Novel Directions of Precision Oncology: Circulating Microbial DNA Emerging in Cancer-microbiome Areas

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Abstract

Microbiome research has extended into the cancer area in the past decades. The microbes can affect oncogenesis, progression and treatment response through various mechanisms, including direct regulation and indirect impacts. Microbiota-associated detections and agents have been developed to facilitate cancer diagnosis and therapy. Additionally, the cancer microbiome has been recently re-defined. Identifications of intra-tumoral microbes and cancer-related circulating microbial DNA (cmDNA) promoted novel research directions in cancer-microbiome areas. In this review, we define the human system of commensal microbes and cancer microbiome from a brand-new perspective and emphasize the potential values of cmDNA as a promising biomarker in cancer liquid biopsy. We outlined all existing studies on the relationship between cmDNA and cancer, and outlooks for potential preclinical and clinical applications of cmDNA in cancer precision medicine and critical problems to be overcome for this burgeoning field.

Keywords: Circulating microbial DNA; Liquid biopsy; Cancer-microbiome-immunity; Intra-tumor microbiome; Cancer precision diagnosis and therapy;

Introduction

Genetic and environmental factors are considered contributing to the initiation, progression, metastasis, variation and evolution of malignancies [1, 2]. In recent years, previously underestimated roles of the microbiome in cancer are being taken seriously as microbiome shows great prospects in cancer prevention, diagnosis and treatment [3, 4]. Actually, the roles of ubiquitous microbes in cancers[5] remain hidden in the biological black box, although some of associations between microbial and tumors have been revealed in many reports on certain cancer-microbial pairs [6-8].

Previous calculations showed that the microbes (mostly bacteria) coexisting in human body represent 1%-3% of the overall body weight, approximately 1 to 3 kilograms (kg) in a 70 kg adult, while the total number of bacteria in the body actually exceeds that of human cells [9, 10]. In a symbiotic relationship, commensal microbiota can regulate many functions of human hosts[11-17].
Typically, they play the important roles in host immunomodulation [18, 19], such as the formation and training of the host immunity [20, 21]. Perturbation of commensal microbiota may result in impaired immune response to infectious and non-infectious factors. Consequently, human microbiota was considered as the largest immune organ of human body. However, they do more than that. Other physiological and pathological functions, including cells proliferation and differentiation, circadian rhythmicity, metabolism, inflammation, tumor invasion and migration as well as cancer treatment response are in their repertoire [18]. Hence, we proposed that commensal microbes of human body deserve to be considered as a separate organ system, named the commensal microbial system (Fig. 1a). Moreover, the underlying interactions of microbial system and the other human systems deserve more explorations (Fig. 1a).

According to the studies of decades, microbes emerge and implement essential roles in tumor occurrence and development, such as human papillomavirus (HPV) in cervical cancer [22, 23], hepatitis B virus (HBV) in liver cancer [24], and helicobacter pylori (HP) in gastric cancer[25]. These microbes and their nucleic acids and proteins can take effects by interacting with tumor signaling pathways [26, 27]. Moreover, host immunity has been regarded as an important facet in the interaction of microbes and tumors [18]. Gut microbiota contributes to shape innate and adaptive immune and is associated with the efficacy and adverse events of ICIs immunotherapy [28, 29]. On the other hand, host immunity impacts on the interaction of microbes and cancer [30, 31]. More recently, certain specific bacteria were identified in previously presumed aseptic tumor tissues and the concept of intra-tumor microbiome (ITM) were being established, which represented a novel direction of the cancer-microbiome-immunity areas [32]. However, the source of intra-tumor microbes remains unclear; the roles that these microbes play on the development of cancer are still in discussion; and the promising applications of ITM in cancer prevention, diagnosis and treatment are even further down the road in this blossoming field [33]. Excitingly, multiple studies identified circulating microbial DNA (cmDNA) in plasma cell-free DNA (cfDNA) of cancer patients and healthy donors after ruling out possible contaminations and have tried to utilize cmDNA in cancer diagnosis training [34, 35]. Identification and studies of cmDNA propose a new direction for cancer liquid biopsies, which will promote cancer precision medicine to blossom.

The new view is that microbes or microbial nucleic acid not only exist in plasma and tumor cells of cancer patients; but also, can be detected in the peripheral blood of healthy individuals [5, 36]. Furthermore, cmDNA can reflect the results of the interactions between microbiome, cancers and immunity, thus, comprehensive and detailed explorations on cmDNA could provide important clues for personalized diagnosis and treatment and facilitate the development of cancer precision medicine. The roles of the gut and intra-tumor microbes in malignancies have been well reviewed previously [5, 6, 37, 38], and the actions of cancer-microbiome-immunity axis have also been summarized comprehensively [39]. Therefore, in this review, we focused on the potential clinical applications of cmDNA. In summary, we specifically aim to: (1) A brief overview of the interactions between microbes, cancers and immunity to provide a background for further cmDNA discussion; (2) highlight the identification, source and research prospects of cmDNA and summarize the exciting translational applications of cmDNA in precision medicine for tumor diagnosis, staging and typing, treatment and prognosis; (3) dissect potential obstacles and critical problems of cmDNA that need to be addressed in the future preclinical research and clinical laboratory applications; and (4) propose the conceptual assumption of tumor microbial burden (TMbB) and list the needed researches of this fields in the future to promote the development of cancer precision medicine.
1. Background of cmDNA liquid biopsy: microbiome-cancer-immunity studies

Long-term investigations have demonstrated human microbiome played crucial actions in cancer susceptibility, development and therapeutic response [40-42]. The complex interactions occur among commensal microbes, cancers and host immunity [43-45].

