REVIEW

Lib Oncol. 2021;49(2-3):124–132
doi: 10.20471/LO.2021.49.02-03.18

MICROBIOME AND CANCER

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Summary

The aim of this review paper is to present the complex interactions between microbiome and the host and the importance of the microbiome in maintaining homeostasis. The ways by which oncomicrobes can influence cancer development, and ultimately the possible impact of the microbiome on the cancer treatment, are reviewed. Microbiome is a community of trillions of microbes and their structural elements, with significant medical potential. It is thought that the microbiome's genome contains approximately 300 times more genes than the human genome. Microbiome is crucial for the homeostasis and well been of an organism. Dysbiosis in the microbiome can lead to developing of various negative impacts on an organism, including carcinogenesis. For some oncomicrobes is has been conclusively proven to be biological carcinogens, and for many others, there is an evidence of their possible involvement in carcinogenesis. Studies have shown that the microbiome can have an impact on every type of medical treatment, including anticancer therapy, by changing its effectiveness and toxicity. Future microbiome research will undoubtedly enable to open new possibilities in the fields of treatments and early diagnosis of cancer.

KEY WORDS: *microbiome, oncomicrobes, cancer*

INTRODUCTION

The human body contains trillions of microorganisms, which are present on all surfaces of organs that are in contact with the external environment, such as the skin, oral and nasal cavities, gastrointestinal tract and vaginal cavity. For example, the concentration of bacteria in the colon is between 10^{11} to 10^{12} /mL, in saliva 10^{9} /mL, on the tooth surface 10^{10} /mL, in the vagina 10^{7}-10^{8}/ mL(1). It is estimated that microorganisms in the digestive system contain over 9 million genes and exceed the human genome 300-fold(2). The number and diversity of microorganisms and the complex interactions between microorganisms and hosts are extremely important for the health and development of the immune system, energy and metabolic homeostasis, nutritional support, and hormonal, immune and inflammatory regulation. This complex chain of interactions between microorganisms and their area of activity is called the microbiome.

All living microorganisms like bacteria, fungi, archaea, small protists and algae in the microbiome are called microbiota. Besides microbiota, the microbiome consists of a whole spectrum of structural elements like proteins, polysaccharides, lipids, nucleic acids, relic DNA, viruses, bacteriophages, toxins, signalling molecules, other organic and inorganic molecules.

Colonization of the digestive tract and the development of the microbiome begins immediately after birth via microbial flora from the moth-

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er's skin, vagina and feces. There are large individual variations in the digestive tract microbiota depending on the type of birth, diet, hygiene conditions, contact exposure, antibiotics/vaccination usage, host genotype.

Fecal microbiota in adults is considered relatively stable in the absence of environmental, developmental, and pathological factors. The gastrointestinal system is colonized by approximately between 3 and 10 different microbes but is predominantly dominated by bacteria from 3 primary phyla: Firmicutes, Bacteroidetes, and Acinetobacteria(3,4).

Rapid advances in microbiome research have been made possible by the discovery of a molecular technique for sequencing bacterial 16S ribosomal RNA because the vast majority of bacteria cannot be cultured. Analysis of ribosomal 16S RNA is a useful phylogenetic marker and allows quantification of the bacterial makeup at the level of the bacterial genus(5). Further deepening of knowledge about the microbiome is enabled by advances in metagenomics or shotgun sequencing, metatranscriptomics and proteomics(6-8).

The microbiome of the gastrointestinal system is essential in maintaining homeostasis and plays an important role in the protection of intestinal epithelium, metabolism and digestion of nutrients, vitamin synthesis and control of potentially pathological microorganisms(9).

Changes in the microbiome due to dietary or environmental factors (e.g. infection, lifestyle) can lead to disruption of the normal intestinal microflora or dysbiosis. Dysbiosis is usually characterized by the loss of beneficial bacteria, the expansion of pathological bacteria, and the general loss of bacterial diversity(10).

Poor oral hygiene leads to oral dysbiosis, which is linked with the development of dental caries, periodontal disease and oral stomatitis(11). According to some studies, there is an evidence linking oral dysbiosis to the development of head and neck cancers and tumors of the digestive system(12).

