoid colon of a CD patient)	APC$^{Min/+}$ mice	SPF	Daily gavage at 10^8 CFU for 8 wk	Mice showed accelerated onset and higher numbers of colonic tumors, but with no sign of colitis	Recruitment of tumor-infiltrating myeloid cells	76
	IL-10$^{-/-}$ mice; T-bet$^{-/-}$Rags$^{-/-}$ mice	SPF	Daily gavage at 10^8 CFU for 8 wk	No increase in tumor load or colitis severity		76
EAVG_002; 7/1	APC$^{Min/+}$ mice	SPF	Transfer from GF to SPF conditions, gavage with SPF microbiota followed by weekly gavage at 10^8 CFU for 20 wk	No increase in tumor load compared to SPF controls gavaged with broth		70
CC53, CC7/3JVN3C1, CC7/5JVN1A4, CC2/3Fmu1, CC2/3FmuA, CC7/4Fmu3 (all clinical isolates from CRC patients)	APC$^{Min/+}$-IL-10$^{-/-}$ mice	GF	Transfer from GF to SPF conditions, gavage with SPF microbiota followed by weekly gavage of 6 bacterial mixtures (10^8 CFU per strain per mouse) for 16 wk	No increase in tumor load compared to SPF controls gavaged with broth		70

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Table 1. Continued

Bacterial strain	Animal models	Housing and pretreatment	Dose and time for bacterial gavage	Outcome	Proposed mechanisms	Reference
	$APC^{Min/+}$ mice	GF	Polyassociation with 6 bacterial mixtures by gavage of 10^8 CFU per strain per mouse, and sacrificed 16 wk later	No increase in tumor load compared to GF controls gavaged with broth		70
Bacteroides fragilis						
Enterotoxigenic *B. fragilis* strain 86-5443-2-2 (a piglet isolate)	$APC^{Min/+}$ mice	SPF; clindamycin/ streptomycin for 3–5 day	Single gavage at 10^8 CFU	Mice displayed rapid colitis and increased tumor numbers	Enterotoxin fragilysin caused DNA damage, activated STAT3/Th17 cell response, and increased protumoral monocytic myeloid-derived suppressor cells to promote carcinogenesis	77-79

CRC, colorectal cancer; IL, interleukin; AOM, azoxymethane; GF, germ free; CFU, colony-forming units; pks, polyketide synthase; APC, adenomatous polyposis coli; DSS, dextran sulfate sodium; SPF, specific pathogen-free; CEACAM, carcinoembryonic antigen-related cell adhesion molecule; COX, cyclooxygenase; TLR, Toll-like receptor; NF-κB, nuclear factor κB.

composed of genotoxin-positive and -negative populations.[89] The pks^+ *E. coli* were not adhesive, whereas the highly adherent *E. coli* were devoid of known genotoxins but caused DNA damage *in vitro*.[89] The findings suggested that aside from causing genotoxicity, the induction of tumor-infiltrating macrophages and other unknown mechanisms may also play indispensable roles in *E. coli*-driven tumorigenesis.

2. *Fusobacterium nucleatum*

Enrichment of *F. nucleatum* was demonstrated in the stool and tissue samples of CRC patients.[43,49,50,66,90-92] *F. nucleatum* is known as a commensal G(–) species residing in the oral cavity, and has been reported as an invasive strain associated with inflammatory disease in the mouth such as gingivitis.[93,94] Invasive strains of *F. nucleatum* were also isolated from inflamed biopsy tissues from patients with CD or acute appendicitis.[63-65]

Recent data showed that orogavage of *F. nucleatum* increased tumor burden in $APC^{Min/+}$ mice or in wild type mice given AOM/DSS (Table 1).[75,76,95] A number of mechanisms were proposed by *in situ* CRC mouse models, including β-catenin and nuclear factor-κB signaling via activation of Toll-like receptor 4 (TLR4) (a cell surface innate receptor for lipopolysaccharide [LPS]),[75,95] and recruitment of tumor-infiltrating myeloid cells.[76] Other studies by using human CRC cell lines and a xenograft model showed that FadA adhesin expressed on *F. nucleatum*, via extracellular binding to E-cadherin, induced nuclear translocation of β-catenin for oncogenic transcription and epithelial hyperproliferation.[96,97] The invasiveness of *F. nucleatum* was not a requirement for its effect on oncogenic transcription but was associated with proinflammatory signals which could be indirectly involved in cancer development.[96] So far, whether *F. nucleatum* infection and its pro-tumorigenic ability are associated with inflammation is still controversial. Although some reported that the histopathological features of colitis and enteritis were not induced,[76] others showed upregulation of proinflammatory cytokines and chemokines following inoculation of *F. nucleatum*.[75] It is noteworthy that the *in situ* CRC model was pre-fed with streptomycin (a bactericidal antibiotic to G(–) bacteria) to disturb the normal flora, followed by inoculation with a very high number of *F. nucleatum* for a long period of time (10^9 CFU by gavage per day for 20 weeks or 10^8 CFU by gavage per day for 8 weeks).[75,76] With such a high bacterial load given continuously, the roles of *F. nucleatum* as a driver or a passenger for tumorigenesis remain in doubt.