1.1 Composition of human microbiome for cancer patients

The broad-sense human microbiome should be defined as the collection of all microbes (bacteria, viruses, fungi, etc.) and their components (DNA, RNA and proteins) located at every part of human body, including gut, lung, oral, vaginal, peripheral blood microbiome, as well as any other microbiomes of parenchymal and interstitial tissues [46]. For cancer patients, the definition should be established as the above concepts plus intra-tumor microbes. Recently, intra-tumor microbiome is considered as a crucial part (Fig. 1b) since Nejman et al. detected more than 1500 human tumors tissues of seven different tumors plus adjacent normal tissues and demonstrated that the living bacteria exist in tumor tissues [33]. They found different types of tumors and different cells in the same tumors, have different bacterial species, DNAs and RNAs [33]. However, the source of the intratumor microbiota is rarely reported and summarized. We proposed and discussed three possible source routes: (i) exogenous source, such as digestive tract, respiratory tract, and urogenital tract. Intra-tumor microbes of intestinal, bronchial and urogenital neoplasms may come from corresponding organs connected to the outside, they are digestive tracts, respiratory tracts, and urogenital tracts; (ii) inborn source, such as parental heredity and intrauterine microbes. This denotes that the microbial fragments existing in human genome or from normal or abnormal intrauterine flora may be present in the offspring and in some tumors; and (iii) peripheral blood source, such as sepsis and bacteremia during infections. This means some microbial fragments may remain in blood or tissues after individuals recover from previous infections during non-tumor-bear or tumor-bear stage. The specific mechanisms can be inferred as follows: first, numerous studies have shown that bacterial translocation may occur between the intestinal mucosa and sterile tissues and organs, as well as tumor tissues [47, 48]. Septicemia or undetected bacteremia can also lead to the location of bacteria at the specific tissues and organs, or mucosal innate immune cells may engulf some bacteria and serve as a shield for them and be carried out to other organs through the lymphatic circulation. Additionally, horizontal gene transfer (HGT), also called lateral gene transfer, is a process by which genetic material is passed between microbes in a non-parent offspring fashion [49]. Bacteria and virus DNA are transferable to human offspring if the HGT occurs in human germ cells. Additionally, genetic materials of the mitochondria are inherited materially to offspring in humans, while it is known that the mitochondria organelle evolved from natural archaea [50]. Thus, we speculate microbiota exists and can change at any time and everywhere in the body and participates in tumorigenesis and development. In addition to intra-tumor microbes, circulating microbial molecules are another important part of human microbiome (Fig. 1b). Emerging evidence demonstrated that cmDNA was significantly distinct between tumor patients and healthy individuals [34]. We will make an in-depth summary and discussion on the identification history, fragment source, basic research and clinical application value of cmDNA later in this article.

1.2 Studies of human microbiome with cancer susceptibility, cancer occurrence and development, cancer treatment efficacy and side-effects.

It’s estimated that 15.4% of human cancers are attributed to microbial infection at early age [51]. There are eleven microbes in the list of group 1 pathogenic carcinogens defined by the International Agency for Research on Cancer (IARC) [52]. Furthermore, some other microorganisms may be
correlated to various tumor carcinogenesis as indicated from studies in animal models or clinical research (Table 1). Microbes can contribute to tumorigenesis, progression, and metastasis via a myriad of mechanisms and signaling pathways in the ten hallmarks of cancer (Fig. 2) [26, 27, 53]. The linkage of carcinogenesis to viral infections is the most studied thus far [54, 55]. Viral infection and genome integration can almost regulate all hallmarks of cancer to promote tumorigenesis, progression, and metastasis [54, 56]. The roles of viruses in cancer initiation and progression are fairly complicated, in which virus subtypes and host states are both critical deciding factors for the fate of host cells. Many studies explored the complex internal mechanisms [23, 57-66]. For example, mechanisms of H-HPVs in cancer have been partly deciphered including viral genome integration and E5, E6, and E7 effector proteins [23]. H-HPVs proteins can regulate intracellular signal transduction that promotes cancer progression [23], such as tumor-associated angiogenesis [57] and immune-related molecular pathways [58-60].

Oncogenic mechanisms linked to bacterial infection are complex multi-step biological processes and involve the alteration of multiple signal transduction pathways, which are yet to be deciphered [67]. It is generally assumed that bacteria multiple-step biological processes and involve the alteration of multiple signal transduction pathways, which are yet to be deciphered [67]. It is generally assumed that bacteria can escape immune defenses and survive via multiple mechanisms, including cellular antigen modification and variation, secretion of cytolytic protein toxins to eliminate immune cells, and antigen mimicry [69, 70]. Long-term recurrent chronic inflammation stimulates cell proliferation that to induce more base pair mismatches, insertion/deletion mutations and the consumption of DNA mismatch repair (MMR) proteins, which increase the malignant transformation potentials of host cells [71, 72]. On the other hand, bacteria effectors and toxic proteins can regulate the ten hallmarks of cancer through activation of STAT3, MAPK and AKT oncogenic pathways [73, 74] and inhibition of P53 tumor-suppressor pathway [68]. Besides, HGT could be another important mechanism. HGT of bacterial DNA was rarely reported until recently. Schroder et al. reported that the bacterial pathogen 

Bartonella henselae can transfer DNA into the genome of human endothelial cell line EA.hy926 [75]. Riley et al. demonstrated that the bacterial DNA integrations in human cells were more common in tumor cells and Pseudomonas-like DNAs integrate into the sites of four oncogenes to induce gastric adenocarcinoma [76]. Multiple teams proposed that genome integration of bacterial DNA is one of the most important oncogenic mechanisms [77-79].