Dysbiosis can be also associated with a number of other diseases, such as chronic liver disease, inflammatory bowel disease, multiple sclerosis, diabetes, obesity, allergies, cardiovascular disease, chronic inflammatory disorders of the skin and cancer(13-20).

The importance of treating and recognizing gut dysbiosis is reflected in the successful treatment of recurrent *Clostridium difficile* infection using a novel fecal microbial transplantation (FMT) treatment(21). FMT increases the diversity of the microbiota and concentration of beneficial bacteria and reduces the concentration of pathogens in order to restore gut homeostasis. By establishing homeostasis, the local permeability of the intestinal epithelium is restricted, its integrity is increased and the systemic and local inflammation is diminished(22). Success in the treatment of *Clostridium difficile* infection has enabled further studies on the use of FMT in the treatment of other conditions associated with dysbiosis such as irritable bowel syndrome, pouchitis, inflammatory bowel disease, eradication of resistant microbes, hepatic encephalopathy, sepsis, neuropsychiatric and hematologic diseases(23).

MICROBIOME IMPACT ON GUT HOMEOSTASIS

An important study using a mouse model of inflammation-induced tumorigenesis has shown the importance of the gut microbiome in protecting against colon tumours. In this study, germ-free mice were exposed to the carcinogen azoxymethane (AOM) and epithelium-damaging and inflammation-inducing substance dextran sulfate sodium (DSS). The results showed that germ-free mice develop more and larger colon tumours compared to control mice. Furthermore, recolonization of germ-free mice by commencal bacteria reduces tumorigenesis(24). Probable mechanisms by which the microbiome can protect against tumorigenesis are biotransformation of numerous chemical compounds that act as a barrier, facilitation of epithelial repair, downregulation of inflammatory pathways that promotion of tumorigenesis, prevention of dysbiosis(25-28).

Gut bacteria also produce numerous metabolites such as short-chain fatty acids, secondary bile acids, alcohols, ammonia, branched-chain fatty acids, amines, sulfur compounds, phenols and indoles, glycerol and choline derivatives(29). Short-chain fatty acids (SCFA) such as acetate, butyrate and propionate, which are released by bacteria through fermentation of resistant starches and fibers, have attracted a lot of attention from re-

searchers. Butyrate plays an important role in the health of the intestinal epithelium and is the main metabolic substrate of colonocytes that meets 60-70% of energy demands needed for proliferation and differentiation(30).

Colonocytes of germ-free mice are deprived of SCFA and in a state of energy deficit and are consequently prone to autophagy(31). Furthermore, SCFAs play an important anti-inflammatory role by inhibiting the interleukins IL-12 and TNFα, suppressing the activity of the NF-κB complex, modulating neutrophil chemotaxis, promoting the release of reactive oxygen species (ROS). Increased NF-κB pathway activity is identified as a cancer promotor that leads to abnormal cell proliferation and differentiation(32). These interactions are enabled because SCFA is a ligand for GPR 41, GPR 43, and GPR 109a receptors. Activation of the GPR 109a receptor by butyrate can cause apoptosis of malignant colon cancer cells and thus inhibit tumor growth(33). This may explain the observation that a high-fiber diet reduces the risk of colon cancer as the concentration of SCFA depends on the amount of fiber-rich food ingested(34).

There is also evidence that other bacterial metabolites such as secondary bile acids play an important role in the onset and progression of cancer. Secondary biliary acids, deoxycholic acid (DCA) and lithocholic acid (LCA), are formed as a result of deconjugation of primary biliary acids by gut bacteria. DCA and LCA may have cytotoxic effect by increasing the production of reactive oxygen and nitrogen species, and that can lead to increased DNA damage and mutations. Bile acids also can activate different oncogenic signals like NF-κB, EGFR, MR3, Cox2 pathways(35).

ONCOMICROBES

To date, only ten carcinogenic microbes have been conclusively proven, according to the International Agency for Cancer Research (IACR). The list is dominated by oncoviruses: Epstein-Barr virus (EBV), Hepatitis B and C virus (HBV, HCV), Kaposi sarcoma herpesvirus (KSHV), Human T-lymphotropic virus (HTLV), Human papillomaviruses (HPV) and Human immunodeficiency virus (HIV)(36).