A recent study demonstrated that gavage of a single strain

or multiple strains of *F. nucleatum* obtained from clinical specimens of CRC patients to gnotobiotic $APC^{Min/+}$ mice or $APC^{Min/+}$-IL-$10^{-/-}$ mice did not increase colitis severity nor enhance colon tumor burden.[70] The germ-free mice were gavaged weekly with a mixture of 6 *F. nucleatum* clinical isolates at 10^8 CFU per strain per mice, or were transferred to SPF microbiota prior to gavage of *F. nucleatum* mixtures and sacrificed 16 weeks later to examine tumor growth. However, no increase in tumors was observed in either protocols.[70] The inconsistent data of *F. nucleatum* suggested that other unidentified mechanisms, such as interaction with other bacteria, may partly contribute to its protumoral characteristics.

3. *Bacteroides fragilis*

A subclass of the human commensal *Bacteroides* species, enterotoxigenic *Bacteroides fragilis* (ETBF), was associated with acute inflammatory diarrheal disease and CRC in patients.[66,98,99] Increased percentage of *B. fragilis* was reported in fecal microbiota.[45,46] The levels of ETBF and enterotoxin gene (*bft*) in tumor and stool samples were significantly higher in late stages (III/IV) of CRC compared to control tissues.[66,67,99] Despite reported as an obligate anaerobe, abundant literature showed invasive traits of the G(–) bacteria *B. fragilis*. The most frequent anaerobe isolated in clinical cases of peritonitis, intra-abdominal abscess or bacteremia is *B. fragilis*, suggesting its aerotolerance and invasiveness.[100-102]

Previous studies have demonstrated that ETBF triggered colitis and accelerated tumor growth in $APC^{Min/+}$ mice (Table 1).[77,78] The colonization of ETBF (10^8 CFU by oral gavage in one dose) was facilitated by pretreatment of clindamycin (an antibiotic against G(+) bacteria) and streptomycin (an antibiotic against G(–) bacteria) or gentamicin (a broad-spectrum antibiotic) to disturb the gut microbiota.[77-79] Following antibiotic manipulation of microbiota, colonization with ETBF but not its nontoxigenic counterparts increased tumor burden in $APC^{Min/+}$ mice.[77,78] The *B. fragilis* enterotoxin (also known as fragilysin) acts as a metalloprotease that causes oxidative DNA damage, E-cadherin cleavage, epithelial barrier damage, and activation of STAT3/Th17 immune responses, and generation of protumoral monocytic myeloid suppressor cells.[77-79,103,104] Studies *in vitro* showed that fragilysin stimulated the production of spermine oxidase in intestinal epithelial cell lines, suggesting a direct role of the enterotoxin on epithelial free radical production and DNA damage.[78] Taken together, the findings indicated that ETBF was a strong colitogenic infectious agent that promoted tumorigenesis through both direct and indirect mechanisms.

UNANSWERED QUESTIONS AND FUTURE DIRECTIONS

A number of hints and questions arose through the review of protumorigenic pathobionts. The protumorigenic bacteria (e.g., *E. coli*, *F. nucleatum*, and *B. fragilis*) identified so far in *in situ* CRC models were all G(–) bacteria with mucosal colonization characteristics. First, the tumor-promoting bacteria are enriched in the mucosa and belong to the category of G(–) microbes, indicating that the juxtaposition of G(–) bacteria and its wall component (i.e., LPS) recognized by mucosal innate receptors are likely to contribute partly to the tumor susceptibility.[33,105-107] The findings re-emphasized the role of abnormal expression or upregulation of epithelial immune receptors in tumor predisposition.[107-109] Second, microbial colonization and active invasion was shown to facilitate other commensal bacteria to be internalized into epithelium,[110-112] and may cause secondary bystanders to pass the epithelial barrier through paracellular spaces.[113-116] The active or passive entry of bacteria could further fuel inflammation-associated tumor growth or may induce DNA damage and hyperproliferative signals in host cells. In addition, the common features of mucosal colonization among protumorigenic bacteria suggest that there may be shared adherence/invasion-associated virulence factors to induce epithelial damages, which may partly underlie bacterial mechanisms of epithelial malignant transformation. Third, physiological mucosal defense against pathogen colonization and invasion included free radical production[117] and autophagy of infected organelles in the epithelial cells.[118,119] The mechanisms underlying the incompentent clearance of intracellular microbes remain poorly understood. A recent paper suggested that increased levels of microRNA 106B caused a reduction of autophagy-related gene transcription in intestinal epithelia of CD may account for the failure of intracellular bacterial clearance.[120] It remains unclear whether epithelial recognition of pathobionts derived from commensals may activate suppressive/tolerant or defensive mechanisms. The involvement of epithelial barrier impairment in tumorigenesis warrants further studies.

In summary, it is widely recognized nowadays that intestinal microbiota is involved in colorectal tumorigenesis. Protumorigenic bacteria (e.g., *E. coli* and *F. nucleatum*) which are not predominant species in fecal microflora are enriched in the cancerous tissues, and may promote tumorigenesis by expression of genotoxins and virulence factors. Further

understanding of the host-microbe interaction and how the bacterial factors fit into the genetic and molecular paradigm of tumor development will shed light to the development of novel microbe-targeting therapy. Specific elimination of pathobionts with sparing of beneficial symbionts would be the next challenge.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTION

Writing - original draft: LCY. Writing - review and editing: LCY, SCW, YHN. Approval of final manuscript: LCY, SCW, YHN.

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