Aside from cancer occurrence and progression, microbes also play crucial roles in modulating the response of cancer treatment [40, 80, 81]. For instance, intestinal 

Bifidobacterium pseudolongum can enhance the efficacy of ICIs immunotherapy and its inosine metabolites modulated response to ICIs [82, 83]. It was reported that gut Bacteroides ovatus and Bacteroides xylanisolvens could increase lung cancer response to erlotinib, a kind of molecular targeted drug [84]. Additionally, the latest reports claimed that intra-tumor microbes of pancreatic adenocarcinoma (PAAD) tissues, containing bacteria predominately from the 

Enterobacteriaceae and Pseudomonadaceae families, can modulate the resistance of PAAD patients to gemcitabine [32]. Intra-tumor Fusobacterium nucleatum and 

Bifidobacterium were found to be related to the response of colorectal cancer chemotherapy [85, 86]. In addition to bacteria, viruses are also closely related to tumor treatment outcomes [87-89]. For example, a Hodgkin’s lymphoma patient developed significant tumor remission after COVID-19 infection without any anti-tumor treatment [90]. Tumor mycoplasma infection can reduce the efficacy of anti-tumor drug gemcitabine via mycoplasma-encoded deaminase [91, 92]. Presently, microbe-based therapies, including oral prebiotics or probiotics, fecal microbiota transplantations (FMT) or dietary interventions, are being examined as adjuvant strategies of cancer treatment in clinical trials (Table 2).
Besides, there are many clinical trials about the safety and efficacy of oncolytic virus in cancer treatment that are detailed in Table 2 [93]. Commensal microbiota can also impact on the adverse effects of cancer therapy, including ICIs immunotherapy [94-96], chemotherapy [44] and radiotherapy [80, 97]. The predictive performance of gut microbiome for radiotherapy-related toxicity is being evaluated by a clinical trial, NCT04638049 [98]. And a clinical trial, NCT03516461, is being conducted by Zhang et al. to assess the efficacy and safety of FMT for radiation-induced enteritis of abdominal radiotherapy [99].

1.3 Human microbiome and host immunity

The human resident microbes are influenced by intrinsic and environmental factors during an individual’s lifetime which shape the host’s immune characteristics [100, 101]. The enormous communities of commensal microbes play a fundamental role in the induction, regulation, training and education of host immune function [19, 102], including innate immunity and adaptive immunity (Fig. 3a). The co-evolution and mutual adaptation between human immunity and commensal microbiota impact the reactivity of immune systems to new-emerging malignant cells [103]. Therefore, effectiveness of anti-tumor immune response also varies during oncogenesis and development due to the action of microbiota, which is closely related to the efficacy of cancer therapy [91, 104], which has been mentioned in previous reviews [39].

After cancer occurrence, human commensal microbiota at different sites of cancer patients can regulate anti-tumor immune response by modulating seven-step events in the “Cancer-immunity Cycle” (Fig. 3b). The first step of effective anti-tumor immune response is the release and recognition of cancer antigens. Previous studies have reported that cancer antigen molecular mimicry mediated by human microbiota is one of the most important mechanisms by which microbes participate in anti-tumor immune response [105-111]. For example, because of the homology and cross-reactivity between Bacteroides fragilis and tumor antigens, Bacteroides fragilis-specific T cells could restore the therapeutic activity of ICIs immunotherapy in mice [109]. Aside from tumor antigens mimicry, commensal microbes originating in the gut [112-116], tumor tissues [86] and other sites of human bodies [117-119] can regulate the function of antigen presentation cells (APCs) and other immune cells [103, 120].

After cancer antigen presentation, T cells are activated and translocate from the lymphatic system and infiltrate into tumor sites. Human microbiota system could impose crucial actions on these steps (Fig. 3b) [121]. It has been reported that gut bifidobacterium, faecalibacterium, Bacteroides fragilis could increase T cells levels and enhance T cells tumor infiltration to promote anti-cancer immune response [83, 122-125]. Human microbiota also can regulate the function of immune cells and stimulate the secretion of cytokines and chemokines [126-131]. For example, it’s reported that intra-tumor salmonella enterica serovar typhimurium can disrupt tumor vascular and can increase TNF-α secretion in TME [132]. Besides, intratumoral bacterial lipopolysaccharide, lipoteichoic acid and 16S rRNA/DNA may modulate immune cells of the TME to influence anti-cancer effects [33]. Third, the potential roles of microbial DNA circulating in peripheral blood or extracellular vesicles remain undiscovered. Circulating microbial DNA as a kind of cfDNA may be just a transient passenger; but in some situations, they play substantive roles, such as immunomodulatory actions [133]. TLR9, PRR, and cGAS-STING signals are all identified DNA-stimulated immune response pathways [134]. In fact, the underlying phenotypes and mechanisms of cmDNA needs more exploration.
2. Novel directions of cancer liquid biopsy: circulating cell-free microbial DNA

Peripheral blood travels throughout the body of cancer patients and carries the certain molecules from the tissues, including messages of cancer microbiome such as DNA, RNA and metabolites [36]. Microbes and viruses from the whole body of a cancer patients were demonstrated to be strongly involved in the cancer development, metastasis, cancer-immune regulation, cancer treatment response and clinical outcomes [135]. As a result, these cancer regulation molecules from cancer microbiome theoretically enter into blood and could be detected through some methods with sufficient sensitivity, for an instance, cmDNA, one of circulating microbial laboratory indexes. Circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) become excellent examples that apply circulating biomarkers of solid tumors to the clinical diagnosis and therapy in recent years. Reasonably, cmDNA has similar potential for clinical applications of cancer liquid biopsies [136].