Parasites with carcinogenic potential include *Schistosoma haematobium, Opisthorchis viverrini* and *Clonorchis sinensis*. Only one bacterium is recognized as a definitive biological carcinogen - *Helicobacter pylori*.

For many other bacteria, there is a strong evidence that they play an important role in carcinogenesis.

The colonization of oncomicrobes alone does not necessarily mean that the affected individual will develop cancer. Only a small proportion of those infected develop cancer which is explained by the influence of genotype on susceptibility to cancer development. EBV is associated with Burkitt lymphoma, B-cell(37,38), T-cell and NK-cell lymphoma(39) and nasopharyngeal carcinoma(40).

EBV has a unique ability to immortalize B lymphocytes. Consequently, the expression of multiple viral proteins (like LMP and EBNA proteins) can lead to the proliferation of infected cells, blocking of apoptosis, cell migration and inducing genomic instability(41). HBV-induced chronic hepatitis and liver cirrhosis is characterized by a vicious cycle of hepatocyte regeneration and necrosis that can eventually lead to mutation accumulation, telomerase reactivation, and consequent hepatocarcinogenesis. Studies show that the smallest HBV protein, Hbx, and PreS/S protein can inhibit p53 and PTEN protein in the cell, disrupt DNA repair mechanisms and stimulate telomerase activity(42).

Similar to HBV, HCV causes chronic inflammation that over time promotes malignant hepatocyte transformation and tumour progression. Viral proteins target important tumor suppressor genes and proto-oncogenes by negatively regulating retinoblastoma protein, promoting proliferation by interfering RAF/MAPK/ERK and Wnt/β-catenin signaling pathways and blocking TNF-α mediated apoptosis(43). Studies have shown that all clinical forms of Kaposi's sarcoma (endemic, classic, HIV-related and iatrogenic) are associated with KSHV infection. Also, data support the connection of KSHV with primary effusion lymphoma and multicentric Castleman disease(44-46). Like all herpes viruses, KSHV enters a latent phase after an acute illness. The key viral proteins that maintain disease in the latency phase are LANA, vCYC, vFLIP, and kaposin A. Latent proteins initiate carcinogenesis by stimulating cell proliferation, antiapoptotic activity, deregulation of the cell cycle, avoidance, and modulation of the im-

mune response. By reactivating the disease, the virus enters its lytic phase in which it synthesizes several lytic proteins such as RTA, MTA and K-bZIP, which allow viral transcription and replication, immune system suppression, angiogenesis and local inflammation(47). Unlike other oncoviruses, HIV cell infection does not cause its malignant transformation and immortalization. HIV indirectly increases the risk of cancer by immunosuppression which in turn enables reactivation of other cancer-related viruses such as EBV, HCV, HBV, HPV and KSHV.

Malignancies in patients with acquired immunodeficiency syndrome generally show more aggressive behaviour and consequently reduced disease-free and overall survival compared to an HIV-negative patient(48-50). There is strong evidence that human papillomaviruses (HPV), especially HPV-16 and HPV-18, are associated with cervical, anal, vaginal, vulvar, penile, oral, tonsillar and laryngeal cancer. The combined presence of viral proteins E6 and E7 readily encourages keratinocytes' immortalisation. Oncoprotein E6 targets p53 protein and thus interferes with apoptosis processes, while oncoprotein E7 targets tumour suppressor protein Rb which in turn leads to proliferation and cell differentiation disruption(51).

Studies have shown that chronic inflammation in the medium-sized or small intrahepatic bile ducts caused by parasites *Opisthorchis viverrini* and *Clonorchis sinensis* causes the development of cholangiocarcinoma(52,53). Similarly, laid eggs of *Schistosoma haematobium* causes a strong inflammatory reaction in the bladder wall. This results in an accumulation of inflammatory cells and increases oxidative stress through the production of oxygen-derived free radicals(54).

Bacteria *H. pylori* infects nearly 50% of the human population and since 1994 is categorised as a biological carcinogen(55). *H. pylori* causes chronic gastritis, duodenal and gastric ulcer, gastric adenocarcinoma and gastric MALT(56). Although *Helicobacter pylori* infection is worldwide spread, only a small number of affected patients will experience malignant transformation.

A combination of specific bacterial strain, host genotype, and environmental factors is thought to be required for cancer development. Among many bacterial proteins, cagA (cytotoxin-associated gene A) and vacA (vacuolating cytotoxin A) are major risk factors. CagA is a highly immunogenic protein that interrelates with various cell signalling and tumour-related pathways which over the years cause dedifferentiation and induce epithelial to mesenchymal transition. VacA protein in vitro showed inhibition of T-lymphocytes activation(57-59).

Among the microbiota, several bacterial strains attract attention and can be potential oncomicrobes. The presence of oral symbiotic anaerobic gram-negative *Fusobacterium nucleatum* in the gut is associated with colorectal cancer. It has the ability to promote carcinogenesis through several viral proteins. *F. nucleatum* expresses key surface protein FadA that enables invasion and adhesion to E-cadherin protein on epithelial, endothelial and cancer cells. E-cadherin activates Wnt/β-catenin pathway which in turn induces expression of T-cell factor (TCF) and thus promotes transcription of Jun, c-*Myc* and Cyclin-D1 oncogenes.

Beta-catenin promotes tumour cell proliferation, survival and progression by suppressing T-cell responses(60). VE-cadherin protein maintains endothelial cell adhesion but when FadA binds on to the VE-cadherin it can disrupt endothelial integrity and increase permeability which allows systemic dissemination(61).

Chronic inflammation is an important factor in CRC genesis. Studies showed that *F. nucleatum* stimulates chronic inflammation with a release of a whole range of proinflammatory cytokines like IL-6, IL-8, IL-10, IL-18, activation of NF-κB pathway, production of reactive oxygen species (ROS), and by increasing expression of miR21. Thus promoting tumour cell proliferation and metastasis (62). Furthermore, two more membrane proteins Fap2 and RadD ease the colonization of even more *F. nucleatum* and the creation of biofilms(63,64). Fap2 has the ability to suppress the immune system by decreasing the killing ability of NK-lymphocyte and activation of T-cells(65,66).

Some bacteria have evolved the ability to damage DNA using toxins and this way facilitate the transformation of a healthy cell by inducing genome instability. For example, Enterotoxic pks+ E.coli is more often isolated from CRC samples then from healthy colon tissue(67). This evidence suggests an important role of pks+ *E. coli* in CRC carcinogenesis. Pks (polyketide synthetase) region of E.coli genome codes genotoxin colibactin. Wil-

son et al. have proven that colibactin alkylates DNA in vivo and generate DNA adducts, thus generating mutations in oncogenes and tumour suppressor genes(68).

Enterotoxigenic *Bacteroides fragilis* has the capability to produce biofilms in the gut and BFT toxin. BFT toxin encourages inflammation, increases intestinal permeability, interferes with intracellular signaling pathways, DNA damage via increased ROS production(69). The Cytolethal Distending Toxin (CDT), produced by some gram-negative pathogenic bacteria, was named by the ability to induce distension of affected cell and DNA damage (single and double DNA strands breakage) that leads to cell death(70). In the literature, there are still numerous bacterial infections and its products that are associated with carcinogenesis such as *Salmonella typhi, Streptococcus bovi, Campylobacter jejuni, Chlamydia trachomatis, Porphyromonas gingivalis*(71-74).

THE EFFECTS OF MICROBIOTA ON CANCER THERAPY

There is gathering evidence that microbiota participates in drug metabolism and influence its toxicity and efficacy. Cyclophosphamide (CP) is an important alkylating cytostatic used in the treatment of numerous hematologic and solid malignancies. Research showed that CP alters intestinal microbiota, hurts intestinal epithelium and allows selective gram+ species to translocate in lymphoid organs. Translocation of microbiota stimulates immune system and activates *pathogenic* Th17 and Th1 cells thus enhancing CP activity. Studying mouse model, germ-free mouse or mouse treated with antibiotics against gram+ bacteria demonstrate CP resistance(75).

Microbiota can also increase the efficacy of oxaliplatin, a cytotoxic drug that form platinum DNA adducts and intraDNA cross-links. The cytotoxic effect of platinum-based agents also depends on the production of ROS. The study showed that mice treated with antibiotics and control mice had similar levels of DNA linked platinum, although antibiotic treated mice displayed reduced DNA damage. This data indicates that microbiota prepares immune cells for stronger pro-inflammatory responses and increased ROS production(76).