2.1 Source of cmDNA in cancer patients

Referring to the previously described knowledge about the source of cell-free DNA (cfDNA), here, we discuss the sources of cmDNA from cancer patients for the first time as follows (Fig. 4): i) passive release of endogenous microbial DNA after cell death, including apoptosis, necrosis, pyroptosis and ferroptosis of cancer cells, immune cells and any other cells; ii) active secretion of cells, including eukaryocytes from cancer patients and prokaryocytes from commensal bacteria; iii) microbe translocation and DNA release; and iv) partially impaired immune clearance of cmDNA [137, 138]. During the process of cancer development, tumor cells grow and proliferate rapidly and compete with each other for relatively-inadequate nutrients. Consequently, large number of tumor cells that died from apoptosis or necrosis released nucleic acids and other molecules. Besides, pyroptosis and ferroptosis are also closely associated with cancer development [139-141]. Various forms of cellular deaths of tumor cells may lead to the release of microbial DNA, RNA and protein which has been demonstrated to exist in tumor tissues in lung cancer, esophageal cancer, colorectal cancer and pancreatic cancer [5]. Interestingly, microbial DNA could be secreted into blood in the forms of extracellular vesicles (EVs). The pathway of viral package, biogenesis and transmission can overlap with secretion and delivery of EVs; EVs and virus particles share common structure, size, and uptake process [142]. Several studies have reported that EVs carry viral genetic materials and envelope proteins [143]. A study on hepatocellular cancer exosome conducted by Yang et al. found that the plasma exosomes containing the HBV DNA and proteins can transfer nucleic acid fragments into other cells including hepatocellular cells and immune cells, and they demonstrated that HBV-positive exosomes induced dysfunction of natural killer cells [144, 145]. Several studies also reported that HCV RNA existed in the plasma exosomes which can modulate immune function by transmitting HCV [146, 147]. Prokaryotes are also able to generate EVs [148], but the EVs from bacteria and viruses can trigger a strong immune reaction and are often cleared rapidly. Microbial DNA and other components need to be wrapped by host-source EVs to escape immune clearance. In theory, microbial DNA in EVs reflects the composition of the human microbiome and cmDNA in blood EVs may serve as a promising biomarker of cancer [138, 149]. Furthermore, microbiota from gut, lung and other organs can translocate into the whole body via blood circulation without causing a systemic infection [150]. Given that Xiao et al. have observed the source of cmDNA was mostly from gastrointestinal genera as well as oral tract microbiome [182], we hypothesize that part of circulating microbial DNA in plasma could derive from translocation of gut microbiota as well as microbiome within tumors which spread into the bloodstream. However, partial or total impairment of host immune clearance function is the decisive condition for cmDNA to survive.
Cancer patients are often in an immune-compromised status and their immune function is often impaired, which allows cmDNA can survive and be detected in tumors and blood.

2.2 The potential clinical applications of cmDNA in cancer diagnosis, staging and prognosis monitoring

Remarkably, emerging evidence in recent years have demonstrated that highly divergent cmDNA showed promising diagnostic and prognostic implications across diverse cancer types (Table 3). Huang et al. [151] and Cho et al. [152] firstly noticed the potential links between cmDNA and cancer through examining the diagnostic performance of cmDNA respectively in early-onset breast cancer (EOBC) and hepatocellular carcinoma (HCC) [151]. Though only five samples were analyzed, Huang et al. found significant differences in bacterial species between breast cancer patients and healthy individuals. EOBC patients had high titer of cmDNA derived from Pseudomonas or Sphingomonas spp., while cmDNA of healthy females were derived from Acinetobacter spp. These results hint that cmDNA from different species of bacteria could serve as indicators in cancer diagnosis. Similarly, Cho et al. [152] evaluated the diagnostic performance of cmDNA in HCC. Through blood metagenomic analyses on 79 HCC patients, 83 cirrhosis patients, and 201 healthy controls, they observed that microbial diversity was reduced in HCC, suggesting that cmDNA characteristics could distinguish HCC from others. Next, they constructed diagnostic model containing 5-genera microbes showing 0.879 AUC (0.729 sensitivity; 0.850 specificity; 0.816 accuracy) in the train set and 0.875 AUC (0.756 sensitivity; 0.797 specificity; 0.798 accuracy) in the test set, indicating great potentials of cmDNA in HCC diagnosis. Besides, Dong et al. [153] found cmDNA from acinetobacter, bacteroides and haemophilus parainfluenzae were enriched in serums of gastric cancer patients.

After that, Poore et al. [34] re-analyzed sequencing data of 18,116 tumor samples across 10,481 patients and 33 cancer types from The Cancer Genome Atlas (TCGA) database. By controlling the possible contaminations and utilizing machine learning (ML), they identified microbial signatures discriminating cancer types after several rounds of modeling. Concerning contamination control as a key link, they identified and excluded external contaminations by using method of sample analyte concentrations described in previous reports and methods of identifying a “blacklist” of microbes from reagents of the same manufacturers and combining the methods of manually reviewed the literature. Besides, internal contaminations during sequencing or data processing were identified by conventional identification method and Bayesian analyses. To further test the performance of cmDNA signatures, they mined blood sequencing data of these samples and found cmDNA patterns performed well on distinguishing early-stage tumors from normal. Then they validated the performance of cmDNA in a separate cohort (prostate cancers vs. healthy individuals: 0.9477 AUC; lung cancers vs. healthy individuals: 0.9716 AUC). The study demonstrated that cmDNA has great feasibility and generalizability as a promising biomarker for cancer liquid biopsy in clinical settings. Following the above studies, Xiao et al. [154] further validated diagnostic value of cmDNA in colorectal cancer (CRC) by performing the whole genome sequencing (WGS) on the plasma samples of 25 CRC patients, 10 colorectal adenoma (CRA) patients and 22 healthy controls. They observed that 127 species showed significant differences between CRC patients and healthy controls. Then they used random forest model to further identify 28 microbial species, and to distinguish CRC/CRA from healthy controls. Additionally, the diagnostic performance of these 28 microbial species was validated via 1X WGS in an additional cohort. Besides, Messaritakis et al. [155] reported the clinical value of cmDNA in predicting outcomes and monitoring treatment efficacy of CRC patients. They found cmDNA, including 16S rDNA, β-galactosidase of E. coli, glutamine synthase of B. fragilis and 5.8S rDNA, was
correlated with diseases progression and survival of CRC patients. The potential value of cmDNA was also reported by Zozaya-Valdés et al. [156] in melanoma, although they claimed that the problem of contaminations should be addressed.