Microbiota can increase the toxicity of irinotecan, a topoisomerase I inhibitor by secreting ß-glucuronidase. Bacterial ß-glucuronidase enzyme causes reactivation of inactive irinotecan form (SN38-G) that was excreted via bile back to active SN38 form thus trigger severe mucositis and diarrhea(77). Severe diarrhea is an indication for dose adjustment or chemotherapy cancelation. Concomitant administration of ß-glucuronidase inhibitor could resolve irinotecan-induced diarrhea(78).

A meta-analysis of randomized controlled trials showed that probiotics containing *B. bifidum*, *L. acidophilus*, *Lactobacillus casei* were associated with lower incidence of radiation-induced diarrhea(79,80).

Anticancer treatments for many cancers is hampered by the ability of the malignant cell to avoid immune surveillance and disorders of the antitumour immune function. A great advance in cancer treatment has been made with immunotherapy that allows the reactivation of the immune function by blocking the immune checkpoints (eg. PD-1 / PD-L1, CTLA-4). Variations in response to immunotherapy have been observed among patients. According to some research, these variations can be explained by the interaction between the microbiota and the immune checkpoint inhibitors(81).

Bifidobacteria, *A. muciniphila*, *Faecalibacterium* and *Bacteroides* are associated with amplified PD-1 blockade, while *B. fragilis* and *Faecalibacterium* are connected with enhanced CTLA-4 blockade. The microbiota appears to play an important ancillary role in antitumor immunotherapy by activating dendritic cells, Th1-cell response and Treg cells(82-84).

CONCLUSION

The microbiome is critical in the development and proper function of the immune system. It is also essential for nutrition and protection against carcinogenesis. Disruption of gut homeostasis leads to dysbiosis, a state of disbalance between microbiota and the host. As shown in this review paper, dysbiosis is a fertile ground for carcinogenesis driven by oncomicrobes. The two main mechanisms by which oncomicrobes can be involved in carcinogenesis include chronic inflammation by affecting complex cellular signalling

pathways and immunosuppression. The microbiome plays an important role in anticancer treatment: chemotherapy, radiotherapy as well as in immunotherapy. The efficacy and toxicity of anticancer treatment can be influenced by the microbiome. Microbiome should be considered as a vast organ with significant medical potential.

Microbiome research holds great promise in developing new methods for cancer screening and prevention, as well as in potential discovery of new antibacterial, antitumour and anti-inflammatory drugs. Existing and/or potentially newly created microbiome can be used to stimulate immune function or deliver drugs. New technologies like cancer bacteriotherapy and synthetic biology are developing. Further investigations are needed to enable better understanding of the complex interactions among microbes, host and the immune system and the role of microbiome in carcinogenesis.

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Sažetak

MIKROBIOM I RAK

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Cilj ovog preglednog rada je prikazati kompleksne međuodnose mikrobioma i domaćina, te važnost mikrobioma u održavanju homeostaze organizma. Prikazani su mogući načini utjecaja mikrobioma na nastanak malignih promjena, kao i na liječenje raka. Mikrobiom je zajednica trilijuna mikroba i njihovih strukturnih elemenata sa značajnim medicinskim potencijalom. Danas se smatra da genom mikrobioma sadrži oko 300 puta više gena nego ljudski genom. Mikrobiom je ključan za održavanje homeostaze i zdravlja organizma. Promjene u mikrobiomu, odnosno disbioza, mogu imati različite negativne učinke na organizam, uključujući i karcinogenezu. Za neke onkomikrobe je dokazano da mogu biti biološki karcinogeni. Studije su pokazale da mikrobiom može utjecati na brojne načine liječenja u medicini, uključujući i liječenje raka, pri tome mijenjajući učinkovitost i toksičnost terapije. Buduća istraživanja mikrobioma će nedvojbno dovesti do novih mogućnosti na području liječenja i rane dijagnostike malignih bolesti.

KLJUČNE RIJEČI: *mikrobiom, onkomikrobi, rak*