Above all, cmDNA showed great performance in precise diagnosis and staging of malignancies, including lung cancer, prostate cancer, colorectal cancer, gastric cancer, early onset breast cancer and HCC (Fig. 5a), which demonstrated that the diagnostic methods targeting cmDNA possess promising clinical potentials. According to these studies, the levels and species of cmDNA in cancer patients could not only distinguish cancer patients from healthy controls but successfully differentiate early-stage from late-stage patients, suggesting underlying value in cancer staging and early diagnosis (Fig. 5b) [157]. Moreover, some researchers have identified the essential microbiota for the distinction of different tumor subtypes on the basis of the optimal features produced from diverse microbiome computational methods, providing the possibility of cmDNA for serving as cancer-subtype biomarker [158-160]. In present clinical applications and explorative studies, the differentiation of tumor subtypes is mainly guided by the combination of pathology, imaging and molecular biology [161]. Actually, the heterogeneity of cancers far exceeds the current criteria for dividing tumor subtypes. Immune phenotypes of tumor microenvironment are novel and effective methods for tumor typing in recent reports. Intra-tumoral microbes and microbial components show regular and statistical differences in different types of immune cells of microenvironment, which hints that intra-tumoral microbes are associated with tumor immunophenotype. As that, various signatures of cmDNA may represent different cancer subtypes. Therefore, the inclusion of patients’ microbiome profiles may bring unexpected assistance in accurate diagnosing and prognosticating for cancer subtypes (Fig. 5c). cmDNA has great advantages for clinical applications, including a non-invasive biopsy and would reflect therapeutic efficacy more easily with prospects for further applications (Fig. 5d). Besides, with cancer progression, the abundance and the detectable rates of cmDNA could be probably higher, indicating the worse prognosis of patients (Fig. 5d). At present, microbial-based therapy of cancer has been one of the emerging cancer treatment modalities during the past few years, yet the cancer therapy strategies based on the cmDNA have not been explored [162]. The use of specific types of microorganisms as cancer treatment is expected to stimulate the immune system for selective elimination of cancer cells could lead to promising results [163]. Targeting microbiota could be used as adjuvant treatments to improve therapeutic efficiency and reduce related toxicity. After the in-depth explorations on the internal roles of cmDNA in tumorigenesis and progression, cmDNA-based cancer therapy combined with exosomes and liposome technologies could also serve as an effective strategy for cancer treatment in the future. In conclusion, cmDNA was reported to show promising potential in diagnostic, subtyping, therapeutic response, and prognostic prediction biomarkers for cancer patients (Fig. 5). Though, additional studies with larger cohorts and functional mechanisms are warranted to validate this hypothesis. In a word, explorations and applications of cmDNA represent novel directions of cancer precision medicine.

3 Problems to be overcome in the preclinical research and clinical application of cmDNA

In spite of the great potential of cmDNA, numerous problems remain to be addressed in this growing field. We will group the potential problems into the following three aspects: preanalytical, analytical and postanalytical phases.

3.1 Preanalytical problems

Before cmDNA detection becomes a routine test in the clinical laboratory, its theoretical reliability and reasonability in cancer diagnosis or prognosis monitoring still need to be confirmed in more
large-sample-size and multicentric studies, and it must be examined in real clinical settings with more complex subpopulations, including different gender, age, ethnicity, racial background and effects induced by many benign diseases. Most notably, traditional theories suggest that only the tumors specifically associated with microbial infection could be diagnosed according to cmDNA, such as liver cancer, gastric cancer and et al. Actually, as for those tumors in which microbial infection is not a necessary condition, such as lung cancer, microbiota signatures could not only be used for cancer diagnosis, but also show some potentials in cancer staging [34]. The underlying mechanisms of intra-tumoral microbes and cmDNA in cancer initiation and progression remain unclear. We hold the view that it is not a simple cause-and-effect relationship between microbes and tumors but the cross-interactions that occur both before and after the tumorigenesis and during cancer progression. Although cmDNA is not as specific as CTCs for cancer diagnosis, the potentials are unable to be underestimated. Based on the present evidence, the preanalytical problems that need to be considered in sample collection and pre-handling can be summarized as four "W" and a "H", who, when, what, why and how. First, “who” refers to intended population of cmDNA detection. In the study of screening tumor-related cmDNA for cancer diagnosis, the cancer patients who present with an infection should be excluded. Besides, the patients with benign disease should be included as controls to reflect the real application value of cmDNA detection, and the impact of subpopulations like gender and age on the clinical application of cmDNA should also be considered. Second, “when” refers to the time point of sample collection and minimum transport time after collection. Sample collection during microbial infection or antibiotic administration (at least one month apart) should be avoided. cmDNA need to be evaluated in different clinical phases, such as phases of newly diagnosed and before treatment, as well as after operation, chemotherapy and radiotherapy, may indicate different clinical problems. The timepoint of sample collection, days or months after treatment, will also matter. Besides, the minimum transport time of samples depends on various factors [133], including the use of a cfDNA preservation solution. Third, “what” refers to the cmDNA detection methods to be implemented. In the research phase, metagenomic next-generation sequencing (mNGS), 16s DNA sequencing and targeted mNGS were usually considered to screen related cmDNA. While in the phase of clinical application, simple, fast and low-cost methods were usually adopted, such as targeted mNGS, qPCR and droplet digital (ddPCR). The last W, “why”, refers to different indications for clinical applications of cmDNA test, including early screening of cancer, diagnosis, therapeutic efficacy monitoring and prognosis evaluation. Finally, the “H”, how, contains all the details of cmDNA detection techniques and procedures in clinical laboratory, including sample volume, selection of anticoagulant, sample storage conditions, sample standard operation procedure (SOP), and all quality control (QC) measurements. Among them, QC measurements are the most important. For example, eliminating the disturbance of cfDNA from other cells, such as leukocytes cfDNA, and other blood components, such as cell debris of hemolysis, can improve sensitivity of cmDNA detection. Furthermore, the microbial contamination control should be taken into consideration during sample collection and processing, for example, cutaneous and environmental contamination should be considered. The use of blank environmental controls is one of the most effective strategies of microbial contamination control. In summary, the corresponding strategies about all of the above problems should be well prepared before the cmDNA detection and analysis.

2 Analytical problems
Preclinical research and laboratory application of cmDNA in cancer diagnosis, cancer stage and prognosis involve multi-steps procedures, taking the mNGS method as an example, this procedure
involve the extraction of plasma cfDNA, preparation before sequencing, mNGS and bioinformatic analyses. First, specificity and sensitivity are significantly crucial for a routine laboratory test. The specificity of cmDNA detection depends on selection of the cmDNA panels of corresponding microbial species in the study. The sensitivity of cmDNA detection depends on various factors, including plasma cmDNA extraction efficiency and detectable rate. Before cfDNA extraction step, cmDNA capture efficiency of various cfDNA extraction kits should be evaluated because cmDNA is more fragmented than other cfDNA [164]. In order to improve the detectable rate of cmDNA, mNGS method with high coverage and resolution is preferable in research phase [34, 35]. For the clinical application phase, simpler methods like PCR, more sensitive ddPCR or lower-cost 16S sequencing methods are more preferable [165]. For mNGS, advancement of library construction methods, such as ssDNA method [166], and increase of sequencing depth, such as depth of 25-30× used in the study of Xiao et al. [35], could improve the sensitivity of cmDNA detection. PCR and ddPCR methods are highly sensitive, and the design of primers and probes, as well as use of the multiplex PCR method, can improve the specificity of amplification and detection. Other strategies also include the combined applications of cmDNA detection with other clinical laboratory inspection items for cancers, such as ctDNA detection and analyses. Microbial contamination induced false positivity is another problem of concern in cmDNA detection and analysis. Microbes are everywhere in human bodies, air environment, instruments, consumables, and reagents, consequently, microbial contaminations can occur from sample processing to testing. Negative controls, mixtures of environmental brushing PBS, consumables-washing PBS, and all reagents, are critically necessary to be tested simultaneously for contamination. Besides, some strategies should be adopted during bioinformatic analyses to remove microbial data from common contamination [34]. We also need to take some measures to avoid false negative results caused by system factors, for example, standard positive control throughout the procedures can be used to avoid false negative and guarantee quantitative accuracy.

### 3.3 Postanalytical problems

Postanalytical procedures covers data interpretation and results reporting. Laboratory physicians should undergo professional training on the cmDNA data interpretation and reporting. Results should be analyzed and reported and combed with the patient’s clinical features and other laboratory data, such as inflammation related items including clustering and counting of white blood cell, C reactive protein (CRP), and erythrocyte sedimentation rate (ESR) to put the data into perspective. Besides, a benchmark database should be established and continuously achieve data accumulation in the laboratory to rule out false positives caused by contamination.

### Conclusions and future perspectives

The definition of cancer microbiome research moves beyond the confines of the traditional gut microbiota into the more systemic microbial research, including intratumor microbiome and cmDNA. In fact, cmDNA reflects an overall situation of microbial burden and the results of interactions between microbes, tumors and immunity in cancer patients. For both in-depth basic research and further clinical applications, this was a revolutionary and explosive change for this fast-growing field. In the future, novel explorations of cmDNA will provide significant clues for malignancy prevention, control, diagnosis and treatment to further promote the development of cancer precision medicine. With the development of detection techniques and in-depth exploration of cmDNA, tumor microbial burden (TMBB), defined as the promising quantitative index of microbiota of cancer patients, may serve as a potential biomarker in cancer diagnosis and therapy. Similar to the concept of the tumor mutation burden (TMB), TMBB is also closely related to host immunity and treatment response of tumor, while
the difference is that the latter is the concept of tumor-related microbiology, and the former is the concept of tumor molecular mutation. We assume that TMB in the future research also could be divided into tumor tissue TMB and blood TMB. Levels of tumor tissue TMB may be measured by the density of microbe cells in the tumor, the proportion of microbial nucleic acid in situ hybridization, or the proportion of microbial proteins in immunohistochemical staining. And the measurement criteria of blood TMB are mostly related to cmDNA. Furthermore, the potential roles of intratumor microbiota in oncogenesis and cancer development may provide other possible explanations for the crucial gaps of knowledge in cancer research, such as mutation genesis and evolution, regulation of cancer immunity and other cancerous signal pathways, which may also be reflected by cmDNA. In this review, we claim that the mechanic and clinical explorations of cmDNA represent an excitingly promising direction, especially in the early-stage diagnosis of solid tumors. However, a great literature gap existed between small-sample-size observations and clinical applications targeting cancer microbiomes. In the future, the value of cmDNA in cancer precision medicine should be tested through more large-sample-size, multicentric, longitudinal studies. Overall, the necessary studies for the future are summarized as follows: 1) the source and production mechanisms of cmDNA; 2) matched detection techniques and methods; 3) the roles and mechanisms of cmDNA in cancer development and invasion; and 4) the exploration of new diagnostic methods, new therapeutic strategies and adjuvant treatment methods targeting human systemic microbiomes. Significant achievements in these directions will provide more possibilities and options for precision medicine in oncology.

Author contributions
WL and BY contributed to design the main ideas of this review and reviewed the manuscript. LY, JZ and ZX retrieved literatures and wrote the manuscript. J. Spencer Hauck reviewed the manuscript and polished English. FN, XZ, ZL and JT contributed to the manuscript revision.

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Conflict of Interests
The authors declare that they have no conflict of interests.

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Figure 1 Definition of human commensal microbial system and the microbiomes of cancer patients. (a) Commensal microbial system, as a novel separate organ system like the other nine systems in the human body, could interact with other human systems to maintain homeostasis; (b) the microbiomes of cancer patients include intratumor microbiome, circulating microbiome and the microbiomes of other organs including gut, lung, skin, oral cavity, etc.
Figure. 2 Interactions between microbes and cancer. Bacteria and viruses can implement important roles on tumorigenesis and progression through modulating the ten crucial hallmarks for cancer development.
Figure 3 Interactions between human microbiome and immune system. (a) An individual is exposed to enormous communities of microbes throughout his or her life, and these microbes could play fundamental roles in the induction, regulation, training and education of host immune function, including innate immunity and adaptive immunity. In reverse, host immune could modify the composition of microbial communities; (b) microbes at different sites of cancer patients can regulate anti-tumor immune response by modulating the seven-steps events of cancer-immunity cycle.
Figure 4 Hypothesized mechanisms by which microbial DNA enters peripheral circulation. The release of tumor microbial DNA following cell deaths in cancer tissues, the secretion of vesicle containing microbial DNA, and translocation of intestinal microbial DNA are all the potential sources of cmDNA.
Figure. 5 The underlying clinical value and research directions of cmDNA in cancer patients. (a) reported cancers in which cmDNA showed excellent diagnostic performance; (b) cmDNA has been revealed to have great values in cancer staging and early diagnosis; (c) the combination of pathological, imaging, and molecular characteristics with microbiome profiles of cancer patients may assist to divide cancer subtypes with more clinical diagnostic and therapeutic significance; (d) intestinal microbes of cancer patients, intra-tumor microbes and cmDNA are influenced by diet and drugs, and are closely associated with efficacy and adverse events of anti-tumor drugs as well as patients outcomes.
Table 1 Microorganisms that were reported to be associated with cancer genesis but not classified as group one pathogenic carcinogens.

| Cancers                      | Microorganisms                                                                 | References |
|------------------------------|------------------------------------------------------------------------------|------------|
| Cholangiocarcinoma           | *Human Polyomavirus 6 (HPyV6)*, *Human Polyomavirus 7 (HPyV7)*, *Merkel cell polyomavirus (MCPyV)* | [167]      |
| Colorectal cancer            | *Bacteroides fragilis*, *Escherichia coli*, *Enterococcus faecalis*, *Streptococcus galloyticus*, *Parvimonas*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella*, *Fusobacterium nucleatum* | [168]      |
| Esophageal cancer            | *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria* | [169]      |
| Gallbladder carcinoma (GBC)  | *Salmonella typhi*                                                           | [170]      |
| Pancreatic cancer            | *Neisseria elongate*, *Streptococcus mitis*, *Porphyromonas gingivalis*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Synergistetes*, *Euryarchaeota*, *Saccharopolyspora*, *Pseudoaxanthomona*, *Streptomyces* | [171]      |
| Lung cancer                  | *Chlamydia pneumoniae*                                                      | [170]      |
| Bladder cancer               | *Staphylococcus aureus*, *Klebsiella spp.*, *Proteus mirabilis*, *Acinetobacter*, *Fusobacteria* | [170, 172, 173] |
| Breast cancer                | *Methyllobacterium radiotolerans*, *Escherichia coli*, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Brevundimonas*, *Mobiluncus*, *Candida*, *Geotrichum*, *Rhodotorula*, *Trichosporon*, *Epidermophyton*, *Trichophyton*, *Trichinella* | [174, 175] |
| Cervical cancer              | *Lactobacillus*, *Atopobium vaginae*, *Dialister invisus*, *Finegoldia magna*, *Gardnerella vaginalis*, *Prevotella buccali*, *Prevotella timonensis* | [176, 177] |
| Endometrial cancer           | *Atopobium vaginae*, *Porphyromonas sp.*                                    | [178]      |
| Ovarian cancer               | *Proteobacteria*, *Firmicutes*, *Brucella*, *Chlamydia*, *Mycoplasma*, *M. genitalium*, *C. trachomatis*, *Neisseria gonorrhoeae* | [179-181] |
| Oral Squamous Cell Carcinoma | *Porphyromonas gingivalis*, *Fusobacterium nucleatum*                         | [182, 183] |
| Prostate cancer              | *C. trachomatis*, *N. gonorrhoeae*                                          | [184]      |
### Active clinical trials of bacterial agents and oncolytic viruses applying to cancer therapy and adjuvant therapy.

| Interventions | NCT number | Cancer types | Purpose |
|---------------|------------|--------------|---------|
| Fecal Microbiota Transplantation (FMT) | NCT03819296, NCT04163289 | Melanoma or genitourinary cancer, Renal cell carcinoma | Effectiveness on medication-induced gastrointestinal complications, Reducing the occurrence of immunotherapy-related adverse events (irAEs) |
| | NCT03772289 | Melanoma | Safety and efficacy on immunotherapy patients |
| Mixed prebiotics or probiotics | NCT03773003 | Various tumors | Investigating the possible Benefit for cancer related fatigue |
| | NCT03552458 | Head-and-neck cancer | Efficacy on oral mucositis of patients undergoing head and neck radiotherapy |
| | NCT04021589 | Metastatic colorectal cancer | Efficacy on cancer patients with chemotherapy |
| | NCT03870607 | Anal cancer squamous cell | Efficacy in increasing the effectiveness of conventional treatment |
| Microbial ecosystem therapeutics strains (MET-4) | NCT03686202 | Solid tumors | Effectiveness on cancer patients with immunotherapy |
| | NCT03836001 | Oropharyngeal Squamous Cell Carcinoma | Effectiveness on cancer patients with chemoradiotherapy |
| | NCT03934827 | Solid tumors | Investigating the possible Benefit for cancer related fatigue |
| | NCT04193904 | Resectable pancreatic cancer | Evaluating the safety and efficacy on preoperative hypo-fractionated radiation |
| | NCT03922035 | Hematopoietic and Lymphoid Cell Neoplasm | Safety and efficacy on patients after hematopoietic stem cell transplant |
| | NCT04079270 | Breast cancer | Effectiveness on breast cancer patients with adjuvant endocrine treatment |

### Oncolytic viruses for cancer therapy and adjuvant therapy

| Interventions | NCT number | Cancer types | Purpose |
|---------------|------------|--------------|---------|
| Adenovirus | NCT02705196, NCT03916510, NCT03740256, NCT03618953, NCT04695327, NCT02798406, NCT04217473, NCT03190824, NCT03259899, NCT03714334, NCT03852511, NCT04097002, NCT03896508, NCT03072134, NCT04685499, NCT03178032, NCT03003676 | Pancreatic cancer, Locally advanced rectal cancer, Solid tumors, HPV-associated cancers, Brain cancer, Metastatic Melanoma, Ovarian cancer, Biliary carcinoma, colorectal cancer, Glioblastoma, Epithelial tumors, Prostate cancer, Glioma, Head and neck squamous cell carcinoma, Diffuse pontine gliomas etc. | To explore the safety, tolerance and efficacy of oncolytic virus agents as a therapeutic or adjuvant therapeutic strategy in treating various cancer patients. |
| Herpes Simplex Virus | NCT03004183, NCT04637698, NCT03866525, NCT03252808, NCT04386967, NCT02779855, NCT04185311, NCT03152318, NCT04735978, NCT04349436, NCT03911388, NCT03767348, NCT02457845, NCT04616443, NCT04348916, NCT02062827, NCT03657576 | Metastatic triple negative breast cancer and non-small cell lung cancer, Pancreatic cancer, Solid tumors, Breast cancer, Glioma, Cutaneous squamous cell carcinoma, Brain tumors, Melanoma, Glioblastoma, biliary carcinoma, Ovarian cancer, Glioma, Diffuse pontine gliomas etc. | To explore the safety, tolerance and efficacy of oncolytic virus agents as a therapeutic or adjuvant therapeutic strategy in treating various cancer patients. |
| Vaccinia Virus | NCT03206073, NCT03954067, NCT03244486, NCT02977156, NCT04301011, NCT02759588, NCT04725331, NCT04226066, NCT03294083 | Colorectal cancer, Solid tumors, Glioblastoma, breast cancer, Ovarian cancer, Renal cell carcinoma | To explore the safety, tolerance and efficacy of oncolytic virus agents as a therapeutic or adjuvant therapeutic strategy in treating various cancer patients. |
| Other Types | NCT04478703, NCT04673942, NCT04448444, NCT03889275, NCT04057193, NCT04215416, NCT04209818, NCT04521764, NCT01846091, NCT02088794, NCT02364713, NCT03043391 | Solid tumors, Breast cancer, Recurrent plasma cell myeloma, Head and neck squamous cell carcinoma, Recurrent ovarian, primary peritoneal or fallopian tube cancer, Fallopian, Urogenital cancer, Children Glioma | To explore the safety, tolerance and efficacy of oncolytic virus agents as a therapeutic or adjuvant therapeutic strategy in treating various cancer patients. |

Table 2: Active clinical trials of bacterial agents and oncolytic viruses applying to cancer therapy and adjuvant therapy.
Table 3 Reported studies evaluating the diagnostic and staging performance of cmDNA in different types of cancers.

| Cancer type | cmDNA signatures | Cohort | Functions of cmDNA |
|-------------|------------------|--------|-------------------|
| Prostate cancer, lung cancer and melanoma [34] | *Aliviobrio* genus using both Kraken and SHOGUN-derived taxonomy assignments | Non-cancer, HIV+, healthy controls (n=69) and 100 patients from three types of high-grade (stage III-IV) cancers: prostate cancer (n=59), lung cancer (n=25) and melanoma (n=16) | Diagnostic performance in distinguishing cancers from healthy patients |
| Early-onset breast cancer [151] | Early-onset breast cancer patients had high titers of cmDNA derived from *Pseudomonas* or *Sphingomonas* spp. | Early-onset breast cancer patients (n=3) and healthy females (n=2) | Potential diagnostic and prognostic value of the cmDNA profiles |
| Hepatocellular carcinoma [152] | 5-genera microbiome signature (*Pseudomonas, Streptococcus, Staphylococcus, Bifidobacterium, and Trabulsiella*) | Patients with HCC (n=79) and cirrhosis (n=83), and matching healthy controls (n=201) | Potential diagnostic value in distinguishing HCCs from healthy controls |
| Gastric cancer [153] | Enriched acinetobacter, bacteroides and hemophilus parainfluenzae in gastric cancer | Gastric cancer (n=71), atypical hyperplasia (n=6), chronic gastritis (n=11), and healthy controls (n=13) | Potential value of cmDNA in diagnosis, progress evaluation and prognosis prediction of gastric cancer |
| Colorectal cancer [154] | 28 microbial species (e.g. *Eubacterium rectale, Bifidobacterium adolescentis, Ruminococcus torques, Roseburia intestinalis and Propionibacterium freudenreichii*) | CRC patients (n=25), colorectal adenoma (CRA) patients (n=10) and healthy controls (n=22) | Potential non-invasive biomarkers in early diagnosis of CRC |
| Colorectal cancer [155] | DNA coding for 16S rRNA, β-galactosidase of *E. coli*, glutamine synthase of *B. fragilis*, DNA coding for 5.8S rRNA of *C. albicans* | CRC patients (n=397) and healthy controls (n=32) | Promising prognostic (PFS and OS) biomarkers of CRC patients |
| Melanoma [156] | *Castellaniella*, the only one differentially abundant amplicon sequence variant | Stage IV melanoma patients (n = 15) and healthy controls (n = 15) | cmDNA can serve as a potential biomarker after removal of contamination |